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Long-term altered immune responses following fetal priming in a non-human primate model of maternal immune activation

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Abstract

Infection during pregnancy can lead to activation of the maternal immune system and has been associated with an increased risk of having an offspring later diagnosed with a neurodevelopmental disorders (NDD) such as autism spectrum disorder (ASD) or Schizophrenia (SZ). Most maternal immune activation (MIA) studies to date have been in rodents and usually involve the use of lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I:C). However, since NDD are based on behavioral changes, a model of MIA in non-human primates could potentially provide data that helps illuminate complex behavioral and immune outputs in human NDD. In this study twenty-one pregnant rhesus macaques were either given three injections over 72 hours of poly I:C-LC, a double stranded RNA analog (viral mimic), or saline as a control. Injections were given near the end of the first trimester or near the end of the second trimester to determine if there were differences in immune output due to the timing of MIA. An additional three non-treated animals were used as controls. The offspring were followed until 4 years of age, with blood collected at the end of their first (year 1) and fourth (year 4) years to assess dynamic cellular immune function. Induced responses from peripheral immune cells were

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Conflicts of interest

All authors declare they have no conflicts of interest.

measured using multiplex assays. At one year of age, MIA exposed offspring displayed elevated production of innate inflammatory cytokines including: interleukin (IL)-1β, IL-6, IL-12p40, and tumor necrosis factor (TNF)α at baseline and following stimulation. At four years of age, the MIA exposed offspring continued to display elevated IL-1β, and there was also a pattern of an increased production of T-cell helper type (T_H)-2 cytokines, IL-4 and IL-13. Throughout this time period, the offspring of MIA treated dams exhibited altered behavioral phenotypes including increased stereotyped behaviors. During the first two years, stereotyped behaviors were associated with innate cytokine production. Self-directed behaviors were associated with T_H2 cytokine production at year 4. Data from this study suggests long-term behavioral and immune activation was present in offspring following MIA. This novel non-human primate model of MIA may provide a relevant clinically translational to help further elucidate the role between immune dysfunction and complex behavioral outputs following MIA.

Keywords

Fetal priming; maternal immune activation; autism; schizophrenia; behavior; cytokines; non-human primate

1.0 Introduction

The recent Zika virus outbreaks have fostered public concerns about the impact that infections during pregnancy can have on fetal development (Rasmussen et al. 2016). Research over the last several decades has found associations between prenatal infections with an increased risk for altered neurodevelopmental trajectories. For example, epidemiological studies focusing on maternal infections during gestation showed associations of increased risk for developing a neurodevelopmental disorders (NDD), such as autism spectrum disorders (ASD) in the child (Atladottir et al. 2010, Brown 2012) or schizophrenia (Brown 2012) later in life. Furthermore, these studies suggest that this phenomenon is not specific to any particular infectious agent, but instead, is driven by the maternal immune response (Atladottir et al. 2010). Translational animal models investigating maternal immune activation (MIA) have found that in the absence of an actual infectious agent, immune stimulation alone, either by bacterial or viral products, or specific cytokines trigger an active immune response in the pregnant dam that elicits abnormal behavior, including anxiety, impaired social and repetitive behaviors in the offspring (Shi et al. 2005, Smith et al. 2007).

Most MIA studies to date have been in rodents and involve the use of lipopolysaccharide (LPS) or polyinosinic:polycytidylic acid (poly I:C); although several models have also generated MIA with live influenza virus or by injection of inflammatory cytokine, interleukin (IL)-6 to produce similar outcomes (Shi et al. 2005, Smith et al. 2007, Meyer et al. 2009, Patterson 2009, Meyer and Feldon 2010). The major findings of these studies were changes in offspring behavior, altered brain development and immune dysfunction in the offspring (Meyer et al. 2006b, Patterson 2009, Shi et al. 2009, Ito et al. 2010, Garbett et al. 2012, Hsiao et al. 2012, Malkova et al. 2012, Garay et al. 2013, Meyer 2014). Behavioral changes have varied between studies but have included such phenomena as reduced or

altered ultrasonic vocalizations, reduced sociability, increased repetitive behaviors loss of latent inhibition, reduced open field exploration, deficits in reversal learning and impairments in pre-pulse inhibition (Meyer et al. 2006a, Meyer et al. 2006b, Meyer et al. 2008, Han et al. 2011, Hsiao and Patterson 2011, Malkova et al. 2012, Schwartzer et al. 2013). In human studies, immune abnormities are often observed in individuals with NDD (Ashwood et al. 2008, Potvin et al. 2008, Muller and Schwarz 2010, Ashwood et al. 2011a, Ashwood et al. 2011c, Ashwood et al. 2011b, Onore et al. 2012, Di Nicola et al. 2013, McAllister 2014, Rose and Ashwood 2014, Careaga et al. 2015). Alterations in immune function have also been observed in rodent offspring of MIA treated dams, including increased production of IL-6 and IL-17 from mononuclear cells, increased granulocyte and monocyte populations, increased production of IL-12p40 and Chemokine (C-C motif) ligand (CCL)-3 from macrophages, an increased T-helper (T_H)-17 cell skewing, and altered profiles of fetal, juvenile, and adult brain cytokine and chemokine levels (Meyer et al. 2006b, Meyer et al. 2008, Mandal et al. 2011, Hsiao et al. 2012, Garay et al. 2013, Mandal et al. 2013, Onore et al. 2014, Choi et al. 2016). While rodent models of MIA provide researchers with a useful initial model to begin to investigate the intricate pathways and interactions between the developing nervous and immune systems, the disparity between human and rodent social structures limits some of the translational aspects of this model in regard to certain complex human behaviors. Moreover, since NDD are, so far at least, based solely on behavioral criteria, non-human primates may be better suited to explore the complexities of behavioral driven disorders due to their closer relationship to humans (Watson and Platt 2012, Chang et al. 2013, Meyer 2014).

To date few studies have explored MIA in non-human primates (Short et al. 2010, Willette et al. 2011, Bauman et al. 2014, Machado et al. 2015, Weir et al. 2015). In one study, maternal influenza infection during early third trimester, led to offspring with smaller brain volume and reduced gray matter, particularly in the cingulate and parietal areas (Short et al. 2010). Prenatal LPS exposure during early third trimester led to increases in global white matter in the brains of the offspring and a trend for larger brain volume, which was accompanied by altered behaviors including reduced response to prepulse inhibition acoustic startle (Willette et al. 2011). However, we do not know of any study that has looked at immune responses in the MIA model in non-human primates.

In this study we sought to examine the effects of MIA on offspring immune activation in a non-human primate model. Previously we reported finding increased repetitive behaviors, motor stereotypies, decreased affiliative vocalizations, and abnormal social behaviors in offspring of non-human primate dams injected with poly I:C to induce MIA (Bauman et al. 2014); offspring also had abnormal gaze patterns when presented with various rhesus monkey facial expressions (Machado et al. 2015). In addition, we also reported findings of altered dendritic morphology of increased number of oblique dendrites and narrower apical dendritic diameter, in MIA offspring compared to saline controls. (Weir et al. 2015). In the present study, we examined plasma cytokine concentrations and dynamic induced cellular responses of peripheral immune cells from offspring of MIA treated dams during the first (year 1) and fourth (year 4) year of life and found elevated production of innate cytokines and chemokines at year 1, and a pattern of elevated T_H2 cytokines during year 4. Moreover, many of the measured cytokines correlated with the emergence of repetitive behaviors in

MIA exposed offspring and may provide insight into observations of increased immune activation and increased impairment in symptoms of NDD (Ashwood et al. 2011a, Ashwood et al. 2011c).

