The inflammatory response to birth requires MyD88 and is driven by both mother and offspring

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Abstract

Birth is an inflammatory event for the newborn, characterized by elevations in interleukin (IL)-6, IL-10, and tumor necrosis factor (*TNF*)-*α* peripherally and/or centrally, as well as changes in brain microglia. However, the mechanism(s) underlying these responses is unknown. Toll-like receptors (TLRs) play crucial roles in innate immunity and initiate inflammatory cascades upon recognition of endogenous or exogenous antigens. Most TLR signaling depends on the adaptor molecule myeloid differentiation primary response 88 (MyD88). We independently varied *MyD88* gene status in mouse dams and their offspring to determine whether the inflammatory response to birth depends on MyD88 signaling and, if so, whether that signaling occurs in the offspring, the mother, or both. We find that the perinatal surges in plasma IL-6 and brain expression of *TNF-α* depend solely on *MyD88* gene status of the offspring, whereas postnatal increases in plasma IL-10 and TNF-α depend on *MyD88* in both the pup and dam. Interestingly, *MyD88* genotype of the dam primarily drives differences in offspring brain microglial density and has robust effects on developmental neuronal cell death. Milk cytokines were evaluated as a possible source of postnatal maternal influence; although we found high levels of CXCL1/GRO*α* and several other cytokines in ingested post-partum milk, their presence did not require MyD88. Thus, the inflammatory response previously described in the late-term fetus and newborn depends on MyD88 (and, by extension, TLRs), with signaling in both the dam and offspring contributing. Unexpectedly, naturally-occuring neuronal cell death in the newborn is modulated primarily by maternal *MyD88* gene status.

Keywords: *Toll-like receptors, MyD88, birth, parturition, neuroinflammation, cytokines, microglia, cell death, milk*

1. Introduction

A mammalian birth is an inflammatory event for both the mother and offspring. The initiation of labor is triggered by a state of "sterile inflammation," characterized by immune cell migration and an increase in proinflammatory cytokines in the uterus and myometrium (Mackler et al., 1999; Thompson et al., 1999; Golightly et al., 2011; Shynlova et al., 2013a, 2013b; Mendelson et al., 2017). Late in gestation, cytokines are also increased in tissues with more direct access to the fetus, including the placenta, decidua, and amniotic fluid (Young et al., 2002; Osman et al., 2003; Shynlova et al., 2013b; Keelan, 2018), and at parturition the newborn experiences stimuli such as microbial colonization that could elicit an immune response. Indeed, cytokines are elevated in the umbilical cord and peripheral circulation of human newborns (Levy, 2007; Marchini et al., 2000; Tutdibi et al., 2012; Denihan et al., 2013).

We recently reported that birth also triggers an immune response in the perinatal mouse, and that this response extends to the brain. For example, we found a striking increase in the pro-inflammatory cytokine interleukin (IL)-6 in fetal plasma just before parturition and a surge in IL-10 at 3 hours post-partum (Castillo-Ruiz et al., 2022). In the brain, tumor necrosis factor (TNF)-α expression and the density of microglia, the innate immune cells of the brain, increase markedly one day after parturition (Castillo-Ruiz et al., 2022). When birth is advanced by a day, increases in each of these markers are also advanced, indicating that birth is, in fact, causal to the inflammatory response. The mechanism(s) underlying the inflammatory response to birth is unknown. Similarly, the extent to which the offspring's response is driven by endogenous inflammatory signaling, versus maternal inflammatory signaling, is not known. The fetus and mother share a blood supply prenatally and maternal immune signals may be transferred to the newborn postnatally via milk (Field, 2005). Thus, it remains unclear whether cytokines measured in neonatal circulation arise from maternal and/or offspring sources.

Given the relative immaturity of the adaptive immune system, inflammatory responses in the fetus and newborn depend heavily on innate immunity (Levy, 2007). Innate immune responses are often triggered by the activation of toll-like receptors (TLRs), which leads to the release of proinflammatory cytokines such as IL-6, IL-1β, and/or TNF-α. TLRs detect an array of microbe-associated antigens and are also activated by endogenous signals such as heat-shock proteins and other markers of cellular stress (Matzinger, 2002; Bianchi, 2007). Expression of TLRs is ubiquitous throughout the body's immune cells and in many cell types within the brain throughout development (Bsibsi et al., 2002; Poulain-Geodofroy et al., 2010; Kaul et al., 2012; McClure & Massari, 2014).

TLRs are transmembrane proteins located at the cell surface or on endosomes, and most signal exclusively through the cytosolic adapter protein myeloid differentiation primary response 88 (MyD88; Hultmark, 1994; Medzhitov et al.,1998; Deguine & Barton, 2014). Thus, mice with a deletion of the *MyD88* gene (*MyD88-*) lack most, but not all, TLR-mediated cytokine production in response to immune activation (Kawai et al., 1999; Hou et al., 2008; Browne et al., 2009). Importantly, even a single copy of *MyD88* (designated here as *MyD88+*) confers largely normal immune and inflammatory responses (e.g., Campbell et al., 2006; Archer & Roy, 2006; Asquith et al., 2010; Okugawa et al., 2011). Here, we took advantage of this observation to independently vary TLR-dependent signaling in the mother and offspring.

MyD88+ dams were bred to *MyD88-* males, or vice versa, to generate four groups of progeny: *MyD88+* pups born to *MyD88+* dams; *MyD88-* pups born to *MyD88+* dams; *MyD88+* pups born to *MyD88-* dams, and *MyD88-* pups born to *MyD88-* dams. Offspring were collected at several time points before and after birth, and peripheral and central markers of immune activation were examined. To probe for a possible functional consequence of altered inflammatory signaling, we determined the extent to which maternal and/or pup *MyD88* gene status altered developmental cell death, a widespread phenomenon that prunes neuron number

and peaks around the time of birth in mice (Ahern et al., 2013; Mosley et al., 2017). Finally, cytokines were measured in recently ingested milk to test for a potential postnatal source of maternal immune signaling.

2. Methods

2.1 Animals and breeding scheme

MyD88 homozygous null mice (*MyD88*-) on a C57BL/6J background were purchased from the Jackson Laboratory (Jax stock: 009088; Bar Harbor, ME, USA), and were paired with wildtype C57BL/6J mice to generate *MyD88* heterozygotes with a single functional copy of the gene (*MyD88*+). *MyD88*+ adult females were then paired with *MyD88*- males, or *MyD88*- females were paired with *MyD88*+ males, to generate the four groups of offspring shown in Fig. 1A. For timed pregnancies, mice were paired overnight and males were removed the next morning; this was designated embryonic day (E) 0. The normal gestation length of C57BL/6J mice is 19.3 days (Murray et al., 2010). Inflammatory responses were examined in fetuses of both sexes just before birth (E18.5) or in pups at 3h or 3 days postnatal (P3) (Fig. 1B). These time-points were chosen based on the inflammatory responses to birth reported previously (Castillo-Ruiz et al., 2022). Each group was represented by at least three litters, with the exception of pups born to *MyD88*+ dams at P3 (n=2 litters). Offspring of all genotypes developed normally and body weights were equivalent among the four groups at all ages examined (Fig. 1C). In addition, to confirm that immune signaling in *MyD88* heterozygotes (*MyD88*+) was similar to that in wild-type mice (*MyD88*++), we collected a group of wild-type C57BL/6J pups on E18.5. Animals were maintained at 22°C on a 12:12 light-dark cycle with food and water available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at Georgia

State University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

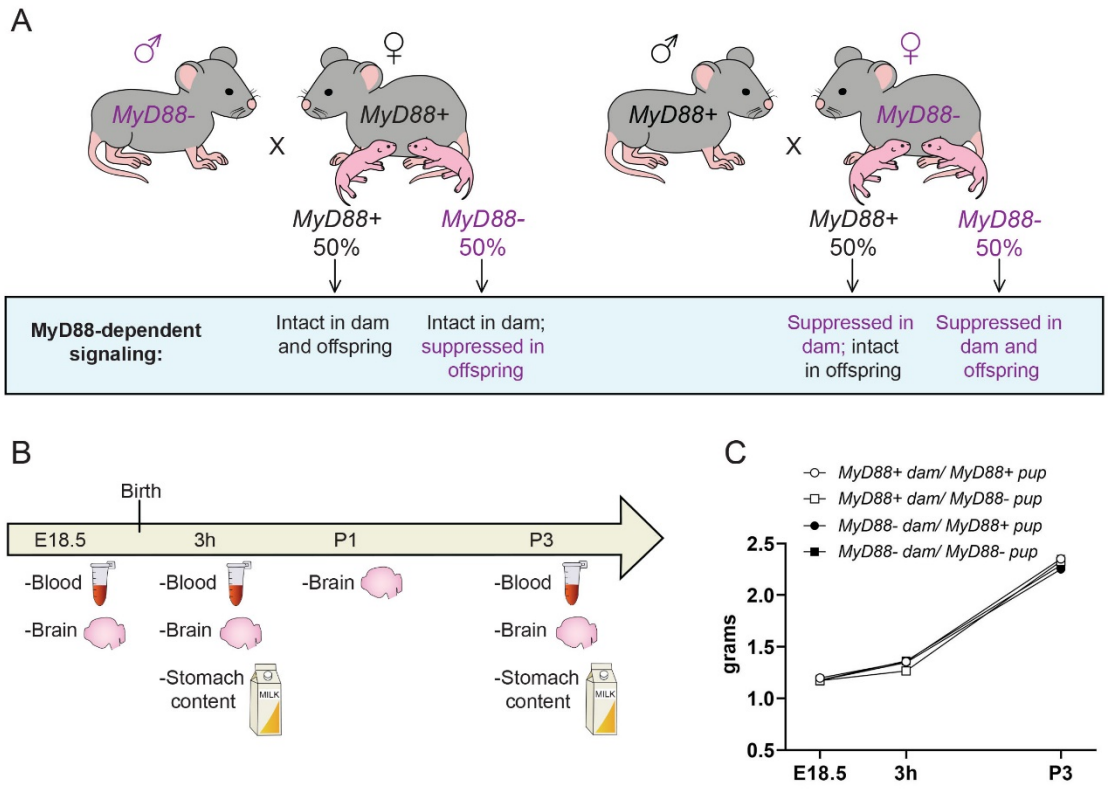

Fig. 1. Experimental design and offspring gross development. (A) *MyD88*- males were bred to *MyD88*+ females, and vice versa, to produce four groups of offspring: *MyD88*+ pups born to *MyD88*+ dams; *MyD88*- pups born to *MyD88*+ dams; *MyD88*+ pups born to *MyD88*- dams; and *MyD88*- pups born to *MyD88*- dams. (B) Offspring were collected at E18.5 or postnatally at 3h, one day (P1) or three days (P3) of age and the indicated tissues were collected for the analysis of inflammatory markers and quantification of neuronal cell death. (C) Pups of all four genotypes exhibited normal gross development, and neither maternal nor pup genotype affected pup body weight. Data are mean + SEM; *n* = 3-39 per data point.

