Published in final edited form as:

Brain Behav Immun. 2010 January ; 24(1): 64–71. doi:10.1016/j.bbi.2009.08.001.

Differential monocyte responses to TLR ligands in children with autism spectrum disorders

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Abstract

Autism spectrum disorders (ASD) are characterized by impairment in social interactions, communication deficits, and restricted repetitive interests and behaviors. Recent evidence has suggested that impairments of innate immunity may play an important role in ASD. To test this hypothesis, we isolated peripheral blood monocytes from 17 children with ASD and 16 age-matched typically developing (TD) controls and stimulated these cell cultures *in vitro* with distinct toll-like receptors (TLR) ligands: TLR2 (lipoteichoic acid; LTA), TLR3 (poly I:C), TLR4 (lipopolysaccharide; LPS), TLR5 (flagellin) and TLR9 (CpG-B). Supernatants were harvested from the cell cultures and pro-inflammatory cytokine responses for IL-1 β , IL-6, IL-8, TNF α , MCP-1, and GM-CSF were determined by multiplex Luminex analysis. After *in vitro* challenge with TLR ligands, differential cytokine responses were observed in monocyte cultures from children with ASD compared with TD control children. In particular, there was a marked increase in pro-inflammatory IL-1 β , IL-6 and TNF α responses following TLR2, and IL-1 β response following TLR4 stimulation in monocyte cultures from children with ASD ($p < 0.04$). Conversely, following TLR9 stimulation there was a decrease in IL-1 β , IL-6, GM-CSF and TNF α responses in monocyte cell cultures from children with ASD compared with controls ($p < 0.05$). These data indicate that, monocyte cultures from children with ASD are more responsive to signaling via select TLRs. As monocytes are key regulators of the immune response, dysfunction in the response of these cells could result in long-term immune alterations in children with ASD that may lead to the development of adverse neuroimmune interactions and could play a role in the pathophysiology observed in ASD.

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Keywords

monocytes; Toll-Like receptors; autism; ASD; cytokines; inflammation

Introduction

Autism Spectrum disorders (ASD) are complex neurodevelopmental disorders appearing in the first few years of life and are characterized by deficits in communication and social interaction, as well as specific stereotypical behaviors. ASD develops in approximately 1.3 per 1000 children, with a disproportionate (4:1) number of males affected (Fombonne, 2005). At present, the etiology of ASD for the majority of cases is not known and is most likely a combination of genetic and environmental factors. Dysregulated immune function is a recurrent finding in ASD, including evidence of brain reactive antibodies; skewed T cell responses to mitogens; altered cytokine levels in the brain, CSF, and periphery; and altered function of innate immune cells such as monocytes and NK cells (Ashwood et al., 2009; Ashwood and Wakefield, 2006; Cabanlit et al., 2007; Denney et al., 1996; Enstrom et al., 2009b; Garbett et al., 2008; Gupta et al., 1998; Li et al., 2009; Molloy et al., 2006; Sweeten et al., 2003; Vargas et al., 2005; Wills et al., 2007). In addition, several potential genetic mechanisms that impact immune function and responses have been implicated, such as complement C4B null allele (Odell et al., 2005; Samano et al., 2004), MET (Campbell et al., 2007), MIF (Grigorenko et al., 2008) reelin (Persico et al., 2001; Skaar et al., 2005), and certain human leukocyte allele (HLA) haplotypes (Torres et al., 2001; Warren et al., 1996). However, any underlying immune mechanism resulting in dysregulation has not to date been elucidated.

Based on the pivotal role innate immune cells play in initiating, directing and prolonging the immune response, a primary dysfunction of innate immunity may explain the widespread immunological abnormalities reported in ASD. During initial responses to newly encountered pathogens, innate immune responses predominate and direct tissue responses and adaptive immunity. Chief among innate immune cells are monocytes, which differentiate into macrophages in tissues and digest and present antigens to T cells, which in turn can stimulate antibody-producing B cells. Unlike adaptive responses mediated by T and B cells, innate monocyte responses are rapid but are not antigen specific and instead rely on the recognition of highly conserved motifs common to microorganisms termed “PAMPs” (pathogen-associated molecular patterns) that serve as ligands for the toll-like receptors (TLRs) (Fleer and Krediet, 2007). Recognition of PAMPs by specific TLRs on innate immune cells leads to the production and release of pro-inflammatory cytokines including interleukin 1-beta (IL-1 β), IL-6, and tumor necrosis factor alpha (TNF α). These cytokines can affect neurodevelopment in a number of ways, for example IL-1 release during neonatal hypoxia correlates to abnormal neurological outcome (Aly et al., 2006); whereas TNF α is capable of regulating either dopaminergic differentiation or apoptosis of neurons in a developmental period specific manner (Doherty, 2007). In a murine study, maternal IL-6 administration results in behavioral changes and prepulse inhibition and latent inhibition in the adult offspring (Smith et al., 2007). These findings suggest the cytokines produced by innate immune cells, such as microglia or monocytes can affect neuronal function and neurodevelopment and may ultimately impact behavior. Thus, the qualitative and quantitative nature of innate immune responses by an individual could have a major influence not only on the overall immune response but also affect neuronal function and neurodevelopment.