3.0 Methods and Materials

The experimental methods used were developed in consultation with the California National Primate Research Center veterinary staff. The University of California, Davis Institutional Animal Care and Use Committee approved all protocols used. All attempts were made (in terms of social housing, enriched diet, use of positive reinforcement strategies, and minimizing the duration of daily training/testing sessions) to promote the psychological well-being of the animals that participated in this research. Detailed methods on the administration of polyinosinic:polycytidylic acid stabilized with poly-L-lysine (poly I:C-LC), rearing conditions and behavioral observations have been previously published (Bauman et al. 2014) and are described below:

2.1 Maternal Administration of Poly ICLC

A total of 24 rhesus monkeys with timed pregnancies were placed into two main treatment groups, namely; controls or MIA and each dam gave birth to a single infant (supplemental Table 1). Maternal injection of poly I:C-LC or saline took place at 8 am on gestational days 43, 44, and 46; a total of 10 dams received injections of either poly I:C (n=6) or saline (n=4), in the first trimester group. Second trimester dams were injected at 8 am on gestational days 100, 101 and 103 where 11 dams received injections of poly I:C (n=7) and saline (n=4). For animals that were in the MIA cohort, three injections of 0.25 mg/kg of poly IC-LC (Oncovir, Inc., Washington, DC) were administered. Three untreated (no administration of saline or poly I:C) animals were also included to determine whether there was an effect of saline on behavioral and immune outcomes.

2.2 Rearing conditions

Mother and infant were housed in individual cages with continual visual access to other animals. Enrichment and species-typical social development was facilitated by placing four mother-infant pairs and an adult male in large chain link enclosures for three hours a day. Each of these socialization groups consisted of both control and treatment males and females. Infants were weaned at 6 months of age, but continued daily peer group interactions through approximately 2 years of age. At the time of the current study, all animals were housed indoors in social pairs 24 hours per day, 7 days per week.

2.3 Behavioral Observations

Offspring participated in a series of behavioral and cognitive experiments from birth through four years of age, as described in our previous publications (Bauman et al. 2014, Machado et al. 2015). Here we focus specifically on the emergence of stereotypic behaviors observed in captive macaques, including whole body motor stereotypies (i.e., pacing, bouncing, swaying, circling, and somersaulting) and self-directed or self-injurious behaviors (i.e., saluting, self-biting, self-hitting, and head-banging). Stereotypic behaviors were quantified at three timepoints (i) Post-weaning (10 months of age), (ii) Juvenile (22 months of age) and (iii)

Subadult (46 months of age). Animals were placed in a large, unfamiliar cage alone and observed for two 5 minute focal samples on 2 separate days. Observations were collected and analyzed by trained observers, blind to the experimental conditions of the animals. At 10 and 22 months of age, the MIA animals displayed an array of abnormal, repetitive behaviors (i.e., whole body, self-directed etc.), thus we initially utilized a composite score of all repetitive behaviors, rather than sub-categories for these analyses (Bauman et al. 2014). As the animals aged, the presence of district categories of repetitive behaviors became apparent. Thus, the four year old data is presented as whole body (i.e., repetitive pacing, bouncing, flipping, spinning, swinging) versus self-directed (i.e, self-clasp, salute, self-bite and other abnormal self-directed behavioral patterns) behaviors as defined previously by our laboratory (Bauman et al. 2008) and others (Pomerantz et al. 2012, Spinelli et al. 2012).

2.4 Blood Collection and Peripheral Blood Mononuclear Cell (PBMC) stimulation

Peripheral blood was collected via an intravenous arm draw at 1 year of age and again at 4 years of age (see Supplemental Table 2 for a timeline of Behavioral assays and blood draws). 4 mLs of peripheral blood was collected into acid-citrate-dextrose Vacutainers. Blood was centrifuged at 2100 rpm for 10 mins and plasma was collected and aliquoted into 2 mL cyrovials and stored in −80 °C until cytokine analysis was performed. The remaining blood cells were layered on lymphocyte separation medium (Corning; Manassas, VA) and PBMC were separated by gradient centrifugation. PBMC were washed twice with 50 mLs each with Hanks Balanced Salt Solution (Corning; Manassas, VA). Cells were counted, excluding dead cells based on trypan blue staining, and PBMC concentrations were adjusted to a concentration of 1×10^6 cells/mL in complete media (RPMI 1640 (Invitrogen; Carlsbad, CA) with 10% Fetal Bovine Serum (Corning; Manassas, VA), 100 IU/ml penicillin (Invitrogen; Carlsbad, CA) and 100 IU/ml streptomycin (Invitrogen; Carlsbad, CA), 1% L-glutamine (Invitrogen; Carlsbad, CA)). Cells were stimulated with TLR4 agonist (LPS; 50 mg/mL) (Sigma-Aldrich; St. Louis, MO) or TLR3 agonist (Poly I:C; 100 mg/mL) (Sigma-Aldrich; St. Louis, MO). After 48 hours, supernatants were collected and stored at −80 °C until analysis.

2.5 Cytokine Analysis

Cytokine concentrations in the supernatants of stimulated cells were determined by a multiplexing bead immunoassays assay designed specifically to analyze non-human primate cytokines (Millipore, Billerica, MA). Quantification of Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Interleukin (IL)-1β, IFNγ, IL-2, IL-4, IL-5, IL-6, chemokine (C-X-C motif) ligand (CXCL)-8, IL-10, IL-12p40, IL-13, IL-17, chemokine (C-C motif) ligand (CCL)-2, CCL-3,CCL-4 and tumor necrosis factor(TNF)α was analyzed in the plasma and collected supernatants. Samples were run according to manufacturer's protocol and guidelines. 25 μL of sample was incubated with antibody-immobilized beads, following incubation wells were washed and then samples were incubated with detection antibodies, followed by the addition of streptavidin-phycoerythrin. After streptavidin-phycoerthrin incubation, wells were washed again and beads were resuspended with sheath fluid. Analysis of the bead sets was run on a Bio-Plex 200 system (Bio-Rad Laboratories, Inc.). Unknown sample cytokine concentrations were determined by Bio-Plex Manager software; calculations were based on

a standard curve of known concentration provided in the kit by the manufacturer. Values of sample cytokines are expressed in pg/mL and have the following detection range: IL-4: (4.9 – 20,000 pg/mL); IL-10 (12.2 – 50,000 pg/mL); GM-CSF, G-CSF, IFNγ, IL-1β, IL-2, IL-5, IL-6, CXCL-8, IL-12p40, IL-13, IL-17, CCL-2, CCL-3, CCL-4, TNFα (2.4 – 10,000 pg/mL). Sample concentrations that were below the limit of detection were given a proxy value as half the limit of detection for statistical comparisons.