2.2 Tissue collection

For 3h and 3 day postnatal collections, cages were checked hourly for births starting on the eve of E19, with checks in the dark performed under red light illumination. For E18.5 collections, timed-pregnant dams were exposed to 4% CO_2 and euthanized. An abdominal

incision was made to expose the uterine horns, and fetuses were rapidly removed. All offspring were weighed, and trunk blood and brains collected. Tail clippings were obtained under sterile RNAase-free conditions for genotyping by TransnetYX® (Memphis, TN, USA) to confirm the presence or absence of the *MyD88* exon 3 deletion. Mice with this deletion do not produce MyD88 protein (Hou et al., 2008). To analyze samples of recently ingested milk, the stomach contents of a subset of the postnatal pups were collected, weighed, immediately flash frozen, and stored at -80°C until processing. Brains were bisected at the midbrain with a razor blade under sterile RNAase-free conditions. The forebrains were used for histological analyses and were fixed in 5% acrolein for 24h, then transferred to 30% sucrose and stored at -4°C before sectioning. The mid/hindbrains were flash-frozen before storage at -80°C and used for the detection of cytokine gene expression by PCR. In a follow-up experiment, separate forebrain and mid/hindbrain samples were collected from a new cohort of pups on P1 (n=5 litters; Fig. 1B) for analysis of brain cytokine expression.

2.3 Plasma cytokine assay

Trunk blood was collected in sterile 40 µl sodium heparin-coated capillary tubes (Sanguis Counting, Numbrecht, Germany). Samples were centrifuged within 20 minutes of collection at 4°C for 15 minutes at 3800g, and plasma was collected and stored at -80°C. Electrochemiluminescence assays were performed at the Emory Multiplex Immunoassay Core (Atlanta, GA, USA) using U-PLEX Biomarker Scale Assays by Meso Scale Discovery (Rockville, MD, USA). We initially cast a broad net by probing for ten targets: interferon (IFN) -γ, IL-1β, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-27p28/IL-30, CXCL2 (C-X-C motif chemokine ligand 2, previously, macrophage inflammatory protein 2), and TNF-α (all validated by Meso Scale Discovery). Of these, IL-6, IL-10, and TNF-α were reliably detected; for all others, values were below the limit of detection for most samples and these cytokines are therefore not analyzed

below. Samples were run in duplicate, and all samples were corrected against a standard curve. If a plasma sample from an individual animal did not reach the required minimum volume (20 µl per duplicate), as was often the case, it was combined in equal parts with one or two other samples of the same sex, pup genotype, and maternal genotype.

2.4 Reverse transcription polymerase chain reaction (RT-PCR) for brain cytokine expression

Frozen brain tissue was homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA was precipitated and tested for purity using standard methods. Primers used were for IL-1β, *IL-6, IL-10, TNF-α*, and the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase (GAPDH*; all validated primers from Qiagen Inc., Valencia, CA, USA). Reverse transcription was performed with a Superscript IV kit (Invitrogen) in a thermal cycler (Applied Biosystems Inc., Foster City, CA, USA), and real-time PCR was performed in the LightCycler 96 System (Roche, Mannheim, Germany) using FastStart Essential DNA Green Master Kit (Roche) according to the manufacturer's instructions. As in our previous study, results for IL-1β were not interpretable due to extreme variability between biological replicates (Castillo-Ruiz et al., 2022) and therefore are not discussed further below. Group values were expressed relative to the expression levels of the E18.5 *MyD88*+ pup/*MyD88*+ dam group using the Pfaffl method of relative quantification (Pfaffl, 2001).

2.5 Milk cytokine assay

Stomach contents of newborns were assayed at the Centers for Disease Control and Prevention in Atlanta, GA, using an Intelliflex assay (Luminex XMAP Intelliflex DRSE) for a customized panel of 11 inflammatory cytokines and chemokines (Procartaplex-PPX-11): CXCL1 (chemokine ligand 1; also known as GROα), IFN-β, IFN-γ, IL-1β, IL-6, IL-12/IL-23p40, CXCL10

(also known as IFNγ-induced protein 10), CCL2 (C-C motif chemokine ligand 2; also known as monocyte chemoattractant protein 1), macrophage inflammatory protein (MIP)1β, CCL5 (also known as RANTES), and TNF-α. A positive control consisting of lung tissue from mice infected with influenza virus was included in the assay, as all of the measured cytokines are significantly elevated in this tissue (Hofstetter et al., 2016). Lung tissue from uninfected mice was also included for comparison. Briefly, as described by the assay kit manufacturer (Life Technologies Corporation, Grand Island, NY), 1X fluorescent capture beads were incubated for 2 h with 25 μL of standard mix, test samples, or positive controls in a 96-well plate. After washing the plate (1X Wash Buffer), 25 μL of biotinylated detection antibody was added to all the wells followed by 1 h incubation. The assay plate was then washed, and streptavidin-PE was added to the wells for 30 min. Aftrer washing, 120 μL of reading buffer was added to each well and the plate was read on Luminex XMAP instrument. For analyses, the data were exported in .csv format and analyzed using the ProcartaPlex analysis software (https://apps.thermofisher.com/apps/procartaplex). All samples were run in duplicate. Values (pg/ml) were corrected for initial size of the ingested milk sample (mg) and are expressed as pg/ml/mg.

2.6 Immunohistochemistry

Brains were sectioned coronally into four series at 40 μm and stored in cryoprotectant at -20°C until use. One series was processed for the immunohistochemical detection of ionized calcium binding adaptor molecule 1 (Iba1) to label microglia, and a second series for activated caspase-3 (AC3) to label dying cells. Tissue was washed between steps in 1X tris buffered saline for 30 minutes (TBS; pH 7.6), and all steps were carried out at room temperature. Epitope retrieval was performed with 0.05 M sodium citrate and unreacted aldehyde blockade with 0.1 M glycine

for 1h and 30 minutes, respectively. Tissue was then incubated in a blocking solution [20% normal goat serum (NGS), 1% H_2O_2, 0.3% Triton X in TBS] for 30 minutes, followed by overnight incubation with the appropriate primary antibodies: rabbit anti-Iba1 (Wako, Chuo-Ku, Osaka, Japan; 1:3,000) or rabbit anti-AC3 [cleaved caspase-3 (Asp175) antibody; Cell Signaling, Beverly, MA, USA; 1:10,000; 2% NGS, 0.3% Triton X in TBS]. Sections were washed in a dilute blocking solution (1% NGS, 0.02% Triton X in TBS), incubated for 1h in a goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:1,000; 0.32% Triton X in TBS), washed in 1X TBS-0.2% Triton X, and incubated for 1h in an avidin-biotin solution (Vector Laboratories; 1:500 in 1X TBS). Tissue was washed in acetate buffer (pH 7.2) for 30 minutes and incubated in 0.02% diaminobenzidine tetrahydrochloride, 2% nickel sulfate, and 0.0025% H_2O_2 made in the same buffer. Sections were mounted onto gelatin-coated slides and dehydrated. AC3 slides were then counterstained with thionin before coverslipping. Slides were scanned at 40X using a Hamamatsu Nanozoomer slide scanner (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and analyzed using Aperio Image Scope software (Leica Biosystems Inc., Buffalo Grove, IL, USA).

2.7 Brain regions examined for Iba1 and AC3 quantification

The paraventricular nucleus of the hypothalamus (PVN) and hippocampus play important roles in the brain's response to peripheral immune activation (Sawchenko et al., 1996; Elmquist et al., 1996; Frenois et al., 2007; Tarr et al., 2012; Whylings et al., 2020). The PVN and CA1 oriens layer of the hippocampus also show marked changes in neuronal activation, microglial density, and cell death at birth (Mosley et al., 2017; Castillo-Ruiz et al., 2018; 2020; Hoffiz et al., 2021). Counts of microglia (Iba1+) and dying cells (AC3+) were therefore performed in the PVN and CA1 oriens of the dorsal hippocampus. Region contours were drawn bilaterally in all sections using previously defined criteria (Castillo-Ruiz et al., 2022), and positive cells were counted

within each contour. The sum of labeled cells across all sections per animal was divided by the total area sampled and multiplied by section thickness to obtain the density of labeled cells per mm^3. All counts were performed by an investigator blind to experimental conditions.

2.8 Data Analysis

Statistical analyses were conducted using SPSSv28 (IBM Corp, Amonk, NY, USA; milk cytokines) or GraphPad Prism (Graphpad Software LLC, San Diego, CA, USA; all other measures). Males and females were combined in all analyses presented below. In initial analyses we found no effects of sex on any dependent measure, although we note that for some measures we were not sufficiently powered to reliably detect sex differences. The effects of age (E18.5, 3h, P3) were examined using one-way ANOVAs. We then used two-way ANOVAs within each age to examine the effects of pup genotype, dam genotype, and their interaction on cytokine levels, Iba1+ cell density, and AC3+ cell density. A Bonferroni correction was used for post hoc tests. Milk cytokines/chemokines were analyzed by three-way ANOVA to test for effects of age, pup genotype, dam genotype, and their interaction.