Several lines of evidence suggest ASD may be linked to innate immune cell dysfunction. Two prior investigations have indicated significantly increased monocyte counts in children

with ASD compared with healthy control children (Denney et al., 1996; Sweeten et al., 2003). In addition, increased CSF and brain tissue levels of cytokines associated with innate cells, such as IL-1 β , IL-6, TNF α , TNFR1, and TNFR2, MCP-1 and MIP-1 β have been reported in ASD (Vargas et al., 2005). Furthermore, brain specimens from individuals with ASD exhibit signs of active, ongoing neuroinflammation including marked activation of microglia and astrocytes, up-regulation of HLA-DR and increases in pro-inflammatory cytokines levels (Vargas et al., 2005). Notably, a recent study on gene transcription in post-mortem human brain specimens found that several pathways associated with innate immune function and activation, including pathways for NF- κ B, MAP kinase, MET, caspase, TOLL and cytokine receptors (IL-1R, IL-6, TNFR2) were more prominently affected in autistic brains compared with typically developing controls (Garbett et al., 2008).

As critical regulators of immune responses in the periphery and in the brain, dysfunction in the monocyte cell lineage may play a key role in the generation of immune abnormalities observed in ASD, including potential autoimmune responses (Cabanlit et al., 2007; Connolly et al., 2006; Connolly et al., 1999; Kozlovskaya et al., 2000; Silva et al., 2004; Singh and Rivas, 2004a; Singh and Rivas, 2004b; Todd et al., 1988; Wills et al., 2009). In addition, as the phenotype of stimulated microglia described in brain tissues from individuals with ASD (Vargas et al., 2005) closely resembles activated monocytes/macrophages (Hess et al., 2004), the study of *in vitro* monocyte responses following stimulation may increase our understanding of the mechanisms underlying altered innate immune responses in the brain of individuals with ASD. To improve our understanding of the potential role of the innate immune response in ASD we examined the response of monocyte cultures to environmental relevant pathogens. In the present study we stimulated monocytes with well-characterized ligands for TLR 2, TLR 4, TLR 5 and TLR 9 to determine if there are differential responses to these ligands in ASD. Furthermore, as many potential animal models of ASD are increasingly using stimulation of immune responses with various TLR ligands (Fortier et al., 2007; Gilmore et al., 2005; Meyer et al., 2007; Smith et al., 2007), this work may help to assess which pathways warrant further investigation and to guide future experiments using such models.

Materials and Methods

Subjects

17 children with autism spectrum disorders (ASD, 14 males) and 16 typically developing control children (TD, 13 males) participated in this study. The average age (range) of children with ASD included in the study was 3.9 (2.2-5.0) years and 3.3 (2.3-4.8) years for TD. All children were recruited through the CHARGE (Childhood Autism Risks from Genetics and Environment) project being conducted at the UC Davis M.I.N.D. (Medical Investigation of Neurodevelopmental Disorders) Institute. The CHARGE study patient diagnostic criteria have previously been discussed in detail (Enstrom et al., 2009a; Hertz-Picciotto et al., 2006). In brief, children diagnosed with ASD fulfilled gold-standard diagnostic criteria based on Diagnostic Statistical Manual (DSM)-IV, Autism Diagnostic Observation Schedule (ADOS) and Autism Diagnostic Interview-revised (ADI-R) criterion. The controls were screened for autism traits using the Social Communication Questionnaire (SCQ), which is adapted from the ADI-R (Rutter et al., 2003). Typically developing children were included as controls only if they scored less than 15 on the SCQ. As an additional inclusion requirement, TD control children had to score within 2 standard deviations of the mean on the Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Scales (VABS) cognitive assessments. All assessments were administered by qualified UC Davis M.I.N.D. Institute clinicians. To minimize factors that may affect immune function, children who were ill at the time of the study, had a temperature above 98.9° F, or were prescribed immunomodulatory medication or anti-psychotics were excluded from the study.