2.6 Statistical Analysis

Analysis of the data using a Shapiro-Wilk test indicated that distribution of the cytokine data was not normally distributed. Therefore, the Kruskal-Wallis rank sum test was used to compare cytokine levels between groups. Data are expressed as median values (interquartile ranges). A Mann-Whitney non-parametric U-test (with a Holm step down procedure to correct for multiple comparisons) was used in post-hoc analyses to compare cytokine levels between groups and adjusted p values < 0.05 were considered statistically significant. Outliers were removed using Robust regression and outlier removal (ROUT). Correlations were analyzed using Spearman's Rank-Order Correlation, p values <0.05 were considered statistically significant. Initial analysis of data revealed no differences in cytokine measures between first trimester injected MIA offspring and second trimester MIA offspring, or between the first and second trimester saline offspring and untreated controls; therefore, the MIA offspring where combined into one group (MIA) and the saline and untreated animals were combined into one group (control).

4.0 Results

3.1 Year 1

Analysis of plasma cytokine levels for the year 1 offspring revealed elevated TNFα ($p = 0.046$), IL-2 ($p = 0.047$), IFNγ ($p = 0.017$), IL-13 ($p = 0.046$) and G-CSF ($p = 0.006$) in MIA offspring compared to controls (Table 1). We also observed elevated cytokine production by PBMC in the MIA group following cell culture compared to controls (Table 2). Cultured PBMC from offspring of MIA treated dams produced significantly more cytokines associated with the innate immune response at baseline (media alone), including: IL-6 ($p = 0.043$), IL-12p40 ($p = 0.026$) and TNFα ($p = 0.015$) and chemokines: G-CSF ($p = 0.002$), GM-CSF ($p = 0.007$), CCL-3 ($p = 0.035$) and CCL-4 ($p = 0.007$). The cytokines IL-2 ($p = 0.034$), IFNγ ($p = 0.022$) and IL-10 ($p = 0.007$) were also elevated in the MIA offspring compared to controls (Table 2). Furthermore, stimulation of cultured PBMC from MIA offspring with a TLR3 agonist induced a significant increase in cytokine production including IL-1β ($p = 0.001$) (Figure 1A), IL-6 ($p = 0.005$) (Figure 1B), IL-2 ($p = 0.002$), IL-10 ($p = 0.023$), IL-12p40 ($p = 0.023$) (Figure 1C) and TNF-α ($p = 0.001$) (Figure 1D), as well as the chemokines G-CSF ($p = 0.007$), GM-CSF ($p = 0.007$), CXCL-8 ($p = 0.010$), CCL-2 ($p = 0.030$), CCL-3 ($p = 0.006$) and CCL-4 ($p = 0.001$) compared to controls. Following TLR4 stimulation, cultured PBMC from MIA offspring produced significantly more G-CSF ($p = 0.0004$), GM-CSF ($p = 0.0016$), IL-12p40 ($p = 0.0121$) and IL-2 ($p = 0.004$) compared to controls.

3.2 Year 4

The analysis of plasma at year 4 showed that several cytokines were again elevated from MIA offspring compared to controls, although this profile differed from that observed at year 1 (Table 1). Plasma concentrations of CCL2 ($p = 0.005$) and IL-6 ($p = 0.050$) were higher in MIA offspring compared to controls. In addition, there were trends for increased IFNγ ($p = 0.087$), CXCL8 ($p = 0.091$) and IL-10 ($p = 0.094$) in MIA compared to controls that did not reach statistical significance after correction for multiple comparisons. Cytokines were increased in cultured PBMC, at baseline (media alone) in MIA offspring compared to controls. These included increased G-CSF ($p=0.022$) IL-2 ($p = 0.029$) and IL-10 ($p = 0.060$) (which were also increased at year 1), as well as increased CXCL-8 ($p = 0.001$) and CCL-2 ($p = 0.025$). There was also a trend for increased IL-1β ($p=0.085$) when compared to saline controls that did not reach significance (Table 3). Following stimulation with a TLR-3 agonist, there was increased production of G-CSF ($p = 0.003$) and a trend for elevated CCL-2 ($p = 0.083$) in MIA offspring compared to controls. Notably in year 4, following TLR3 stimulation of PBMC isolated from MIA offspring there was increased production of T_H2 associated cytokines, including elevated production of the canonical T_H2 cytokines, IL-4 ($p = 0.006$) (Figure 2A) and IL-13 ($p = 0.041$)Figure 2C), compared to control offspring. In cell cultures stimulated with a TLR-4 agonist, increased production of the T_H2 cytokine IL-4 ($p = 0.022$) (Figure 2B) was also observed in MIA offspring compared to controls. Increased levels of G-CSF ($p = 0.009$), IL-1β ($p = 0.036$) and IL-17 ($p = 0.023$), as well as a trend for elevated CCL-2 ($p = 0.076$) and IFNγ ($p = 0.078$) were also noted in MIA offspring compared to controls after TLR-4 stimualtion.

3.3 Associations between behavioral and immune responses

As previously reported, the MIA treated offspring exhibit stereotyped behaviors more frequently than controls during the first two years of life (Bauman et al. 2014). Here we first carried out an exploratory analysis of immune profiles from blood samples collected at 12 months of age with individual stereotpy data from each animal collected before, at 10 months of age (labeled as postweaned) (Table 4), and after, at 22 months of age (labeled as junenile) (Supplemental Table 2) the first year blood collection/processing. Positive correlalations were found between several innate immune cell cytokines and chemokines and stereotyped behaviors observed at 10 months of age, including TLR-4 induced production of G-CSF (Spearman = 0.642; $p=0.001$) (Figure 3A), CCL3 ($r = 0.438$; $p = 0.032$) (Figure 3B) and CXCL8 ($r = 0.461$; $p = 0.023$). TLR-3 induced innate immune cytokines, including IL-1β ($r = 0.618$; $p = 0.004$) (Figure 3C), IL-6 ($r = 0.448$; $p = 0.032$) and IL-12p40 ($r = 0.524$; $p = 0.015$) (Figure 3D) were found to have a positve association with stereotyped behaviors. Year 1 cytokines were also found to associate with stereotyped behaviors observed at the juvenile (22 months) age. A positive association was observed between the production of G-CSF ($r = 0.490$; $p = 0.015$) and IL-6 ($r = 0.408$; $p = 0.048$) in media cultures and stereotyped behaviors (Supplemental Table 3). Induced IL-12p40 cytokine production following TLR-3 stimulation was also associated with increased stereotypy ($r = 0.425$; $p = 0.038$). Following TLR-4 stimulation, increased G-CSF production was associated with increased juvenile stereotyped behaviors ($r = 0.531$; $p = 0.007$). Behavioral observations at 4 years of age were expanded to include subcategories of self-directed and whole body stereotyped behaviors. We found associations in year 4 blood

analysis, primarily between increased T_H2 cytokines and more impaired behaviors (Table 5). Of note, following TLR-3 stimulation, the PBMC production of G-CSF (r = 0.544; p = 0.009), IL-4 (r = 0.445; p = 0.033), IL-5 (r = 0.645; p=0.016) and IL-12p40 (r=0.499; p=0.015) at year 4, was associated with increased self-directed behaviors. Analysis of whole body stereotypies also revealed an association with increased IL-6 production following TLR3 stimulation (r=−0.415; p=0.049). Following TLR-4 stimulation, PBMC production of IL-4 (r=0.447; p=0.032) (Figure 4A), IL-10 (r = 0.516; p = 0.012) (Figure 4B) and IL-13 (r=0.581; p=0.004) (Figure 4C) was associated with increased self-directed behaviors.