3. Results

3.1 Pup and maternal MyD88 signaling drive the peripheral cytokine responses

To test whether the inflammatory response to birth depends on MyD88-dependent signaling in the dam, the offspring, or both, we first measured plasma cytokine levels in offspring just before birth (E18.5), and postnatally at 3h and P3. Many of the cytokines/chemokines we measured (IFN-γ, IL-1β, IL-12p70, IL-13, IL-17A, IL-27p28/IL-30, and CXCL2) were below the

level of detection. IL-6, TNF-α, and IL-10 were reliably detected, as reported previously in perinatal mice (Castillo-Ruiz et al., 2022), and are the focus of our analyses.

We previously observed a sharp increase in the proinflammatory IL-6 between E16.5 and E18.5 in plasma from wild-type fetuses (Castillo-Ruiz et al., 2022). We confirm the elevation in IL-6 at E18.5 here, and show that it requires *MyD88* solely in the fetus: IL-6 levels were almost five times higher in pups with a functional copy of *MyD88* compared to *MyD88*- pups at E18.5 ($F_{1, 23}$ = 39.09, p< 0.0001), with no effect of dam genotype and no interaction (Fig. 2A).

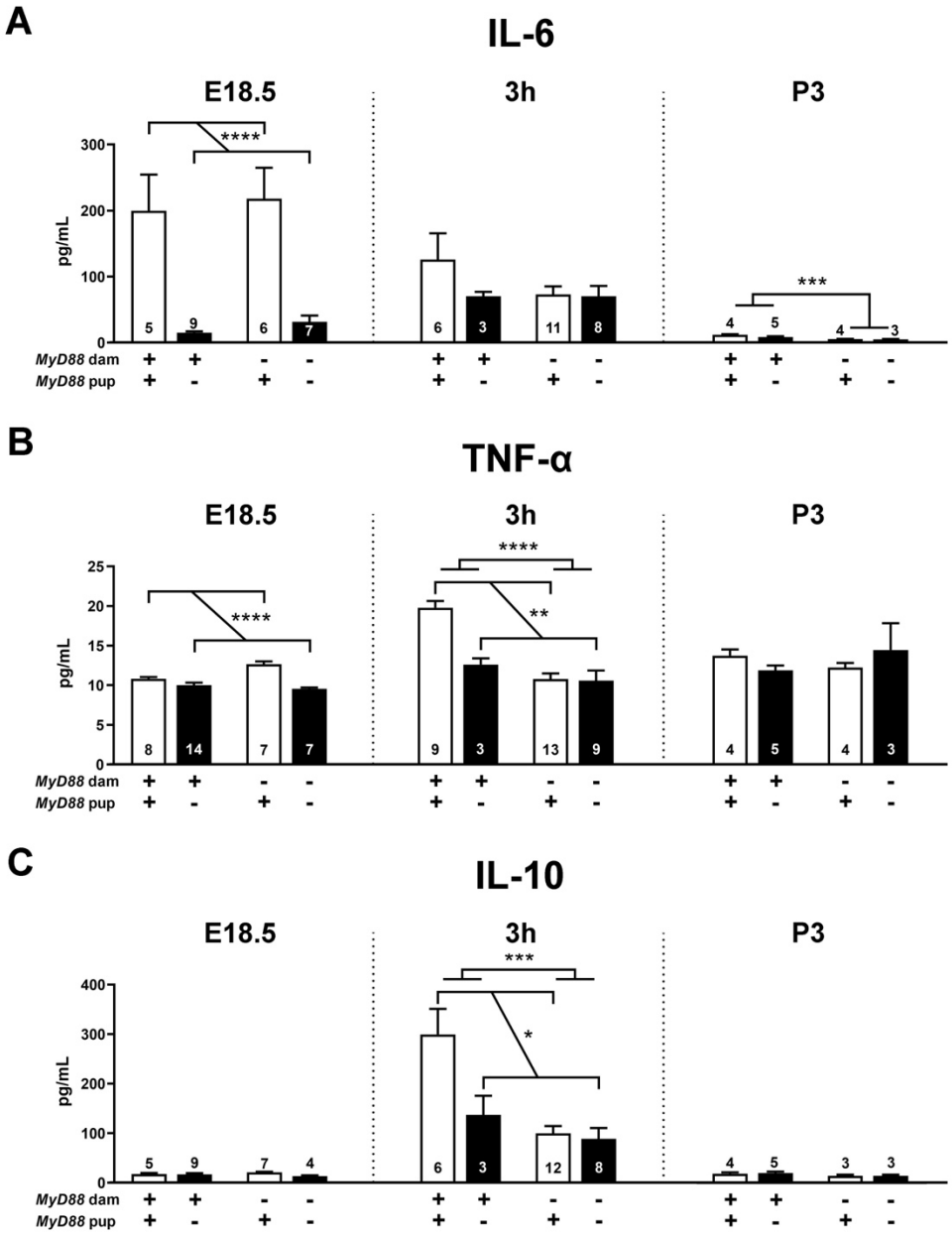

Fig. 2. Maternal and fetal MyD88 signaling contribute to dynamic changes in plasma cytokines. (A) *MyD88*+ pups experienced a surge in plasma IL-6 at E18.5 that was absent in *MyD88*- pups (main effect of pup genotype). There was also an effect of maternal *MyD88* genotype on IL-6 at P3, although levels were very low at this age. (B) TNF-α was higher in *MyD88*+ pups than in *MyD88*- pups at E18.5 (main effect of pup genotype). At 3h postnatal there were main effects of pup genotype, dam genotype, and an interaction, such that TNF-α levels were highest in *MyD88*+ pups born to *MyD88*+ dams. (C) There was a large surge of plasma IL-10 at 3h after birth that depended on both pup and maternal *MyD88* genotype. There were main effects of pup and dam genotype, as well as an interaction, such that *MyD88*+ pups born to *MyD88*+ dams exhibit the highest IL-10 plasma levels. *p< 0.05; **p< 0.01; 1****p< 0.0001. Data are mean + SEM; *n* individual or pooled samples per group is indicated at the base of each bar.

IL-6 levels declined rapidly after birth ($F_{2, 56} = 12.22$, $p < 0.0001$ for main effect of age) for all groups. Although levels at P3 were extremely low, pups born to *MyD88+* dams had slightly higher IL-6 than those born to *MyD88-* dams ($F_{1, 12} = 26.21$ $p = 0.003$) (Fig. 2A).

TNF-α plasma levels did not change significantly across the three perinatal ages, consistent with previous findings (Castillo-Ruiz et al., 2022). However, effects of both maternal and pup *MyD88* genotypes were observed. TNF-α levels were slightly higher in *MyD88+* pups compared to *MyD88-* pups at E18.5 (main effect of pup genotype: $F_{1, 32} = 32.86$, $p < 0.0001$), although this effect was larger in *MyD88+* pups born to *MyD88-* dams (pup-by-dam genotype interaction: $F_{1, 32} = 11.07$, $p = 0.002$) (Fig. 2B). At 3h, we observed effects of maternal genotype (*MyD88+* > *MyD88-*; $F_{1, 30} = 22.85$, $p < 0.0001$), pup genotype (*MyD88+* > *MyD88-*; $F_{1, 30} = 10.18$, $p = 0.003$) and an interaction ($F_{1, 30} = 9.06$, $p = 0.005$), such that TNF-α levels were highest in *MyD88+* pups born to *MyD88+* dams (Fig. 2B).

IL-10 is generally anti-inflammatory and often increases after an inflammatory challenge. We previously showed a striking increase in plasma IL-10 at 3h after birth in wild-type offspring (Castillo-Ruiz et al., 2022). We replicated that finding here (main effect of age: $F_{2, 58} = 37.22$, $p < 0.0001$) (Fig. 2C) and found that both pup and dam MyD88 signaling play a role. Specifically, there were main effects of maternal genotype (*MyD88+* > *MyD88-*; $F_{1, 25} = 15.11$, $p = 0.0007$), pup genotype (*MyD88+* > *MyD88-*; $F_{1, 25} = 7.39$, $p = 0.012$) and an interaction ($F_{1, 25} = 5.60$, $p = 0.03$), such that IL-10 levels at 3h postnatal were highest in *MyD88+* pups born to *MyD88+* dams (Fig. 2C). IL-10 levels were low at E18.5 and P3 and did not differ by maternal or pup genotype.

Mice with a single functional copy of *MyD88* are indistinguishable from mice with two copies of the gene in response to most immune challenges (Campbell et al., 2006; Archer & Roy, 2006; Asquith et al., 2010; Okugawa et al., 2011). We confirmed that in a limited sample of

E18.5 pups here, as there were no significant differences between *MyD88+* and wild-type (*MyD88++*) mice for any of the plasma cytokines measured (Supplementary Fig. 1).

Thus, MyD88 signaling is required for the inflammatory response to birth in the periphery, and depends on at least one functional copy of the *MyD88* gene in the pup (IL-6) or both the pup and dam (TNF-α and IL-10).

3.2 Pup MyD88 genotype influences brain TNF-α expression

We next evaluated *IL-6*, *TNF-α*, and *IL-10* gene expression in perinatal mid and hindbrain at E18.5, 3h and P3 using RT-PCR (forebrains were used for the histological analyses described below). We found significant effects of age for all three cytokines (IL-6: $F_{2, 46} = 20.52$, $p < 0.0001$; *TNF-α:* $F_{2, 46} = 5.84$, $p = 0.006$; IL-10: $F_{2, 45} = 4.74$, $p = 0.01$) (Fig. 3). Specifically, brain expression of *IL-6* ($p < 0.0001$) and *TNF-α* ($p = 0.004$) increased between E18.5 and 3h after birth (Fig. 3A,B), whereas *IL-10* was moderately higher at E18.5 relative to P3 ($p = 0.01$).