None of the children had any other known medical disorder or primary diagnosis (e.g. Fragile X or Rett syndrome). None of the TD controls were related to the ASD cases or had siblings with ASD. Informed consent was obtained from the parents of each participant, and the study was approved by the UC Davis Institutional Review Board.

Monocyte Isolation

Fresh peripheral blood was collected in sodium citrate tubes. Within 1 hour of collection, blood was delivered to the laboratory and peripheral blood mononuclear cells (PBMC) isolated using density gradient centrifugation (Cellgro; Manassas, VA). The blood was centrifuged at 2100 rpm for 10 minutes and plasma removed. The remaining cell containing fraction was then resuspended in Hanks' Buffered Salt Solution (HBSS) (Mediatech; Manassas, VA) before being carefully layered over 15ml of Lymphocyte Separation Medium (Mediatech) and centrifuged at 2100 rpm for 30 minutes. After centrifugation, cells were washed twice in HBSS and resuspended in PBS supplemented with 0.5% BSA (VWR; West Chester, PA), and 2 mM EDTA (Sigma-Aldrich; St. Louis, MO). It has previously been reported that positive selection of monocytes as compared with negative selection of monocytes, results in higher purity of monocytes and limits the possible contamination by other cytokine producing immune cells such as dendritic cells, that are able to respond to TLR stimulation and could affect the interpretation of the results (Mao et al., 2005). As such, we believe that positive selection of monocytes using anti-CD14 antibody provides a better and more accurate assessment of monocyte responses to TLR ligand stimulation. In addition, we previously showed that positive selection of monocytes that were then cultured in media for 24 hours produced negligible levels of cytokines, suggesting there was a minimal stimulatory affect due to isolation process (Mao et al., 2005). Monocytes were separated from the PBMC by incubating with CD14-labeled magnetic beads at 4°C for 15 minutes before passing through a magnetic column (Miltenyi Biotec; Auburn, CA). The purity of the monocytes was assessed by flow cytometry and was >98%. Cell viability was also assessed by trypan blue exclusion and was >98%. Following isolation, cell concentration was adjusted to 2×10^6 cells/ml in RPMI 1640 medium (Invitrogen; Carlsband, CA) containing 10% endotoxin-undetectable, heat-inactivated FBS (Omega Scientific; Tarzana, CA) and supplemented with 100 IU/ml penicillin, 100 IU/ml streptomycin (Sigma).

Monocyte Stimulation

To induce cytokine secretion, 100,000 isolated monocytes were plated in 50 μ l RPMI per well in 96 well plates (VWR) with 50 μ l TLR ligands diluted in complete RPMI medium containing 10% FBS and antibiotics (as above). Final ligand concentrations based on previously demonstrated values were 10 μ g/ml lipoteichoic acid (LTA, *Staphylococcus aureus*, Sigma), 50 μ g/ml poly I:C (InvivoGen; San Diego, CA), 10 μ g/ml lipopolysaccharide (LPS, *Escherichia coli* serotype 0111:B4, Sigma), 1 μ g/ml flagellin (Invivogen), and 2 μ M CpG-B (InvivoGen) (Mao et al., 2005). Cells were incubated for 24 hours at 37°C in a 5% CO₂ controlled humidified incubator. Following incubation, cells were centrifuged at 2100 rpm for 10 minutes and 75 μ l supernatant harvested by careful pipetting, divided into aliquots and stored for a maximum of 2 months at -80°C until analyzed.

Quantification of Cytokine Concentration

50 μ l of stored supernatants from cultured monocytes that were stimulated with or without TLR ligands were used to quantify the production of cytokines using a human Biosource multiplex kit according to manufacturer's protocol (Invitrogen; Carlsband, CA). This approach allowed for the simultaneous measure of the following cytokines/chemokines: TNF α , IL-1 β , IL-6, IL-8, IL-12p70, macrophage chemotactic protein 1 (MCP-1), and

granulocyte macrophage-colony stimulating factor (GM-CSF). However, the concentration of IL-12p70 measured was consistently close to or below the level of detection of the assay irrespective of the stimulant used and, thus, is not included in the results or discussion as too few results were obtained to be meaningful. Briefly, supernatants were incubated for 2 hours at room temperature with antibody-conjugated beads. At the end of the incubation period, the plate was washed 2 times with wash solution supplied by the vendor, incubated for 1 hour with biotinylated detector antibody, and washed twice. The plate was then incubated for 30 minutes with streptavidin-RPE and then washed 3 times prior to analysis on a Luminex 100TM (BioRad; Hercules, CA). Concentration was determined with a 5 parametric curve fitting algorithm using MasterPlex QT Quantitation Software (MiraiBio; Alameda, CA). Concentrations were exported into GraphPad Prism 5 software for further statistical analysis.