4.0 Discussion

Maternal immune activation studies in rodents have reported findings of aberrant behaviors, changes in brain volume, altered neuron morphologies, increases in immune cell populations and immune mediators that have added support to epidemiological findings that associate immune activation during gestation as a potentially contributing risk factor for NDD in a subset of individuals. However, the complexity of human behaviors makes it difficult to directly translate many rodent behaviors to those observed in humans, necessitating a better translational animal model, one whose behavior and social structure more closely resembles that of humans. We have demonstrated that MIA induced by poly I:C-LC in rhesus monkeys leads to long-term behavioral changes in the offspring, including behaviors relevant to both ASD and SZ (Bauman et al. 2014, Machado et al. 2015), evidence of altered dendritic morphology (Weir et al. 2015), and in the current study, evidence of elevated plasma cytokine concentrations and increased functional immune responses following immune cell activation. These findings add further validity that this novel non-human model of MIA may have translational relevance to complex human NDD. Long-term immune alterations were observed in the MIA offspring. Assessment of cytokines in plasma showed elevated levels in the MIA group for both years 1 and 4 compared to controls. More specifically, MIA offspring displayed elevated chemokines and IFNγ at both time-points. Dynamic immune cellular responses were also altered over time with increased production of innate immune cell cytokines predominating in MIA offspring at year 1, and with a more skewed T_H2 phenotype at year 4. Moreover, several innate immune cell and T_H2 associated cytokines correlated with emergence of stereotyped behaviors measured at several different time-points. The findings of long-term altered immune responses in the MIA group supports previous findings of immune changes in rodent models of MIA, and perhaps more importantly from studies of immune function in humans with NDD.

In a murine model of MIA, we previously showed increased production of IL-12(p40) and CCL3 from stimulated macrophages derived from adult offspring of poly I:C injected dams (Onore et al. 2014). Hsiao et al. reported findings of persistently altered immune responses in adult offspring of dams treated with poly I:C, including increased production of IL-6 and IL-17 from stimulated T cells and increased numbers of leukocyte subsets, most notably granulocytes (Hsiao et al. 2012). In the present non-human primate model of MIA, we report findings of hyper-responsive leukocytes with elevated production of innate cytokines during year 1. While we continued to see some innate driven responses during year 4, MIA offspring displayed a shift towards a T_H2 phenotype with elevated production of IL-4 and IL-13. These shifts in cytokine production in response to various immune cell stimuli

suggest that while innate cytokines may be driving inflammation early on in life for MIA offspring, T_H2 cytokines may play a more important role later on.

Both innate and adaptive cellular responses have been implicated in human studies of ASD and SZ (MÜller et al. 2000, Onore et al. 2012). We, and others, have reported altered antibody profiles, as well as dysregulated monocyte, NK cell and T cell responses in children with ASD (Singh 1996, Okada et al. 2007, Ashwood et al. 2008, Ashwood et al. 2011a, Suzuki et al. 2011, Al-Ayadhi and Mostafa 2012, Ricci et al. 2013, Careaga et al. 2015). Studies investigating plasma cytokine concentration in children with ASD have generally reported findings of elevated pro-inflammatory but reduced anti-inflammatory cytokine levels (for review see (Rose and Ashwood 2014)). Our laboratory has previously found increased cytokines associated with the innate immune response including IL-1β, IL-6, and IL-12 in plasma of children with ASD compared to typically developing (TD) children and children with developmental delays (Ashwood et al. 2011a). A number of studies have replicated these findings, showing elevated IL-1β, IL-6 and/or IL-12 in individuals with ASD (Emanuele et al. 2010, Suzuki et al. 2011, Ricci et al. 2013). In the current study, at year 1, MIA offspring were also found to produce elevated levels of the innate cytokines IL-1β, IL-6, IL-12(p40) and TNFα at baseline (media alone) and after stimulation with a TLR-3 agonist compared to controls. All four of these cytokines play an important role in innate immunity (Trinchieri 1995, Fearon and Locksley 1996, Jones et al. 2005, Arend et al. 2008) and expression of these cytokines are directly downstream of TLR signaling (Medzhitov 2001). Increased production of IL-1β, IL-6 and TNFα after TLR-2 or TLR-4 stimulation is not only frequently reported cytokine in ASD, but has also been connected with worsening behaviors (Enstrom et al., 2010, Jyonouchi et al. 2014). Interestingly, IL-1β production after stimulation was elevated at both year 1 and year 4 time points in MIA offspring compared to controls, suggesting that this inflammatory cytokine may play an important contributing role to altered behaviors and/or pathology.

In addition to the innate immune cell cytokines, T_H2 cytokines have also been associated with worse behavioral and cognitive outcome in individuals with ASD in multiple studies (Enstrom et al. 2010, Ashwood et al. 2011a, Ashwood et al. 2011c, Al-Ayadhi and Mostafa 2013, Jyonouchi et al. 2014). For example, IL-4, was found to be associated with impairments in nonverbal communication and IL-13, another T_H2 associated cytokine, showed a trend associating with social impairment (Ashwood et al. 2011a). In the current study, elevated innate immune cell cytokines were associated with increased stereotyped behaviors, although we did see a trend for year 1 offspring, TLR-4 induced production of IL-13 positively correlate with stereotyped behaviors at the juvenile (22 months) age. In year 4, there were associations with more impaired behavior and T_H2 cytokines, including IL-4, IL-5 and IL-13. These data are intriguing, and the potential role for T_H2 and innate immune cell cytokines play in behaviors associated with NDD need further study.

Immune perturbations in SZ have also been reported, with both innate and T_H2 processes implicated (Muller and Schwarz 2010, Al-Amin et al. 2013). Studies in patients with SZ have reported an increase in innate immune cell populations including monocytes, neutrophils and $\gamma\delta CD^+8$ T cells (Wilke et al. 1996, Müller et al. 1998), elevated levels of serum or plasma IL-6 (Maes et al. 1995, Frommberger et al. 1997, Lin et al. 1998) and

increased IL-4 concentrations in cerebral spinal fluid (CSF) (Mittleman et al. 1997). Furthermore, similar to ASD, cytokine levels in SZ also seem to be associated with worsening behaviors. For example, one study found plasma IL-6 to be elevated during acute phases of illness but found no difference when the patients were in remission (Frommberger et al. 1997). Another study reported an association between negative behaviors and CSF levels of IL-10, but only in non-medicated patients (van Kammen et al. 1997). Behavioral state, medication, age and duration of illness all contribute to the extent and most likely, the type of immune dysfunction experienced in individuals with SZ making it difficult to clearly elucidate the role of the immune system in this complicated neurodevelopmental disorder.