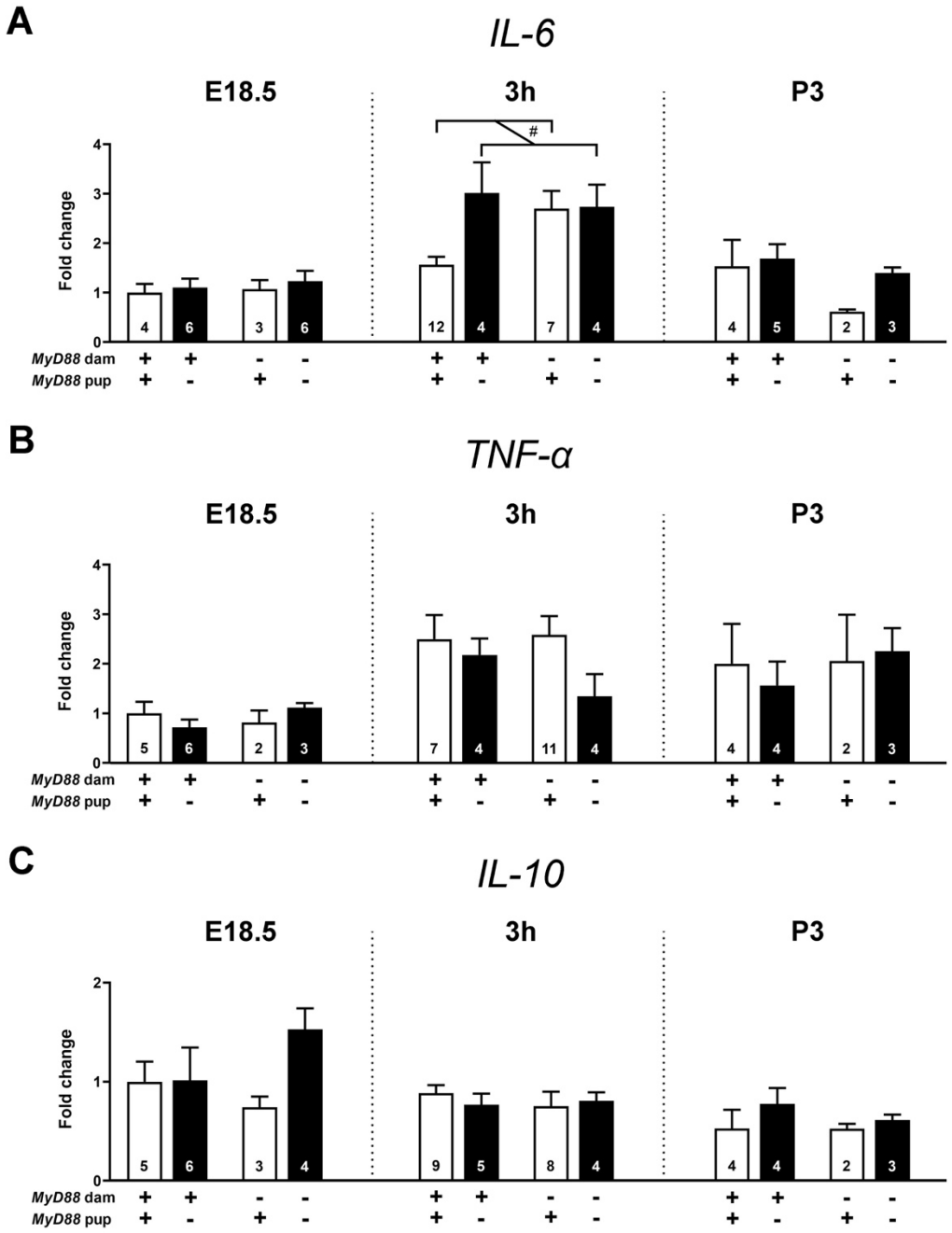

Fig. 3. Perinatal age but not *MyD88* gene status influences cytokine mRNA expression in the brain at E18.5, 3h, and P3. (A) *IL-6* expression increased between E18.5 and 3h postnatal and dropped back down at P3. In addition, there was a marginally significant effect of pup genotype at 3h. (B) *TNF-α* expression increased between E18.5 and 3h and remained elevated at P3. (C) *IL-10* expression was highest prenatally and exhibited slightly lower levels at postnatal time points. Data (mean + SEM) are expressed relative to E18.5 *MyD88*+ pups gestated in *MyD88*+ dams. *n* per group is indicated at the base of each bar.

Analysis of cytokine gene expression within age did not reveal effects of maternal or pup *MyD88* gene status, except for a marginally significant effect of pup genotype on IL-6 at 3h ($F_{1, 23} = 4.46$, p= 0.05).

In our previous work (Castillo-Ruiz et al., 2022), the most striking change in brain cytokine expression in response to birth was a large increase in *TNF-α* mRNA on P1 in the PVN, hippocampus, and whole forebrain. Because P1 was not included in the experiment above, we generated a new cohort of animals and collected the brains of offspring at that timepoint. The mid/hindbrain samples from controls (*MyD88*+ pups gestated in *MyD88*+ dams) collected at E18.5 in the main experiment were run together with the new samples to examine *TNF-α* expression by RT-PCR. We confirm a five- to ten-fold elevation of *TNF-α* on P1 relative to E18.5 and find that it depends on pup *MyD88* genotype, with higher levels in *MyD88*+ pups compared to *MyD88*- pups ($F_{1, 20} = 7.90$, p= 0.01) (Fig. 4). There was no significant effect of maternal genotype or an interaction, although we note a tendency for higher levels in pups from *MyD88*+ dams. We also examined the forebrains of the P1 pups and find the same pattern: a significant effect of pup genotype (*MyD88*+ > *MyD88*-) on *TNF-α* expression, with no effect of maternal genotype or an interaction (Supplemental Fig. 2). The expression of IL-6 and IL-10 was also examined in P1 brains. We did not observe elevations in these cytokines at P1 in our previous study (Castillo-Ruiz et al., 2022), and we found no effect of pup or maternal *MyD88* genotype on either of these cytokines (Supplemental Fig. 3).

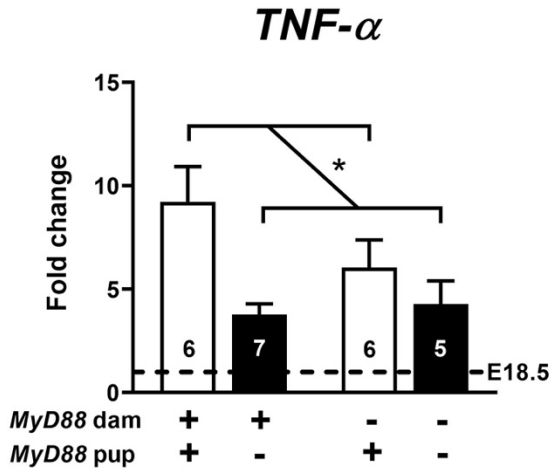

Fig. 4. *TNF-α* expression in the brain is elevated on P1 and requires *MyD88* in the pup. The dotted line represents the mean TNF-*α* levels for the reference group: *MyD88*+ pups gestated by *MyD88*+ dams at E18.5. Levels were elevated in all groups at P1 relative to E18.5. There was a main effect of pup genotype, with higher levels in *MyD88*+ pups. Data are mean + SEM; *n* per group is indicated at the base of each bar. *p< 0.05.

3.3 Maternal MyD88 genotype alters microglial density in the PVN and hippocampus of offspring

Microglia are the resident immune cells of the brain and, as a major source of brain cytokine production, play an important role in orchestrating neural immune responses (Harry et al., 2013; Schwarz et al., 2013; Hoogland et al., 2015). Microglial numbers increase postnatally in both the PVN and hippocampus, and when birth is advanced by one day these increases are also advanced (Castillo-Ruiz et al., 2022). Here, we found that microglial density in the newborn brain depends on *MyD88*, albeit in a complex fashion.

At E18.5, microglial density in the PVN exhibited a pup genotype-by-dam genotype interaction, with lowest levels in *MyD88*- pups born to *MyD88*- dams ($F_{1, 22}$ = 8.02, p= 0.01). Three hours after birth, microglial density was lower in the PVN of *MyD88*- pups than in *MyD88*+ pups ($F_{1, 31}$ = 5.34, p= 0.03), regardless of dam genotype (Fig. 5A). However, three days later, microglial density in the PVN depended on maternal *MyD88* genotype, with density in pups born to *MyD88*- dams lower than that in pups born to *MyD88*+ dams ($F_{1, 12}$ = 7.33, p= 0.02), regardless of pup genotype. Thus, lower microglial levels in the PVN were consistently associated with the absence of MyD88, but whether MyD88 was required in the pup, the dam,

or both, varied by age. In the hippocampus, microglial density was, surprisingly, lower in pups born to *MyD88*+ dams 3h after birth ($F_{1, 32} = 23.19$, $p < 0.0001$) (Fig. 5B). However, as seen in the PVN, microglial density was lower in pups born to *MyD88*- dams ($F_{1, 13} = 11.07$, $p = 0.006$) at P3, in this case with a marginally significant pup-by-dam genotype interaction ($F_{1, 13} = 4.7$, $p = 0.05$; Fig. 5B).