Flow Cytometry

Briefly, after supernatants were removed, monocytes were resuspended in staining buffer (0.5% bovine serum albumin, 0.05% sodium azide in PBS). The cells were then stained for fluorescein isothiocyanate (FITC)-conjugated anti-human TLR2 (eBioscience, San Diego, CA), allophycocyanin (APC)-conjugated anti-human HLA-DR, and APC-Cy7-conjugated anti-human CD14 (BD Biosciences, CA). The cells were then spun down and washed with staining buffer. Cells were then analyzed on a LSR II flow cytometer (BD Immunocytometry Systems). The data acquired were analyzed with FlowJo software (BD Immunocytometry Systems). CD3⁺CD14⁺ cells were gated on for analysis of surface expression.

Statistical analysis

A Shapiro-Wilk test was used to assess the normality of cytokine levels. T-tests were used to compare the induced cytokine production by monocyte cell cultures from children with ASD and TD controls. Analyzes were Bonferroni corrected for multiple comparisons. All analyzes were two-tailed and p values less than 0.05 were considered statistically significant. For all statistical analyses, GraphPad Prism5 was used to calculate results. Evaluation of induced-cytokine levels and clinical variables as assessed by ADI-R, ADOS and the aberrant behavior checklist (ABC) (Hertz-Picciotto et al., 2006) among children with ASD was determined using Spearman or Pearson correlations depending on the distribution of the clinical data. Associations between potential clinical features such as gastrointestinal symptoms were also assessed (see Hertz-Picciotto et al., 2006 for details on medical assessments in the CHARGE protocol). Using clinical characteristics reported in the Early Development Questionnaire and answers to questions regarding loss of language (Q11) and social skills (Q25) of the ADI-R, the autism population was further divided into two groups based on the clinical onset of autistic symptoms; firstly, children with regression who initially developed but subsequently lost previously acquired language and/or social skills (n = 1) and secondly, children with early onset autism characterized by early deficits in the requisite behavioral domains (n = 16). There were too few subjects with regression to determine differences between the onset of autism and induced cytokine release. Correlation analyzes were performed using the Statistical Analysis System, version 9.1 (SAS Institute Inc, Cary, NC).

Results

Cytokine Production after TLR 2 Stimulation

There were no significant differences in the number of CD14⁺ monocytes isolated from children with ASD ($0.33 \pm 0.04 \times 10^6$ cells/ml; mean $\pm$ S.E.M.) compared with TD controls ($0.36 \pm 0.03 \times 10^6$ cells/ml, p = ns). In addition, there were no differences in the frequencies

of putative monocyte subsets CD14⁺CD16⁺ and CD14⁺CD16⁻ cells in children with ASD compared with TD controls (data not shown). To determine the effects of specific TLR2 ligand stimulation, monocyte cell cultures were stimulated with LTA for 24 hours, after which the supernatants were analyzed for cytokine and chemokine production by Luminex multiplex analysis. Monocyte cell cultures from children with ASD were found to produce significantly increased levels of IL-1 β (652.8 ± 197.6 pg/ml; mean $\pm$ S.E.M.) compared with TD controls (178.4 ± 29.4 pg/ml, $p = 0.02$, Figure 1A). IL-6 production was also significantly increased from monocyte cell cultures from children with ASD that had been stimulated with LTA ($244,300 \pm 80,710$ pg/ml) compared with TD controls ($78,270 \pm 26,100$ pg/ml, $p = 0.04$, Figure 1B). Similarly, TNF α production was significantly elevated in LTA-stimulated monocyte cell cultures from children with ASD ($5,795 \pm 1,786$ pg/ml) compared with TD controls ($2,004 \pm 1,826$ pg/ml, $p = 0.04$, Figure 1C). LTA-induced production of IL-8 ($477,578 \pm 390,757$ ASD vs. $85,982 \pm 18,178$ TD pg/ml), and GM-CSF (51.9 ± 8.1 ASD vs. 42.9 ± 5.0 TD pg/ml) followed a trend towards increased cytokine production in ASD but did not reach statistical significance between ASD and TD controls. The concentration of MCP-1 following LTA-stimulation was not different between ASD and TD ($20,815 \pm 5,405$ ASD vs. $39,952 \pm 10,163$ TD pg/ml).