Immune findings in NDD literature have been mixed with regard to cellular phenotypes or T cell skewing. For instance, some, studies emphasizeT_H1 cell phenotypes while others demonstrated a more T_H2 phenotype. What may seem like inconsistencies in the field most likely reflect the heterogeneity of NDDs that consist of potential multiple subtypes. In a recent study, utilizing a large cohort of subjects from the Autism Phenome Project (APP), evidence suggests that there was a significant subgroup of individuals within the ASD population that had elevated production of inflammatory cytokines following immune cell stimulation, in contrast to a group of children with ASD that did not. The children with enhanced immune activation had more severe core ASD features with more impaired behaviors. Further, cluster analysis suggested that the type of cellular immune response also varied within the group of children with enhanced immune cell activation with, one subgroup exhibiting an innate cytokine profile, a second with a T_H1 cell profile of upregulated production of IFNγ and a third subgroup exhibiting a T_H2 profile with elevated IL-13 production (Careaga et al. 2015). Multiple etiologies (known and unknown) may contribute to NDD or a more central regulatory mechanism controlling all immune responses may be occurring but that certain individuals display different inflammatory profiles in the absence of this regulation. Our current findings of increased innate and T cell responses in the non-human primate MIA model are consistent with the findings of distinct subgroups within NDD that exhibit evidence of immune dysfunction.

The relationship between immune dysfunction and behavioral outcome is still unclear. While there is evidence from the literature as well as our own findings that indicate increased inflammation is associated with increased severity of aberrant behavior, the exact role that immune dysfunction plays remains unknown; it could potentially be additive, synergistic, causal or independent of behaviors. Genetic studies evaluating differential gene expression in both ASD and SZ have found differences in immune related genes (Garbett et al. 2008, Muller and Schwarz 2010). Furthermore, several implicated genes contribute to both neural and immune pathways, and could affect both independently of one another, or combined play an additive or synergistic role. There is also evidence that changes to the current immune state can alter behavior. For example one study reported improved behaviors in children with ASD when they had a fever (Curran et al. 2007). Immune intervention therapies in ASD have reported improvements in behaviors (Asadabadi et al. 2013). However, while we know that inflammation can contribute to/cause behavioral change, such as with sickness behaviors and assoicated loss of appetite, lethargy and social withdrawal (Dantzer et al. 2008), we currently do not know the exact role that cytokines and immune dysfunction play in ASD or SZ associated behaviors. It is highly likely that there are many

factors contributing to NDD behaviors, with the role of the immune system varying based on the individual etiology and subtype of NDD.

The findings presented here add further support to the potential of maternal immune activation during gestation having lifelong effects on the offspring, including altered immune function. The non-human primate model of MIA, due to the sophisticated cognitive and social behavior exhibited by non-human primates, may provide us with new insights into human behavioral disorders. One major limitation of this study was the small sample sizes in each group. Due to the limited offspring numbers we combined offspring from both the first and second trimesters in order to generate more statistical power. While we did not see any significant differences between the first and second trimester MIA offspring, larger studies are needed to probe specific effects of MIA at different exposure time points during gestation. Future studies should also focus on the relationship between immune dysfunction and behaviors and the potential mechanisms that govern them, and ultimately, begin to explore potential immune therapies/interventions.

4.1 Conclusions

Activation of the maternal immune system during gestation can lead to persistent immune dysfunction in the offspring. Dynamic cellular responses from PBMC revealed offspring of MIA treated damsdisplayed elevated production of innate immune cell associated cytokines early in life, shifting to a more T_H2 type response as the animals aged. Furthermore, this study supports the findings of increase inflammation associated with abnormal behaviors seen in previous rodent models of MIA. Taken together with our previous findings of increased ASD and SZ relevant behaviors and altered dendritic morphology, evidence of an altered immune response adds further validity for the use of non-human primate models of MIA to better assess neurodevelopmental and behavioral disorders such as ASD and SZ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Maternal immune activation in a non-human primate model leads to juvenile offspring that exhibit elevated innate immune cell responses at 2 years of age
- At 4 years of age, older offspring exhibit a more T_H2 cytokine phenotype with elevated production of IL-4 and IL-13
- Innate cytokines positively correlate with stereotyped behaviors at year 2, while T_H2 associated cytokines positively correlate with self-directed stereotypies at year 4.

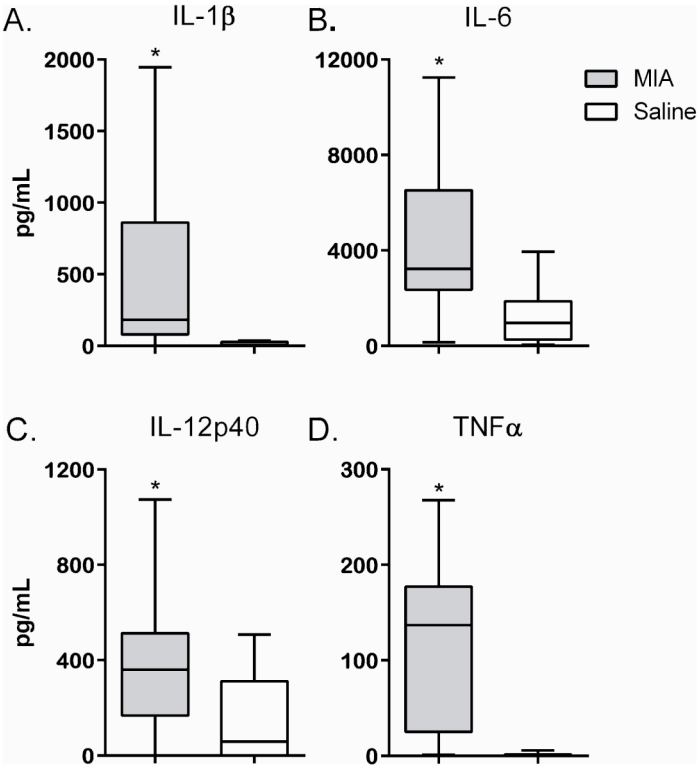

Figure 1. Year 1 Induced cytokine production

Induced cytokine production from year 1 offspring after stimulation with TLR-3 agonist poly IC. Graphs depict innate cytokine levels of IL-1α (a), IL-6 (b), IL-12p40 (c) and TNFα (d) between MIA offspring (grey bars) and control offspring (white bars). Concentrations shown in pg/mL. Data depicted as box showing 25–75 percentiles and whisker percentiles and 5–95percentiles. *denotes p-value < 0.05.

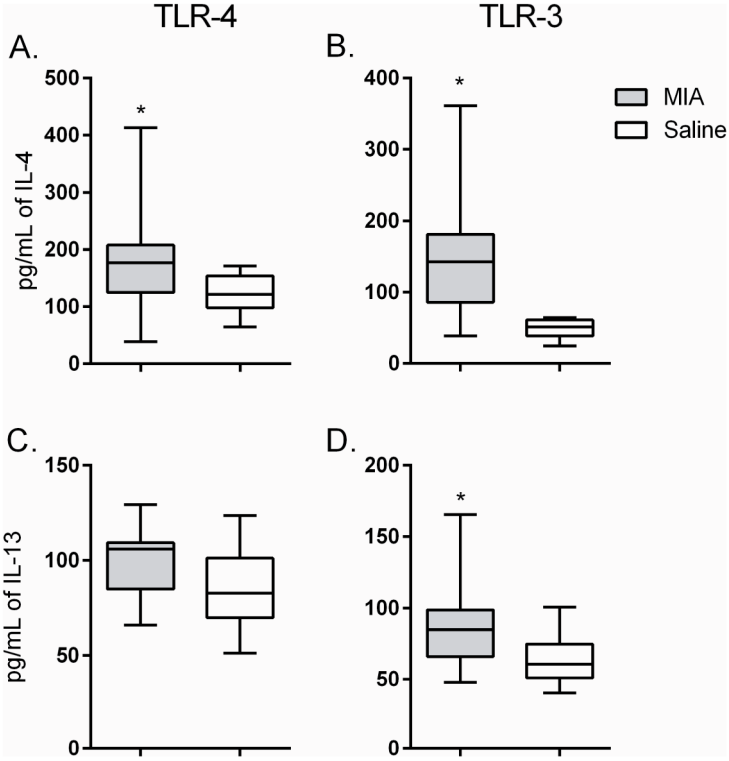

Figure 2. Year 4 induced cytokine production

Induced cytokine production from year 4 offspring after stimulation with either TLR-3 agonist (a. and c.) or TLR-4 agonist (b. and d.). Graphs depict classical T_H2 cytokine levels of IL-4 (a. and b.) and IL-13 (c. and d.) between MIA offspring (grey bars) and control offspring (white bars). Concentrations shown in pg/mL. Data depicted as box showing 25–75 percentiles and whisker percentiles and 5–95 percentiles. *denotes p-value < 0.05.