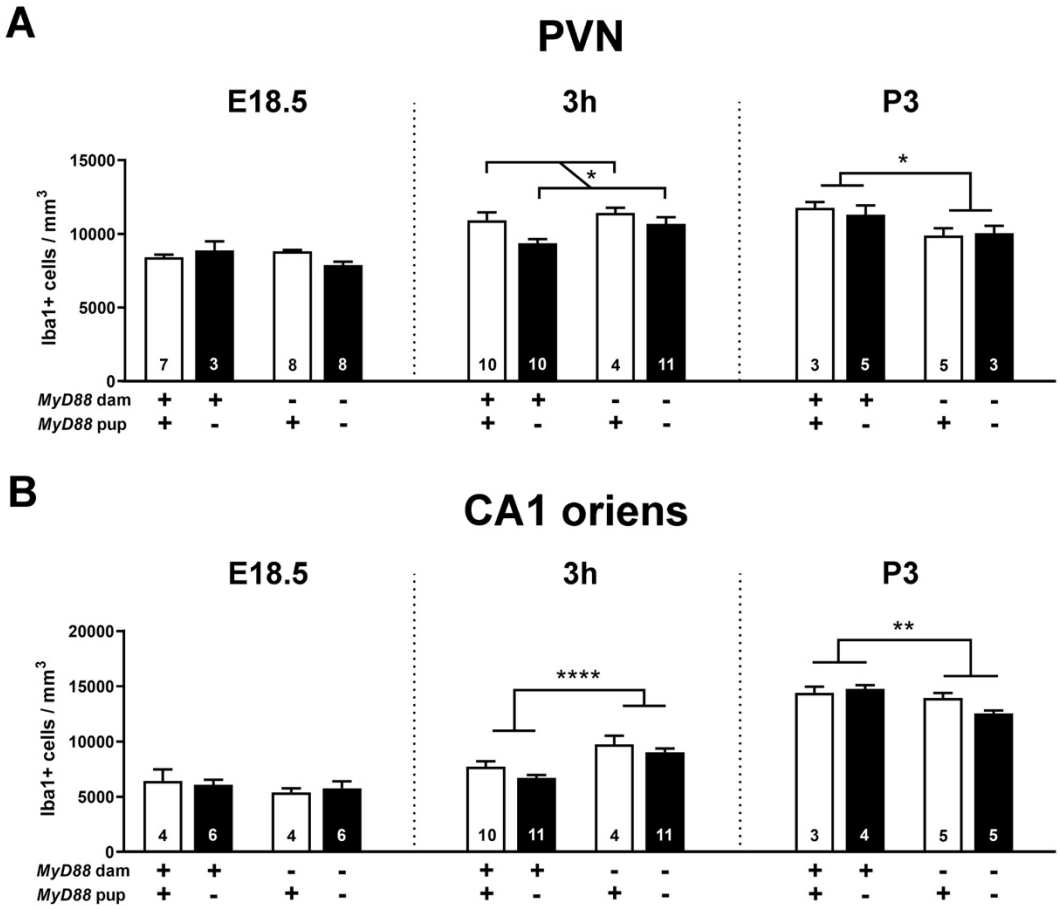

Fig. 5. Maternal and pup *MyD88* gene status affect microglial density in the brains of offspring. (A) In PVN, there were no main effects on microglial density at E18.5, but a pup-by-maternal genotype interaction, such that density was lowest in *MyD88*- pups born to *MyD88*- dams. At 3h, microglial density was lower in *MyD88*- pups than in *MyD88*+ pups (main effect of pup genotype), whereas at P3, microglial density was lower in pups born to *MyD88*- dams (main effect of dam genotype), regardless of pup genotype. (B) In the CA1 oriens of the hippocampus, maternal *MyD88* genotype drove microglial density at 3h and P3, such that pups born to *MyD88*+ dams exhibited lower microglial density at 3h but higher microglial density at P3 (main effects of dam genotype). *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. Data are mean + SEM; *n* per group is indicated at the base of each bar.

3.4 Maternal MyD88 genotype alters perinatal cell death in the brains of offspring

We tested a potential functional consequence of an altered inflammatory response at birth by determining whether maternal or pup *MyD88* genotype influences naturally-occurring cell death. Neuronal cell death eliminates roughly half of the neurons originally produced and peaks at around the time of birth in mice (Ahern et al., 2013; Mosley et al., 2017). Cell death in the PVN, hippocampus, and other areas is altered by changing the timing or mode of birth (Castillo-Ruiz et al., 2018; 2020), and the magnitude of naturally-occurring or injury-induced neuronal cell death can be altered by cytokines or microglial activity (Barker et al., 2001; Sedel et al., 2004; Correale & Villa, 2004; Fontaine et al., 2008; Wakselman et al., 2008; McAdams & Juul, 2012; Jacobs et al., 2019).

The number of cells expressing the apoptosis marker AC3 was examined in the PVN and hippocampus at E18.5, 3h, and P3. Cell death density in the PVN was suppressed in pups born to dams lacking *MyD88* at all three ages (E18.5: $F_{1, 8} = 8.12$, p= 0.02; 3h postnatal: $F_{1, 58} = 15.03$, p= 0.0003; and P3: $F_{1, 20} = 16.13$, p= 0.0007), with no main effect of pup genotype at any age (Fig. 6). There was, however, a significant pup-by-dam genotype interaction at P3 ($F_{1, 20} = 5.34$, p= 0.03), with greatest cell death in *MyD88*- pups born to *MyD88*+ dams (Fig. 6A). Cell death in the CA1 region of the hippocampus was also suppressed in pups born to *MyD88*- dams 3h after birth ($F_{1, 46} = 13.70$, p= 0.0006), with no effect of pup genotype or pup by maternal genotype interaction (Fig. 6B). A similar pattern (lower cell death in pups born to *MyD88*- dams) was seen at E18.5 and P3, but effects of maternal genotype did not reach significance at these ages.

The effects of maternal MyD88 genotype were relatively large, with cell death decreased in pups born to *MyD88*- dams by 26%, 40%, 44%, and 43% in the E18.5 PVN, 3h PVN, P3 PVN and 3h CA1, respectively. Thus, the immune signaling normally occurring in dams around the

time of birth robustly influences developmental cell death in the brains of their offspring during the late prenatal and immediate post-partum period.

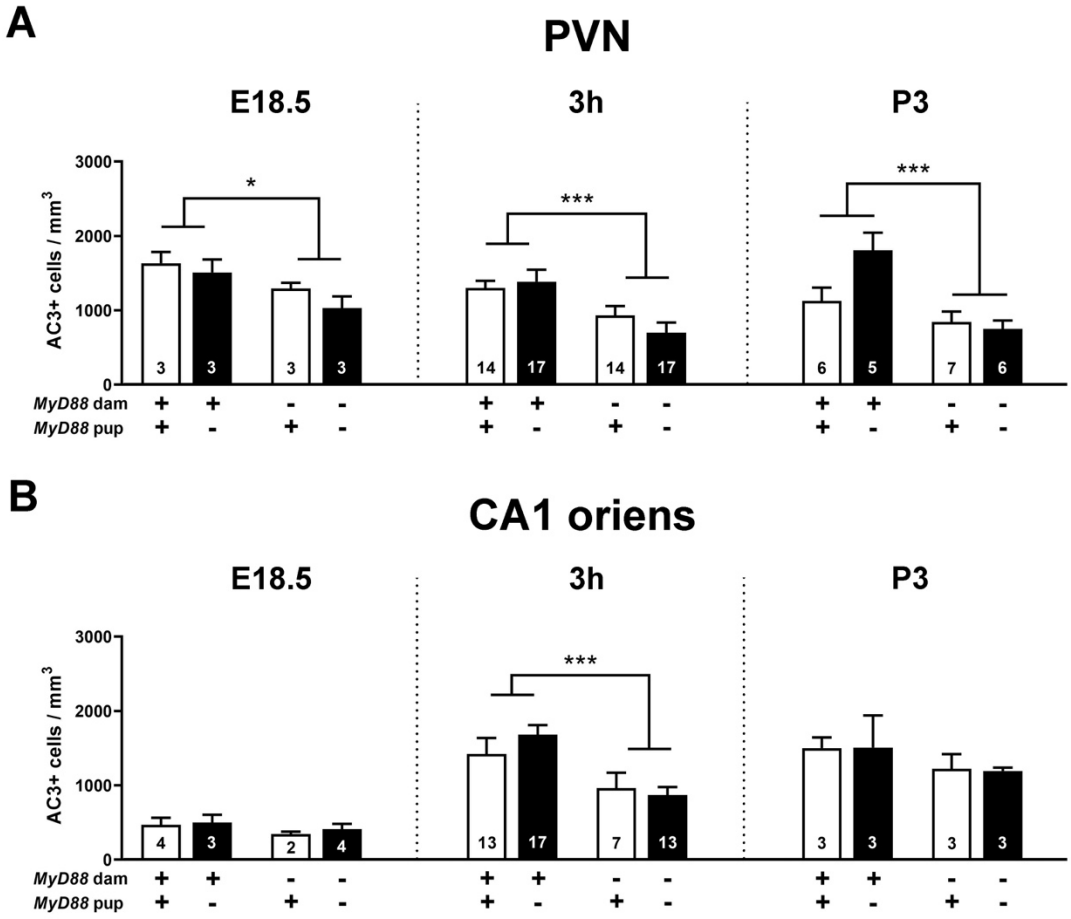

Fig. 6. Maternal *MyD88* increases naturally-occurring cell death in the offspring brain. (A) In the PVN, cell death density was higher in the pups of *MyD88*+ dams at all three ages: E18.5, 3h, P3 (main effects of dam genotype). (B) In the CA1 oriens, cell death density was higher in pups born to *MyD88*+ dams at 3h postnatally (main effect of dam genotype); similar trends at E18.5 and P3 did not reach significance. Data are mean ± SEM; *n* per group is indicated at the base of each bar. *p< 0.05; ***p< 0.001.

3.5 High levels of cytokines in immediate post-partum milk do not require MyD88

The effects of maternal *MyD88* genotype on microglia and cell death in the PVN at P3 were surprising. One possible contributor to such an effect could be breast milk, which contains

cytokines and other immune-related molecules (Mitchie et al., 1998; Maheshwari et al., 2003; Case et al., 2010; Aspinall et al., 2011; Dawod et al., 2020). To test this, we collected the stomach contents of a subset of the animals in the experiment above at 3h or 3 days after birth and measured the levels of 11 cytokines and chemokines. Although levels were quite variable, we found significantly higher concentrations immediately after birth (3h) than at 3 days for almost all cytokines/chemokines assayed, with the differences ranging from 3-fold to 10-fold (Fig. 7).