Flow Cytometry Analysis of TLR 2 and HLA-DR Cell Surface Expression

To determine the effects of TLR 2 ligand stimulation on the cell surface expression of its receptor and the activation marker HLA-DR, CD14⁺ monocytes were evaluated for cell surface expression of, TLR 2 and HLA-DR by flow cytometry after a 24-hour culture in the presence of media alone or the TLR 2 ligand LTA as described above. The frequency of cells that showed positive staining for TLR 2 (Figure 2A) were not significantly different in children with ASD compared with TD controls after incubation with media or with the TLR 2 ligand LTA. Mean fluorescent intensity (MFI) of TLR 2 was not significantly different between children with ASD compared with TD controls following stimulation with media or after TLR 2 ligand stimulation (Figure 2B). As anticipated following 24 hour culture, the majority of CD14⁺ monocytes showed positive staining for HLA-DR from children with ASD and TD controls (Figure 3A). There was an increased frequency of monocytes that had positive staining for HLA-DR in children with ASD compared to TD controls after incubation in media alone (Mean $\pm$ SEM, ASD vs. TD, 78.48 ± 3.43 vs. 68.06 ± 4.98 , $p = 0.01$, Figure 3A) and following TLR 2 stimulation (73.5 ± 4.5 vs. 61.19 ± 4.96 , $p = 0.02$, Figure 3A). The MFI of HLA-DR expression did not differ significantly in children with ASD compared with TD controls (Figure 3B).

Cytokine Production after TLR 4 Stimulation

IL-1 β , IL-6 and TNF α concentrations following monocyte cell culture stimulation with the TLR 4 ligand LPS as measured by Luminex are shown in Figure 1 D-F. Monocyte cell cultures from children with ASD responded to LPS stimulation by producing significantly increased concentration of IL-1 β when compared with TD controls ($2,418 \pm 417$ pg/ml vs. $1,312 \pm 267$ pg/ml respectively, $p = 0.04$, Figure 1D). However, there were no significant differences in IL-6 ($210,100 \pm 64,250$ pg/ml vs. $179,600 \pm 49,440$ pg/ml, ASD vs TD, Figure 1E) or TNF α production ($5,249 \pm 1,126$ pg/ml vs. $6,235 \pm 1,079$ pg/ml, Figure 1F) from LPS-stimulated monocyte cell cultures of children with ASD compared with TD controls. Similarly, there were no differences in the production of IL-8 ($113,741 \pm 20,120$ vs. $82,110 \pm 7,679$ pg/ml) or GM-CSF (88.7 ± 12.9 vs. 116.9 ± 18.3 pg/ml) between ASD and TD following LPS stimulation. However, the concentration of the chemokine MCP-1 was significantly decreased in monocyte cell cultures from ASD children compared with TD controls after LPS stimulation ($2,864 \pm 535$ pg/ml vs. $25,570 \pm 7,123$ pg/ml, $p = 0.005$). We then examined whether there were associations between induced cytokine levels following TLR 4 stimulation and clinical variables among ASD participants. The release of IL-1 β

following LPS stimulation was associated with impairments in social interactions ($\rho = 0.56$, $p = 0.039$) and non-verbal communication ($\rho = 0.87$, $p = 0.006$) as measured using the ADI-R. Similarly, the release of IL-6 following LPS stimulation was associated with non-verbal communication ($\rho = 0.84$, $p = 0.009$). An association between IL-6 release following LPS stimulation with social interactions ($\rho = 0.50$, $p = 0.067$) as measured by ADI-R did not reach statistical significance but there was a statistically significant association with social interactions ($\rho = 0.81$, $p = 0.05$) as measured by the ADOS. These data suggest that more impaired social behaviors and nonverbal communication are associated with increased release of IL-1 β and IL-6 after LPS stimulation. No associations were observed between behavioral assessments and with other cytokines or for induced cytokine responses following stimulation with any other TLR ligands investigated. There were no associations between induced cytokine levels and gastrointestinal symptoms.