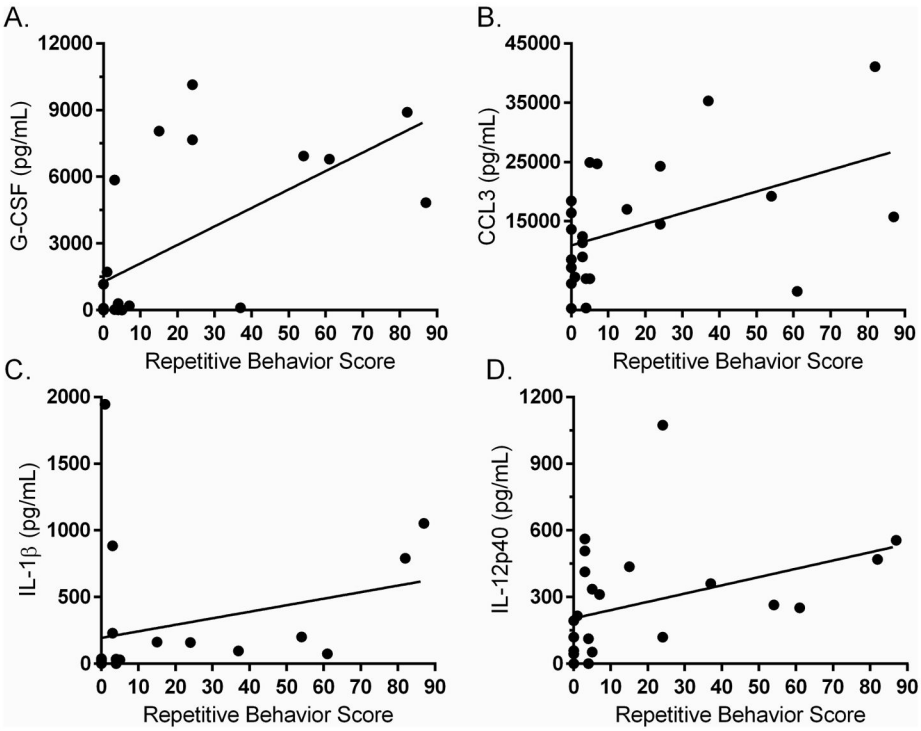

Figure 3. Year 1 cytokine and behavior associations

Year 1 cytokine correlations to repetitive behaviors. Top row (a–c) illustrate relationship between TLR-4 induced chemokine production and post-weaned repetitive behavior scores. Bottom row illustrates relationship between TLR-3 induced innate cytokines and post-weaned repetitive behavior score (d–f). Cytokines concentration is shown as pg/mL.

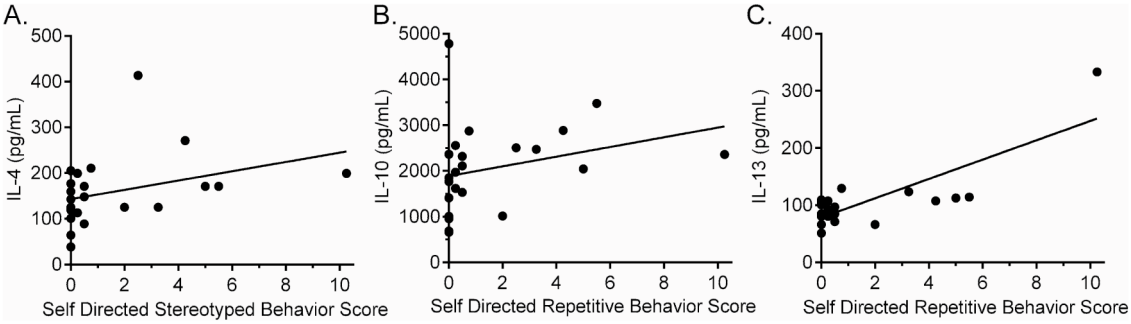

Figure 4. Year 4 cytokine and behavior associations

Year 4 cytokine correlations to self-directed repetitive behaviors. Graphs illustrate relationship between TLR-4 induced cytokine production and Sub-Adult repetitive behavior scores. Cytokines concentration is shown as pg/mL.

Table 1

Year 1 and year 4 plasma cytokine concentrations

Plasma cytokine concentrations (pg/ml) measured at 1 and 4 years of age in offspring of non-human primates exposed to maternal immune activation (MIA) during gestation. IL-6, IL-17, CCL3 and CCL4 were undetectable in plasma for year 2. IL-4 was undetectable in plasma at both time points. Data presented as median with interquartile range; * denotes p-values less than 0.1 and ** denotes p-value less than 0.05.

	Plasma Cytokine Concentrations				
		Year 2		**Year 4**	
		MIA offspring Median (IQR)	**Saline Offspring Median (IQR)**	**MIA offspring Median (IQR)**	**Saline Offspring Median (IQR)**
Innate	IL-1β	0.7 (0.7–0.7)	0.7 (0.7–0.7)	0.87 (0.66–1.71)	0.56 (0.21–1.31)
	IL-6	-	-	1.62 * (0.8–5.1)	0.8 (0.8–1.07)
	IL-12p40	45.37 (36.39–81.25)	70.27 (41.31–75.15)	29.9 (16.02–46.15)	26.75 (0.75–69.99)
	TNFα	1.05 ** (1.05–34.64)	1.05 (1.05–1.05)	62.99 (41.32–83.75)	45.45 (28.88–58.33)
T_{Cell}	IL-2	9.99 ** (4.58–21.23)	2.4 (1.68–6.94)	9.64 (2.2–27.44)	11.33 (2.48–15.72)
T_H1	IFNγ	4.77 ** (1.1–10.21)	1.1 (1.1–2.35)	25.68 * (15.72–32.73)	13.8 (6.43–22.99)
T_H2	IL-4	-	-	-	-
	IL-5	1.22 (0.52–2.54)	1.03 (0.2–1.32)	4.85 (3.3–13.45)	3.225 (2.1–6.77)
	IL-13	1.75 ** (36.39–81.25)	1.75 (41.31–75.15)	26.98 (15.81–35.7)	18.93 (15.61–26.43)
T_H17	IL-17	-	-	1.64 (1.17–3.9)	1.64 (0.73–1.97)
T_{Reg}	IL-10	29.66 (3.1–71.6)	8.99 (3.1–16.31)	40.92 * (31.46–82.87)	31.46 (15.6–49.72)
Growth Factors	G-CSF	3.62 ** (1.05–22.83)	1.05 (1.05–1.05)	130.3 (76.88–234.8)	116.2 (62.41–134.4)
	GM-CSF	0.5 (0.31–1.10)	0.36 (0.31–0.42)	1.62 (1.3–2.07)	1.09 (0.81–1.65)
Chemokines	CXCL8	113.1 (30.89–510)	48.14 (33.16–122)	151.6 * (126.2–279.6)	110.5 (20.86–230.9)