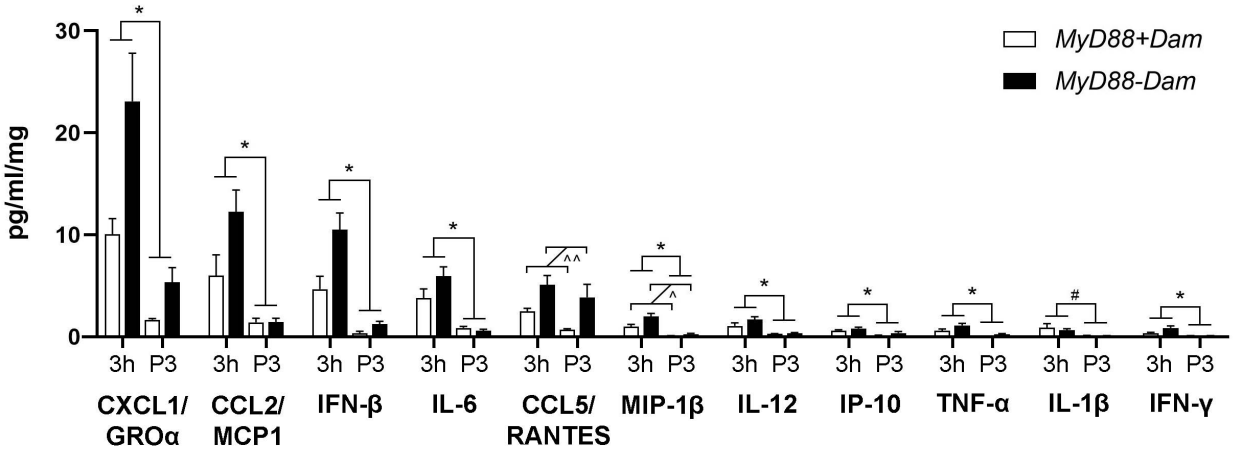

Fig. 7. Cytokine/chemokine levels in stomach contents (ingested milk) of pups born to *MyD88+* and *MyD88-* dams. CXCL1/GROα, CCL2/MCP1, IFN-β, and IL-6 were present at highest levels. Almost all cytokines/chemokines were significantly higher at 3h compared to 3 days postnatal (*p< 0.05; #p= 0.06). There was an effect of maternal *MyD88* genotype on CCL5/RANTES (^^p< 0.01) and MIP-1β (^p= 0.05) with higher levels in *MyD88-* dams. Levels are expressed as pg/ml, corrected for weight of the sample in mg. Data are mean + SEM; *n* = 25 for 3h and n = 10 for P3.

CXCL1/GROα was present in highest amounts, with levels per mg tissue greater than that in influenza virus-infected lung (positive control). CCL2/MCP1, IFN-β, and IL-6 levels on the day of birth were also quite high, with detectable, but lower levels for all other analytes. *MyD88* genotype, however, had only a modest effect on milk cytokine/chemokine concentrations. There were no effects of pup *MyD88* genotype and no pup- by maternal genotype effects for any cytokine/chemokine, as might be expected if stomach content reflects milk produced by the

mother. Maternal *MyD88* genotype influenced CCL5/RANTES (p= 0.003; *MyD88*- > *MyD88*+) and marginally influenced MIP-1β (p= 0.05; *MyD88*- > *MyD88*+), although neither of these was present in high amounts and, unexpectedly, levels in both cases were higher in dams lacking *MyD88*. Thus, immediate post-partum milk contains high levels of several cytokines/chemokines, but these do not appear to require maternal or pup MyD88 signaling and are unlikely to explain the higher levels of inflammatory markers that were associated with a functional copy of *MyD88* in pups and/or dams in the periphery and brain.

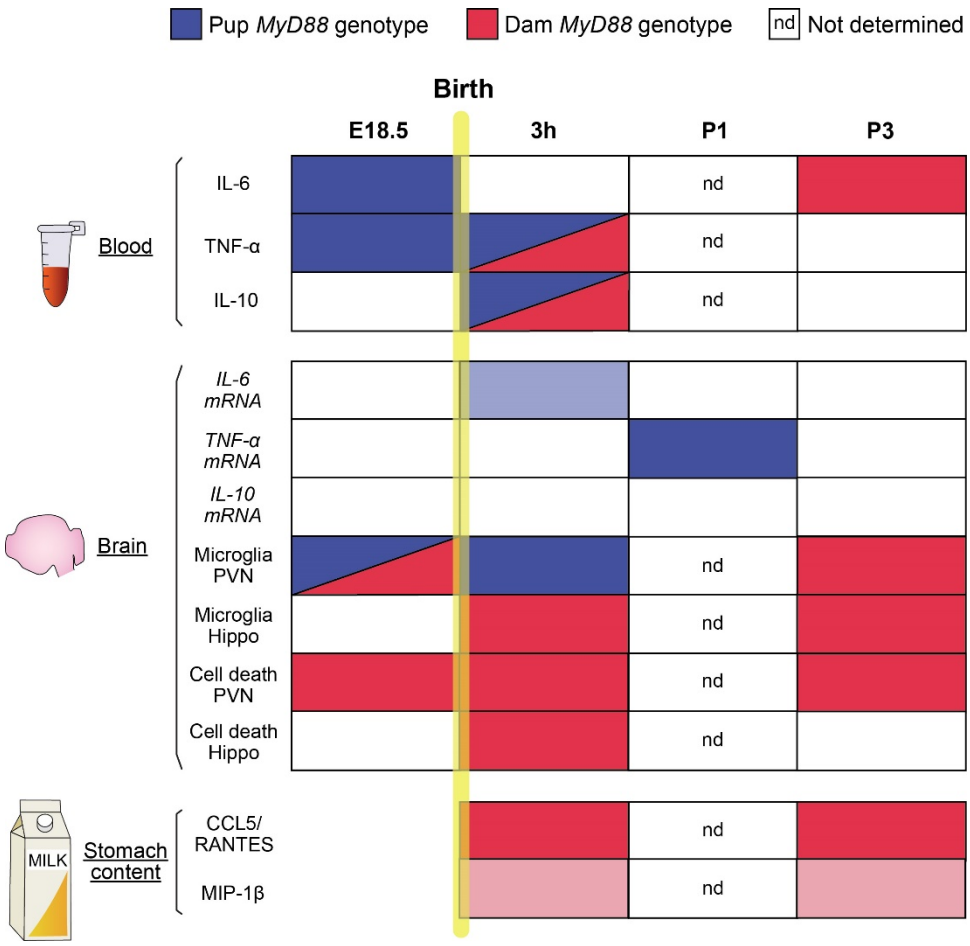

Fig. 8. Summary of findings. Schematic summarizing the interplay of MyD88-dependent signaling in the dam (maternal *MyD88* genotype) and/or offspring (pup *MyD88* genotype) on the dependent measures evaluated in this study. Dark blue or dark red filled boxes indicate significant, substantial effects of pup or maternal genotype, respectively; light-filled boxes indicate minor effects; white boxes indicate no effect. 'nd' signifies measures that were not assessed.

4. Discussion

4.1 Overview

Markers of inflammation are elevated in human babies and mouse fetuses/pups around the time of birth. Although inflammation often has a negative connotation, it may also be adaptive. Indeed, inflammation in the uterine compartment may be required to trigger parturition (Norman et al., 2007) and immune activation in the offspring may prepare them to efficiently clear microbes or microbioal products that cross mucosal barriers during birth or initial colonization (Levy, 2007). MyD88 is required for most TLR signaling and also regulates the IL-1 receptor signaling pathway (Deguine and Barton, 2014). The findings presented here in mice lacking *MyD88* therefore support three main conclusions: 1) many of the inflammatory markers elevated at birth require MyD88-dependent signaling (and, thus, presumably TLR/IL1R-dependent signaling); 2) MyD88-dependent signaling in both the mother and offspring contribute to inflammation in the fetus/newborn; 3) normally-occurring inflammation in the fetus/newborn influences functionally significant processes, such as developmental neuronal cell death. A summary of all findings from this study is depicted in Figure 8.

Although most of the measures we examined were altered by *MyD88* gene status of the mother and/or pup, there were a few exceptions (e.g., the increases in brain *IL-6* and *TNF*-α expression between E18.5 and 3h after birth, which occurred regardless of pup or dam *MyD88* genotype). TLR-independent inflammatory pathways leading to cytokine release may play a role in these cases (e.g., Kawai & Akira, 2006; Nociari et al., 2007). In addition, although 10 of the 12 functional TLRs in mice signal exclusively through MyD88, TLR-3 signals via an alternative pathway involving TIR-domain-containing adapter protein inducing interferon-β (TRIF), and TLR-4 can signal via either MyD88 or TRIF (Akira & Takeda, 2004; Deguine & Barton, 2018).

Thus, for dependent measures where we did not identify an effect of *MyD88* gene status, we cannot rule out a role for TLR signaling.

The activation of TLRs is most commonly associated with exogenous antigens, such as pathogens or microbe-associated molecules (Kawai & Akira, 2006). Newborns encounter a plethora of microbes as they exit the womb, and this may play a role in triggering immune markers. For example, we previously showed that expression of pro-inflammatory cytokines in the hindbrains of newborn germ-free mice (gestated, born, and maintained in sterile conditions) is dampened compared to expression in mice with a normal microbiota (Castillo-Ruiz et al., 2018). However, TLRs can also be activated by endogenous signals associated with stress or cellular damage, which are often referred to as damage-associated molecular patterns (DAMPs) (Matzinger, 2002; Bianchi, 2007; Hou et al., 2008). Because some markers of inflammation are already elevated in the fetus just prior to delivery (e.g., on E18.5, before microbial colonization), endogenous signals likely also play a role. Indeed, birth triggers a stress response and is accompanied by hypoxia in the offspring (Van Woudenberg et al., 2012; Evers and Wellman, 2016; Castillo-Ruiz et al., 2022), so cellular stress with resultant release of DAMPS is likely to accompany parturition.