Cytokine Production after TLR 3, TLR 5, and TLR 9 stimulation

As children with ASD had demonstrated increased sensitivity to signaling via TLR 2 and TLR 4 with resultant cytokine production of pro-inflammatory cytokines, we hypothesized that children with ASD may have a global alteration in TLR signaling to all PAMPs. To determine if monocyte cell culture production of proinflammatory IL-1 β , IL-6, and TNF α was elevated for other TLR ligands, we assessed induced cytokine production by Luminex following stimulation with either flagellin (TLR 5), poly I:C (TLR 3) or CpG-B (TLR 9). In contrast to stimulation with either LTA or LPS, monocyte cell cultures from children with ASD did not produced significantly different levels in the majority of cytokines that were assessed, after stimulation with either poly I:C or flagellin when compared with monocytes cell cultures from TD controls that were similarly stimulated (Figure 4). In contrast, MCP-1 was decreased in ASD children compared to TD controls following poly (I:C) stimulation ($28,630 \pm 4,952$ vs. $52,500 \pm 6,178$ pg/ml respectively, $p = 0.006$). Following TLR-9 stimulation with CpG-B, monocyte cell cultures from ASD children produced significantly decreased levels of IL-1 β (205.6 ± 45.7 vs. 584.8 ± 142.4 pg/ml, ASD vs TD, $p = 0.01$), IL-6 ($9,601 \pm 2,327$ vs. $70,390 \pm 29,050$ pg/ml, $p = 0.05$), and TNF α (668.6 ± 211.0 vs. $2,184 \pm 585.5$ pg/ml, $p = 0.02$), GM-CSF (28.8 ± 7.3 vs. 71.4 ± 18.6 pg/ml $p = 0.04$) and MCP-1 ($9,030 \pm 1,811$ vs. $23,010 \pm 3,608$ pg/ml $p = 0.002$) compared with TD controls (Figure 4C). CpG-B induced production of IL-8 ($91,949 \pm 16,653$ vs. $101,946 \pm 14,410$ pg/ml, ASD vs TD) was not statistically different between monocyte cell cultures from ASD and TD control children. No differences were observed in cell surface expression of TLR 3, 4, 5 and 9 on CD14 $^{+}$ monocytes from children with ASD and TD controls (data not shown). In addition, induced cytokine levels following stimulation with TLR 2, TLR 3, TLR 4, TLR 5 and TLR 9 ligands were not associated with concentrations of T $_{H1}$ (IL-12p70, IFN γ) or T $_{H2}$ (IL-4, IL-5, IL-10) cytokines in plasma samples from the same children with ASD and TD controls as assessed by Luminex assays (data not shown).

Discussion

The current study describes the differential sensitivity of isolated peripheral blood monocytes from children with ASD compared with age-matched TD control children to defined TLR ligands. Our results indicate notable differences in cytokine production following TLR stimulation in monocyte cell cultures from ASD children including increased proinflammatory cytokine production following exposure to the TLR 2 ligand, LTA with increased production of IL-1 β , IL-6 and TNF α (3.3-, 3.1-, and 2.9-fold increases, respectively) relative to TD controls. In addition, there was an almost 2-fold increase in IL-1 β responses following TLR 4 stimulation with its ligand LPS. Our current findings are consistent with previous reports of enhanced innate immune activity in ASD

(Croonenberghs et al., 2002; Jyonouchi et al., 2001), and further indicates that a dysfunctional innate immune response may occur in a number of individuals with ASD.

Proinflammatory cytokines, IL-1 β , IL-6, and TNF α , which are predominantly derived from cells of the monocyte lineage, are of special interest in the study of neuroimmunological contributions to psychiatric disorders. These cytokines can act both locally and centrally to increase neuroinflammatory responses and/or to affect brain function such as the induction of serotonin from the hypothalamus; changes that may affect behavioral responses (Dunn, 2006). Of the TLR ligands analyzed in this study, those specific to induce TLR 2 signalling, elicited the most profound proinflammatory response in monocyte cell cultures derived from children with ASD. TLR 2 is constitutively expressed on the surface of microglial cells (Bsibsi et al., 2002; Kielian et al., 2005; Olson and Miller, 2004) and deficiencies in TLR 2 but not TLR 4, reduce T cell recruitment, microglial proliferation, and cytokine/chemokine expression in a neonatal murine model (Babcock et al., 2006). Previous animal studies have demonstrated that TLR 2 stimulation, leading to proinflammatory cytokine production, is sufficient to induce neuroinflammation and the neuronal degeneration that is characteristic of bacterial meningitis, and that TLR 2 deficient animals are protected from such changes (Hoffmann et al., 2007). In a murine EAE model of multiple sclerosis, the clinical disease course and severity of the condition correlated with increased brain expression of CD14 and TLR 2 transcripts, suggesting that there is an increase in or upregulation of microglial cells and monocytes in this model, and that TLR signaling may be actively involved in neuroinflammation and autoimmune development (Zekki et al., 2002). The induction of an inflammatory cytokine storm, initiated by monocyte activation, could produce downstream effects leading to the generation of neuroinflammatory and/or autoimmune responses. An autoimmune sequelae such as the generation of anti-neuronal antibodies to a wide variety of targets have been described in individuals with ASD and may be a consequence of responses originally started by inappropriate innate immune activity (Cabanlit et al., 2007; Connolly et al., 2006; Connolly et al., 1999; Croen et al., 2008; Kozlovskaja et al., 2000; Silva et al., 2004; Singh and Rivas, 2004b; Singh et al., 1997a; Singh et al., 1997b; Wills et al., 2009).