Plasma Cytokine Concentrations				
	Year 2		**Year 4**	
	MIA offspring Median (IQR)	**Saline Offspring Median (IQR)**	**MIA offspring Median (IQR)**	**Saline Offspring Median (IQR)**
CCL2	144.7 (130.2–181.6)	143.6 (97.32–186.1)	179.8 ** (111.1–208.4)	79.24 (35.44–120.5)
CCL3	-	-	28.01 (20.2–53.08)	33.16 (23.35–45.36)
CCL4	-	-	5.74 (0.8–17.52)	0.85 (0.8–6.83)

* p <0.1;

** p < 0.05;

IL=interleukin; G-CSF=Granulocyte Colony Stimulating Factor; GM-CSF-Granulocyte Macrophage Colony Stimulating Factor; CCL= Chemokine (C-C Motif) Ligand; CXCL= Chemokine (C-X-C Motif) Ligand

Table 2

Year 1 induced cytokine production

Comparison of cytokine production (pg/ml) from peripheral blood mononuclear cell culture after 48 hours of stimulation with either TLR-4 agonist, TLR-3 agonist or in media alone, in offspring at year 1 of non-human primates exposed to maternal immune activation (MIA) during gestation. Data presented as Median with interquartile range; * denotes p-values less than 0.1 and ** denotes p-value less than 0.05.

	Year 2 Induced Cytokines						
		Media		TLR-4		TLR-3	
		MIA offspring Median (IQR)	Saline Offspring Median (IQR)	MIA offspring Median (IQR)	Saline Offspring Median (IQR)	MIA offspring Median (IQR)	Saline Offspring Median (IQR)
Innate	IL-1β	26.63 (1.68–150)	0.7 (0.7–64.51)	2,736 (2,388–8,557)	2,604 (1,035–7,809)	181.9** (80–861.2)	6.16 (0.7–28.1)
	IL-6	1,035** (75.61–10,000)	4.16 (0.8–1,693)	9,488 (6,917–23,596)	9,142 (4,258–10,342)	3,226** (2,357–6,527)	964.6 (265.6–1,866)
	IL-12p40	206.6** (31.82–1,003)	2.89 (0.6–372.6)	630.8** (306.1–763.2)	263.3 (106–429.1)	360.2** (167.4–512.4)	58.31 (0.6–311.4)
	TNFα	22.45** (2.73–96)	1.05 (1.05–12.16)	310.3 (6.68–621.7)	378.6 (1.05–565.5)	136.8** (25.24–177)	1.05 (1.05–1.4)
T_{Cell}	IL-2	3.47** (3.3–4.73)	3.3 (3.3–3.3)	51.86** (3.3–66.89)	3.3 (3.3–3.3)	13.08** (3.3–17.47)	3.3 (3.3–3.3)
T_H1	IFNγ	2.31** (1.1–17.74)	1.1 (1.1–1.1)	7.6 (1.87–73.76)	8.61 (1.1–37.95)	24.24 (2.92–45.64)	4.72 (1.1–23.58)
T_H2	IL-4	4.37** (1.35–18.25)	1.35 (1.35–1.35)	1.35* (1.35–39.62)	1.35 (1.35–1.35)	1.35** (1.35–20.99)	1.35 (1.35–1.35)
	IL-5	1.15 (1.15–1.15)	1.15 (1.15–1.15)	1.15 (1.15–1.15)	1.15 (1.15–1.15)	1.15 (1.15–1.15)	1.15 (1.15–1.15)
	IL-13	1.75 (1.75–1.75)	1.75 (1.75–1.75)	1.75 (1.75–1.75)	1.75 (1.75–1.75)	1.75 (1.61–2.85)	1.75 (1.75–1.75)
T_H17	IL-17	0.85 (0.85–0.85)	0.85 (0.85–0.85)	0.85 (0.85–0.85)	0.85 (0.85–0.85)	0.85 (0.22–0.85)	0.85 (0.85–0.85)
T_{Reg}	IL-10	35.75** (3.1–64.83)	3.1 (3.1–3.1)	3,420 (2,890–5,725)	4,338 (1,139–7,468)	94.75** (3.1–656.3)	3.1 (3.1–13.07)
Growth Factors	G-CSF	5.98** (1.91–9.15)	1.1 (1.1–1.1)	5,854** (637.9–7,863)	1.1 (1.1–135.8)	69.54** (268–717.9)	1.1 (1.1–1.1)
	GM-CSF	1.31** (0.7–1.79)	0.7 (0.7–0.7)	20.68** (3.61–127.2)	0.7 (0.7–0.7)	7.9** (0.7–15.47)	0.7 (0.7–0.7)

	Year 2 Induced Cytokines					
		Media		TLR-4		TLR-3	
		MIA offspring	Saline Offspring	MIA offspring	Saline Offspring	MIA offspring	Saline Offspring
		Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Chemokines	CXCL8	3,024 (747–10,976)	469.3 (125.4–9,223)	46,498 (37,590–59,898)	34,844 (23,255–42,839)	10,243 ** (6,315–14,499)	279 (130.4–5,274)
	CCL2	387.1 (71.98–8,063)	62.24 (0.8–7,108)	25,420 (22,919–27,856)	26,531 (25,332–31,097)	6238 ** (4,551–7,468)	507.9 (157.4–6,689)
	CCL3	9.79 ** (1.85–4,289)	1.85 (1.85–1.85)	14,498 (5,442–21,740)	11,374 (5,323–18,447)	1,532 ** (34.98–4,485)	17.41 (1.85–83.94)
	CCL4	12.83 ** (2.55–121.6)	2.55 (2.55–2.55)	168.5 (87.1–266.6)	35.46 (2.55–262.5)	58.94 ** (26.21–164.3)	2.55 (2.55–18.05)

* p <0.1;

** p < 0.05;

CCL= Chemokine (C-C Motif) Ligand; CXCL= Chemokine (C-X-C Motif) Ligand; G-CSF=Granulocyte Colony Stimulating Factor; GM-CSF- Granulocyte Macrophage Colony Stimulating Factor; IFN= interferon; IL=interleukin; T cell= T lymphocyte; T_H= T helper; TLR= toll like receptor; TNF= tumor necrosis factor; T_{reg}= regulatory T cell

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Table 3

Year 4 induced cytokine production

Comparison of cytokine production (pg/ml) from peripheral blood mononuclear cell culture after 48 hours of stimulation with either TLR-4 agonist, TLR-3 agonist or in media alone, in offspring at year 4 of non-human primates exposed to maternal immune activation (MIA) during gestation. Data presented as Median with interquartile range; * denotes p-values less than 0.1 and ** denotes p-value less than 0.05.