The DAMPs known to signal through TLRs are quite variable and include, for example, heat-shock proteins, high mobility group box 1, and saturated fatty acids acting through cell surface TLRs including TLR2 and TLR4 (Chen and Nunez, 2010; Huang et al., 2012), as well as microRNAs signaling through endosomal TLRs such as TLR7 and TLR8 (Chen et al., 2013). In the brain, the microRNA let-7b can be released from dying neurons and activate TLR7 in neighboring cells (Lehmann et al., 2012). Whether any of the known DAMPs are released at birth and which TLR(s) they activate is currently unknown, but is a rich area for future study.

Overall, we did not find evidence of sex differences in this study, with the caveat that sample sizes were too small for meaningful comparisons in many cases. However, we also did

not find differences between males and females in our previous study on the inflammatory response to birth in wild-type mice (Castillo-Ruiz et al., 2022). Thus, while we cannot rule out sex differences in the inflammatory response to birth, or the role of MyD88 in this process, if sex differences exist, they are presumably subtle.

4.2 Peripheral immune responses

Umbilical cord blood levels of IL-6 are elevated in normal, uninfected human babies during labor (Jokic et al., 2000). However, because IL-6 can cross the placenta (Dahlgren et al., 2006), the source of the cytokine is unknown. We recently showed that fetal mice experience a marked increase in plasma IL-6 between E16.5 and E18.5 (Castillo-Ruiz et al., 2022). Levels at E18.5 in the pup are 7-fold higher than circulating levels in their dams, suggesting that the source of the IL-6 is the fetus itself (Castillo-Ruiz et al., 2022). The current study supports this inference by demonstrating that *MyD88+* fetuses, but not their *MyD88-* siblings in the same litters, experience high levels of IL-6 at E18.5 independent of dam genotype. We also found that TNF-α plasma levels were elevated in *MyD88+* fetuses compared to *MyD88-* fetuses prenatally (E18.5), and TNF-α levels were highest in MyD88+ pups born to MyD88+ dams at 3h after birth. Thus, peripheral elevations of pro-inflammatory cytokines in the offspring depend on MyD88 in the fetus itself (IL-6), or both the offspring and dam (TNF-α).

Plasma IL-10 is often elevated following an inflammatory event and may act to contain inflammation (Lin et al., 2015). Here, we replicated the previously reported surge in plasma IL-10 at 3h after birth in mice (Castillo-Ruiz et al., 2022) and find that both maternal and offspring inflammatory signaling contribute: *MyD88+* pups born to *MyD88+* dams exhibited the highest IL-10 plasma levels. Previous studies show that impairment of neonatal IL-10 signaling following immune challenge leads to heightened neuroinflammatory states and behavioral abnormalities

later in development, while overexpression of IL-10 may be neuroprotective following immune activation or brain injury (Spera et al., 1998; Meyer et al., 2008; Lin et al., 2015). Thus, the IL-10 surge immediately after birth is a product of MyD88 (and, thus, presumably, TLR) signaling in both the dam and pup and may protect against the array of inflammatory stimuli and consequent immune responses encountered by the offspring at birth.

4.3 Brain cytokine expression

TLRs, as well as MyD88, are expressed in the mouse brain during development and throughout life (Lathia et al., 2008; Kaul et al., 2012), so direct effects of *MyD88* deletion on the brain are possible. In addition, peripheral inflammation is communicated to the brain via several routes (Dantzer, 2018), although the peripheral and central responses to an inflammatory event often differ in terms of the specific cytokines involved and the time-course of the responses (e.g., Qin et al., 2007; Bossu et al., 2012).

An elevation of *TNF-α* expression is arguably the most common central response to inflammation (Nadeau & Rivest, 1999; Qin et al., 2007; Bossu et al., 2012; Shin et al., 2019). We previously demonstrated an increase in *TNF-α* in the PVN and hippocampus one day after birth in mice, and a similar elevation in whole forebrain homogenates, suggesting that the response is widespread (Castillo-Ruiz et al., 2022). The exposure to microbes at birth may be an important contributor to this response, because neonatal mice born into germ-free conditions have a blunted expression of *TNF-α* in hindbrain homogenates compared to conventionally colonized mice (Castillo-Ruiz et al., 2018). Other stimuli associated with birth, such as hypoxia, may also affect neural *TNF-α* gene expression (Szaflarski et al., 1995; Kaur et al., 2013).

Here we found that both forebrain and mid/hindbrain *TNF-α* expression on P1 depends primarily on *MyD88* genotype of the pup, with greater levels in *MyD88*+ than in *MyD88*- pups.

Although other cell types contribute, microglia are the main producers of cytokines in the developing brain (Schwarz et al., 2013; Williamson et al., 2011), and may be the source of the *TNF-α* reported here. Microglia also express a wide array of TLRs (Olson & Miller, 2004), so effects of *MyD88* gene deletion on brain cytokine expression could be via microglia. It would be interesting to test that in the future by, for example, using cell-type specific knockouts of *MyD88* in microglia (as in Rivera et al., 2019) or other cell types.

In addition to their inflammatory roles, brain cytokines also function in normal development. For example, neural development is altered in mice with whole-body deletions of the genes for *IL-6*, *IL-10* or *TNF-α* (Barker et al., 2001; Golan et al., 2004; Pratt et al., 2013). In particular, TNF-α plays a role in neuronal differentiation, cell death, synaptogenesis, and synaptic scaling (Sedel et al., 2004; Stellwagon & Malenka, 2006; Bernardino et al., 2008; Deverman and Patterson, 2009; Bilbo and Schwarz, 2012). Thus, the elevated *TNF-α* seen postnatally may play play a role in these functions. One limitation of our approach is that we measured cytokine mRNA expression in the brain. Although we made this choice to avoid contamination from any peripheral cytokines that crossed the blood-brain barrier, it should be kept in mind that mRNA may not fully parallel the cytokine protein levels present in neural tissue.

4.4 Microglial density

Microglia proliferate perinatally and microglial number increases rapidly after parturition in both rats and mice (Dalmau et al., 2003; Sharaf et al., 2013; Castillo-Ruiz et al., 2022). We found that *MyD88* gene status impacts this measure in the PVN and hippocampus. Results were mixed immediately after birth (3h), but by P3 microglial density was elevated in both brain regions of the offspring of *MyD88+* dams, regardless of pup geneotype. An appreciation of the

roles that microglia play in neural development has increased rapidly in recent years. As the macrophages of the brain, it has long been known that microglia phagocytose dead and dying cells during naturally occurring neuronal cell death (Perry et al., 1985; Ashwell, 1990). However, it is now clear that microglia also regulate the number of neural progenitor cells, actively contribute to neuronal cell death, and prune synapses, among other developmental roles (see Bilbo & Schwarz, 2012; Michell-Robinson et al., 2015, for reviews). Thus, by influencing microglial cell number in the offspring, MyD88 signaling in the dam could affect each of these functions.

4.5 Neuronal cell death

Cell death patterns in multiple regions throughout the newborn brain respond to the stimulus of birth (Castillo-Ruiz et al., 2020). Although naturally-occuring neuronal cell death is commonly viewed as an autonomous cell-suicide program, we found that maternal *MyD88* gene status significantly impacted cell death density in both the PVN and hippocampus of her pups. In each case where a difference was found, cell death was greater in the pups of *MyD88*+ dams. The importance of neuronal cell death for sculpting developing neural circuits has been known for decades, but as far as we are aware this is the first suggestion that the normal inflammatory signaling occurring in the mother around the time of birth modulates this process in her offspring. Because changes in perinatal cell death lead to lasting changes in neuronal number (e.g., Ramlall et al., 2021), the effects found here are likely to have functional consequences.

Interestingly, Schroeder and colleagues (2021) recently reported greater neuronal cell density in the neocortex and hippocampus of *MyD88-/-* mice compared to wild-type controls at P4 and/or in adulthood. Although not explicitly stated, it appears that all *MyD88-/-* pups in that

study were born to *MyD88-/-* dams, with all controls born to wild-type dams. We observed reduced cell death in the offspring of *MyD88-* dams, independent of offspring genotype, suggesting that the effects observed by Schroeder et al. (2021) may have been mediated by maternal *MyD88* genotype, and supporting the prediction that the suppression of developmental cell death we observed in the offspring of *MyD88-* dams would have enduring consequences for neuronal cell number. The stress response is altered in adult *MyD88* null mice (Hosoi et al., 2021), and behavioral abnormalities, including hypo-locomotion, deficits in hippocampal-dependent learning and memory, and altered depression-like behavior are also reported (Drouin-Ouellet et al., 2012; Schroeder et al., 2021; Hosoi et al., 2021). Thus, altered neural development due to the suppression of MyD88-mediated inflammatory signaling may have life-long neuroanatomical, physiological, and behavioral consequences.

It is not known how maternal *MyD88* genotype influences microglia and cell death in her offspring, but several routes are possible. Cytokines circulating in the mother can reach the fetus (e.g., Dahlgren et al., 2006), and we observed significant effects of maternal *MyD88* genotype on plasma TNF-α, IL-6, and and IL-10 in the pups immediately after birth (in all cases, elevated in pups born to *MyD88+* dams). It is possible that these cytokines are, in part, maternally-derived and influence microglia and cell death in the offspring. Other inflammatory signaling molecules (cytokines, chemokines or interferons) not measured here could also be passed from mother to pup, as could immune components in milk that we did not measure. In addition, although TLR/MyD88 signaling is usually associated with innate immunity, it can also regulate cells of the adaptive immune system (in particular, T-cells; Duan et al., 2022). Some T-cells pass from mother to fetus during gestation and influence immune responses in the neonate (Stelzer et al., 2021). It will be of interest to explore each of these avenues in the future to understand how maternal TLR/MyD88 signaling affects offspring brain development.