Evidence from animal studies, also suggests that innate immune cell activation following TLR stimulation with LPS or poly I:C can influence early brain development and result in neuronal and behavioral changes. In such models it has been demonstrated that maternal inflammation caused by administration of LPS or poly I:C results in abnormal behavior in the offspring (Borrell et al., 2002; Fortier et al., 2007; Gilmore et al., 2005; Hava et al., 2006; Meyer et al., 2007), an effect that can be partially replicated with maternal administration of recombinant IL-6 directly (Smith et al., 2007). Prenatal murine exposure to LPS resulted in decreased prepulse inhibition of the acoustic startle reflex and increased circulating IL-2 and IL-6 in adult offspring of exposed mothers (Borrell et al., 2002; Fortier et al., 2007). Abnormal TLR signaling and induced cytokine production may also affect post-natal early brain development in humans. Interestingly, both increased concentrations of IL-2 and IL-6, among other inflammatory cytokines, have been reported in ASD (Croonenberghs et al., 2002; Jyonouchi et al., 2001; Molloy et al., 2006; Singh et al., 1991; Vargas et al., 2005). Although animal studies are not likely to directly model several facets of ASD, they may reflect or provide clues as to how immune mediated responses may lead to early brain changes and alterations in behaviors. Furthermore, our data would suggest that models of maternal infection that are directed towards TLR 2 pathways warrant further investigation.

By utilizing isolated monocytes in this study, we minimized the potential contributions of cytokines produced by other immune subsets, most notably dendritic cells (Mao et al., 2005) that could impact on our results and data interpretation. This allowed us to determine if the initial recognition of TLR ligands by monocytes specifically results in a differential

inflammatory cytokine response in ASD and TD children. In this study, we demonstrated that there is differential signaling in monocytes through different TLRs in children with ASD compared to TD controls. For instance, while LTA induced an increased proinflammatory IL-1 β , IL-6 and TNF α response and LPS induced increased IL-1 β in ASD compared to TD, exposure to poly I:C or flagellin produced similar responses between cases and controls, and CpG produced a significantly lower monocyte response in ASD compared to TD. This may mean that signals generated through different TLR by the recognition of distinct PAMPS expressed by specific bacteria or viruses may lead to differential innate immune activity in ASD. For example, in the current study, signaling through TLR 9 by CpG stimulation was notable for resulting in significantly lower IL-1 β , TNF α , MCP-1 and GM-CSF release in ASD compared with TD. Typically, TLR 9 ligand recognition induces downstream anti-viral responses, mainly through interferon α/β production (Kawai and Akira, 2007). The clinical significance of this is unknown but may suggest that children with ASD respond poorly to TLR 9 stimulation that may lead to an ineffective anti-viral interferon response and may lead to inappropriate responses which could lead to infection, chronic inflammation and tissue destruction and could hence expose the individual to increased levels of autoantigens. In contrast, signaling through TLR 2 and TLR 4 leads to the marked release of proinflammatory cytokines. The pronounced increase in the production of these cytokines in response to LTA and LPS ligation warrants further investigation to elucidate the signaling cascade generated from TLR 2 and TLR 4. A previous report indicated that in the first month of life, children that later develop ASD have more infections than their counterparts (Rosen et al., 2007). These previous findings documenting the presence of increased bacterial and viral infections in conjunction with our observations that children with ASD are hyper-responsive to LTA and LPS stimulation could suggest that aberrant signaling through TLR 2 and TLR 4 may participate in this disorder. Inappropriate stimulation of innate immune responses during critical neurodevelopmental junctures, such as early childhood, could contribute to alterations in neurodevelopment and potentially lead to changes characteristic of ASD (Rosen et al., 2007).