	Year 4 Induced Cytokines						
		Media		TLR-4		TLR-3	
		MIA offspring	Saline Offspring	MIA offspring	Saline Offspring	MIA offspring	Saline Offspring
		Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
Innate	IL-1β	125.7 * (97.99–208.3)	63.85 (24.57–138)	1,916 ** (1,145–2,856)	1,086 (992.4–1,523)	690.6 (314.2–1,382)	541.3 (302.8–874.4)
	IL-6	1,053 (763.8–1,473)	1,112 (479.9–1,345)	5,200 (4,270–6,505)	5,515 (4,693–6,117)	3,467 (2,430–5,447)	3,693 (2,802–6,518)
	IL-12p40	29.53 (22.17–38.98)	22.17 (13.74–32.21)	147.6 (88.51–164.1)	98.18 (80.31–123.3)	101.4 (60.22–144.6)	58.51 (13.19–110.7)
	TNFα	161.4 (83.18–201.7)	108.8 (78.16–128)	1,314 (999.9–2,174)	1,540 (1,188–2,169)	1,255 (697.5–1,913)	1,413 (728.1–1,809)
T_{Cell}	IL-2	7.89 ** (5.74–10.13)	5.12 (3.26–8.57)	50.36 (39.16–59.83)	41.71 (34.32–53.05)	33.56 (21.89–40.88)	24.33 (14.91–35.64)
T_H1	IFNγ	5.31 (2.06–12.79)	2.64 (1.29–4.38)	147.1 * (134.9–183.5)	134.9 (122.6–165.3)	192.5 (128.5–299.8)	159.2 (116.2–302.2)
T_H2	IL-4	22.64 (17.83–38.76)	21.45 (12.23–27.92)	176.9 ** (125–207.8)	122.1 (98.22–154)	142.4 ** (85.95–181.2)	51.61 (38.51–61.18)
	IL-5	1.02 (0.22–1.74)	1.27 (0.36–1.27)	5.08 (1.77–11.57)	6.33 (1.08–6.93)	3.83 (1.08–8.72)	2.455 (1.08–3.83)
	IL-13	21.52 (18.81–29.92)	20.12 (17.26–22.52)	105.9 (84.96–109.3)	82.8 (69.97–101.2)	84.96 ** (65.92–98.74)	60.58 (51.16–74.82)
T_H17	IL-17	1.56 (1.36–2.47)	1.48 (1.25–1.86)	8.17 ** (7.11–9.88)	5.84 (3.96–7.78)	3.86 (3.05–8.36)	3.05 (2.23–4.46)
T_{Reg}	IL-10	184.2 * (116.2–306.4)	95.33 (67.54–172.8)	2,359 (1,514–2,714)	1,871 (994.9–2,356)	785 (383.3–933.4)	591 (437.9–705.1)
Growth Factors	G-CSF	217.2 ** (101.5–490.4)	82.5 (56.16–118.9)	5,492 ** (921.3–7,129)	269.5 (207.1–366.1)	1,198 ** (248.3–1,392)	176.8 (111.7–324.6)
	GM-CSF	5.31 (2.06–12.79)	2.64 (1.293–4.375)	54.69 (32.07–130.8)	44.97 (31.29–73.2)	19.11 (11–50.94)	19.3 (12.15–45.3)
Chemokines	CXCL8	6,634 ** (3,701–10,233)	1,764 (1,143–3,134)	40,834 (18,878–62,749)	25,855 (20,162–36,203)	18,352 (11,489–21,108)	8,696 (7,890–16,700)

	Year 4 Induced Cytokines					
	Media		TLR-4		TLR-3	
	MIA offspring	Saline Offspring	MIA offspring	Saline Offspring	MIA offspring	Saline Offspring
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)
CCL2	1,497 ** (463.7–5,276)	416.7 (164.4–775.5)	19,253 * (6,309–27,919)	7,262 (5,259–9,319)	10,051 * (2,812–23,877)	2,688 (1,920–6,144)
CCL3	232.4 (45.74–593.3)	245.9 (54.64–444.6)	3,257 (2,206–4,588)	3,164 (2,438–3,747)	1,469 (611.2–2,151)	1,390 (1,106–3,111)
CCL4	96.12 (64.6–135.1)	89.26 (61.76–113.5)	337.2 (215.8–432.2)	282 (211.8–483.6)	296.8 (179.6–372.2)	222.5 (133.1–355.6)

* p <0.1;

** p < 0.05;

CCL= Chemokine (C-C Motif) Ligand; CXCL= Chemokine (C-X-C Motif) Ligand; G-CSF=Granulocyte Colony Stimulating Factor; GM-CSF- Granulocyte Macrophage Colony Stimulating Factor; IFN= interferon; IL=interleukin; T cell= T lymphocyte; T_H= T helper; TLR= toll like receptor; TNF= tumor necrosis factor; T_{reg}= regulatory T cell

Table 4
Year 1 cytokine and behavior associations

Associations between cell culture cytokine production after 48 hours of stimulation with TLR-4, TLR-3 agonist or in media and stereotyped behaviors measured around 1 year of age. Data presented as Spearman's rank correlation coefficient and p-value. Only significant correlations are shown.

Postweaned Stereotyped Behaviors			
	Cytokine	**Spearman's r**	**p-value**
Media	IL-1β	0.735	0.001
	IL-6	0.601	0.002
	IL-10	0.570	0.014
	IL-12p40	0.590	0.003
	TNFα	0.584	0.006
	CCL2	0.547	0.006
	CCL4	0.603	0.005
	CXCL8	0.508	0.011
	G-CSF	0.656	0.003
	GM-CSF	0.500	0.035
TLR3	IL-1β	0.618	0.004
	IL-6	0.448	0.032
	IL-10	0.524	0.015
	IL-12p40	0.583	0.003
	CCL2	0.461	0.023
	CCL4	0.534	0.010
	CXCL8	0.524	0.009
	G-CSF	0.452	0.046
TLR4	IL-1β	0.524	0.010
	IL-6	0.539	0.010
	IL-10	0.471	0.031
	IL-12p40	0.465	0.025
	CCL3	0.438	0.032
	CXCL8	0.461	0.023
	G-CSF	0.642	0.001
	GM-CSF	0.441	0.046

CCL= Chemokine (C-C Motif) Ligand; CXCL=Chemokine (C-X-C Motif) Ligand; G-CSF=Granulocyte Colony Stimulating Factor; GM-CSF-Granulocyte Macrophage Colony Stimulating Factor; IL=interleukin; TLR= toll like receptor; TNF= tumor necrosis factor

Table 5
Year 4 cytokine and behavior associations

Associations between cell culture cytokine production after 48 hours of stimulation with TLR-4 agonist, TLR-3 agonist or in media and self-directed stereotyped behaviors measured around 4 years of life. Data presented as Spearman's rank correlation coefficient and p value. Only significant correlations are shown.

Sub-Adult Stereotyped Behaviors			
Self Directed Stereotypies			
Cytokine		**Spearman r**	**p-value**
	IL-4	0.445	0.034
	IL-5	0.645	0.016
	IL-12	0.499	0.015
	IL-17	0.482	0.023
TLR3	G-CSF	0.543	0.009
	IL-4	0.447	0.032
	IL-10	0.516	0.012
TLR4	IL-13	0.581	0.004

G-CSF=Granulocyte Colony Stimulating Factor; IL=interleukin; TLR= toll like receptor; TNF=tumor necrosis factor