4.6 Cytokines in millk

It was somewhat surprising that maternal *MyD88* gene status affected microglial density and neuronal cell death in her offspring postnatally. It is possible that maternal inflammatory signaling programs events in the offspring *in utero* that emerge after birth. However, newborns may also be affected by maternal immune status via breast milk. We measured 11 analytes in the stomach contents of newborns to estimate cytokine/chemokine content of milk. Although stomach contents do not necessarily reflect what is present in milk, they may arguably be more physiologically relevant from the point of view of the pup, as they reflect what survives the initial steps of digestion and therefore has the potential to signal in the newborn's gastrointestinal tract.

We found higher levels of almost all cytokines/chemokines 3h after birth compared to P3, consistent with what has been reported for human colostrum versus mature milk (Michie et al., 1998; Maheshwari et al., 2003). In addition, we found very high levels of several cytokines, especially CXCL1/GROα in newborn stomach contents. CXCL1 is often regarded as the rodent homolog of human IL-8 (Cacalano et al., 1994), which is present at high levels in human milk (Michie et al., 1998). Interestingly, IL-8 survives gastric digestion, its receptors are expressed in infant gut, and the activation of these receptors increases proliferation and differentiation of intestinal epithelial cells *in vitro* (Maheshwari et al., 2002; 2004; 2005). Thus, IL-8 / CXCL1 may be an especially important cytokine in peri-partum breast milk. Recently, Dawod et al. (2020) reported reduced levels of some milk cytokines in mouse dams with a knockout of *TLR2*, but CXCL1 was not examined in that study. We find that the presence of CXCL1 did not require a functional a *MyD88* gene in the dam or pup, suggesting that it is either constitutively added to milk, or its presence depends on MyD88-indpendent signaling. However, sample sizes were limited, so these findings should be interpreted with caution. In addition, there are many other

bioactive substances in milk (for review see Ballard & Morrow, 2013) not measured in this study, that could be affected by maternal MyD88 and affect pups postnatally.

5. Conclusions

Early life immune activation via bacterial or viral infection of the mother significantly and persistently impacts offspring immune and nervous system function and behavior and may underlie behavioral dysfunction (Dammann & Leviton, 1997; Bilbo & Schwarz, 2012). Our work indicates that immune activation occurring as a normal part of parturition also shapes the brain. This has clinical implications, as it suggests that any practices altering inflammation at birth could affect offspring brain development.

Inflammatory markers previously reported to be elevated in perinatal mice were reproduced here, indicating that the response is robust and replicable. We also find that most of the inflammatory markers examined require MyD88-dependent signaling, and that there is cross-talk between MyD88 signaling in the pup and the dam. Expression of TLRs is ubiquitous throughout the body's immune cells and in most cell types within the brain in both humans and mice (Bsibsi et al., 2002; Hou et al., 2008; Bolane et al., 2012; Kaul et al., 2012; Poulain-Geodofroy et al., 2010; McClure & Massari, 2014). Thus, it will be of interest in future studies to identify what signals and cell types contribute to the MyD88-dependent signaling. We also identified at least one dependent measure – developmental cell death – that is impacted by the normal inflammatory response to birth. There may be many others, which could be explored using the *MyD88* knockout approach employed here, or additional means to alter inflammatory responses at birth.

Acknowledgements

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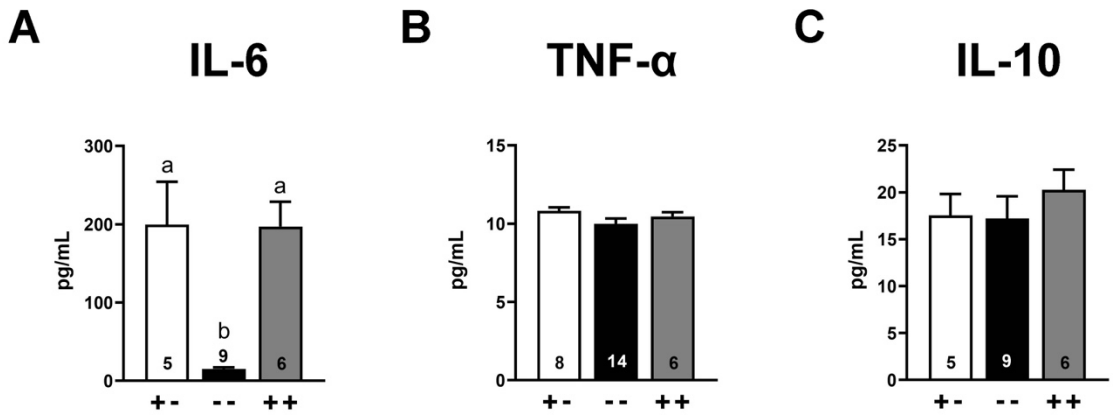
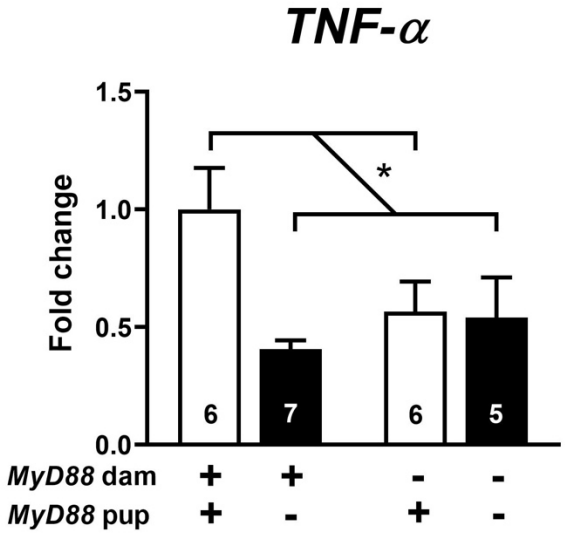
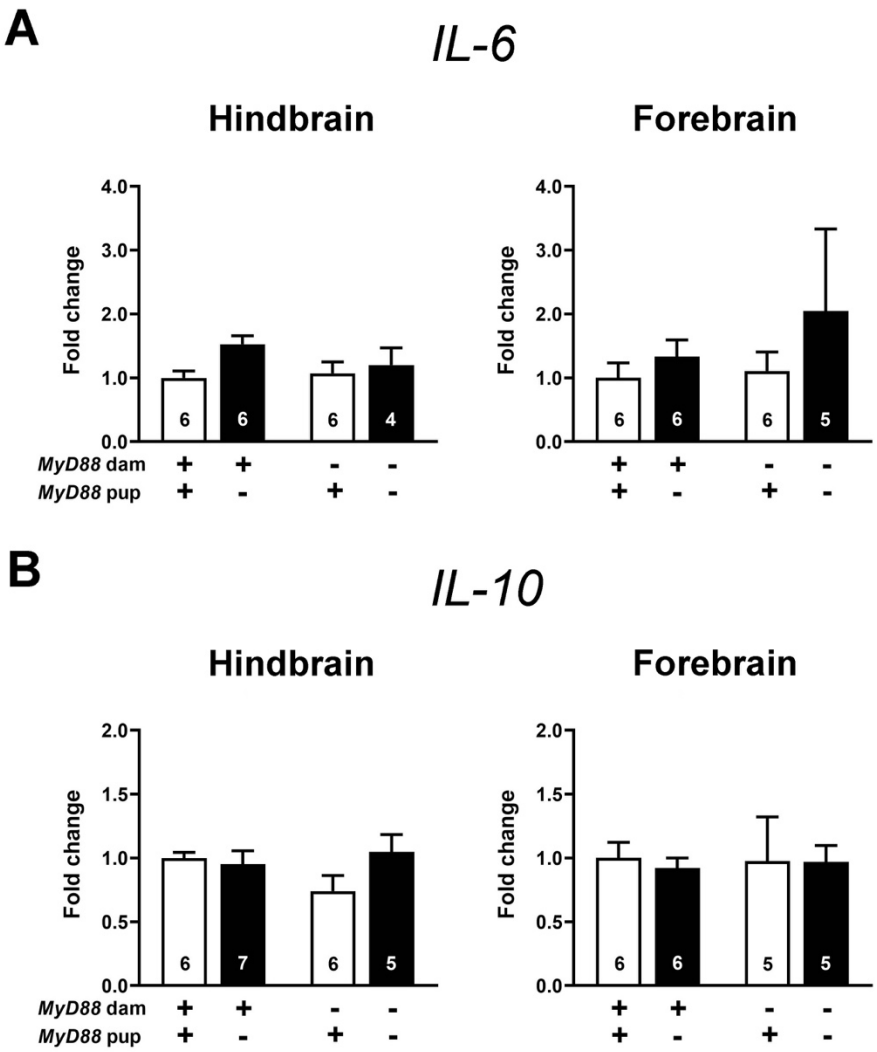


Supplementary Fig. 1. Plasma cytokine levels at E18.5 in *MyD88* wild-type (++) pups did not differ from levels in pups with a single copy of the MyD88 gene (+-) for any of the cytokines measured. In (A) group means with different letters are significantly different from each other. *n* per group is indicated at the base of each bar.



Supplementary Fig. 2. *MyD88* pup genotype influences *TNF-α* expression in the forebrain on P1. There was a main effect of pup genotype, with higher levels in *MyD88*+ pups. Effects of maternal genotype and the interaction of pup and maternal genotype did not reach significance. Data (mean + SEM) are expressed relative to P1 *MyD88*+ pups born to *MyD88*+ dams. Note that this comparison group differs from that in Fig. 4; no forebrain samples at E18.5 were available to allow for a similar analysis. *n* per group is indicated at the base of each bar. *p< 0.05.



Supplementary Fig. 3. The expression of IL-6 and IL-10 in the P1 hind- and forebrain was not affected by maternal or pup *MyD88* genotype. Values are expressed relative to *MyD88*+ pups born to *MyD88*+ dams. *n* per group is indicated at the base of each bar.

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