Overall these data indicate that there is a differential innate immune response to TLR 2, 4 and 9 from monocyte cell cultures derived from ASD children when compared to age-matched TD controls. These data suggests that an underlying dysfunction in monocyte pathogen recognition and/or TLR signaling pathways is altered in young children with ASD. This altered innate immune response may have widespread effects on the activation and response of other immune cells and may also impact on neuronal activity given the extent of cytokine receptors present on neuronal and glial cells (Gladkevich et al., 2004). Furthermore, altered innate responses may ultimately play a role in the initiation and perpetuation of autoimmune responses that are present in some individuals with ASD. Our observations might also reflect genetic alterations in TLR signaling pathways, or pathways that control monocyte function, such as the MET pathway, and ultimately lead to monocyte activation and cytokine production. MET is a pleiotropic receptor tyrosine kinase and is a key negative regulator of immune responses (Beilmann et al., 1997; Beilmann et al., 2000; Ido et al., 2005; Okunishi et al., 2005) that exerts its effects through engagement of its ligand, hepatocyte growth factor (HGF). Notably, MET signaling induces a tolerogenic phenotype in innate immune cells without affecting their antigen presenting capabilities (Okunishi et al., 2005; Rutella et al., 2006). Interestingly, the gene encoding MET carries a common polymorphism, the rs1858830 'C' allele, which is functional and increases the relative risk for autism approximately 2.25-fold (Campbell et al., 2006). Thus, the *MET* 'C' variant may predispose to the absence of down-regulation of innate immune cell activation in ASD, and that the combination of a MET polymorphism and increased response to TLR ligands could combine to increase susceptibility to loss of self-tolerance and increased immune responsiveness. These data further support earlier studies that have noted innate

immune abnormalities in ASD (Enstrom et al., 2009b; Garbett et al., 2008; Jyonouchi et al., 2005; Jyonouchi et al., 2002; Sweeten et al., 2003; Vargas et al., 2005) and the potentially critical role of the innate immune response in ASD. Such findings warrant further investigation not only in children with ASD but also in other neurodevelopmental disorders where they may help towards our understanding of the initiation and progression of these disorders.

Acknowledgments

This study was funded by the NIEHS Children's Center grant (P01 ES011269), US EPA STAR program grant (R833292 and R829388), NIEHS CHARGE study (R01ES015359), Cure Autism Now Foundation, the Ted Lindsay Foundation, Peter Emch Foundation and a generous gift from the Johnson Family. We thank Isaac Pessah for his careful review and suggestions in the completion of this manuscript. We would like to thank the staff of both the UC Davis M.I.N.D. Institute and the CHARGE study for their technical support. The commitment of the families who took part in these studies, at both the M.I.N.D Institute and as part of the CHARGE study, is also gratefully acknowledged.

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Figure 1.

Induced cytokine concentrations of IL-1 β (A, D), IL-6 (B, E), and TNF α (C, F) in monocyte cell culture supernatants following stimulation with the TLR 2 ligand LTA (A-C) or TLR4 ligand LPS (D-F). Responses are shown for supernatants from children with an autism spectrum disorder (white bars) or typically developing controls (grey bars) * $p < 0.05$.

Figure 2.

Frequency (%) of CD14⁺ monocytes that demonstrate positive TLR-2 staining in children with ASD (white bars) or TD controls (grey bars) following 24-hour incubation with media alone or LTA (A). Mean fluorescent intensity (MFI) of TLR-2 staining on CD14⁺ monocyte cell cultures from children with ASD (white bars) compared with TD controls (grey bars) following incubation with media alone or with LTA (B).

Figure 3.

The frequency (%) of CD14⁺ monocytes that demonstrate positive staining for HLA-DR in children with ASD (white bars) or TD controls (grey bars) following 24-hour incubation with media alone or LTA (A). The frequency of HLA-DR⁺ CD14⁺ monocytes is increased in children with ASD compared to TD following 24-hour incubation with media alone ($p = 0.01$) or after LTA stimulation ($p = 0.02$, A). There were no difference in mean fluorescent intensity (MFI) of HLA-DR staining on CD14⁺ monocyte cell cultures in ASD compared with TD controls following incubation with media alone or LTA (B).

Figure 4.

Induced cytokine concentrations in monocyte cell culture supernatants following stimulation with the TLR 3 ligand, poly I:C (A), the TLR 5 ligand, flagellin (B) and the TLR 9 ligand, CpG (C). Responses are shown for supernatants from children with ASD (white bars) or TD controls (grey bars) (* $p < 0.05$, ** $p < 0.005$).