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Review Article

Innate immune dysfunction and neuroinflammation in autism spectrum disorder (ASD)

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ABSTRACT

Autism spectrum disorder (ASD) is a highly heterogeneous neurodevelopmental disorder characterized by communication and social behavior deficits. The presence of restricted and repetitive behaviors often accompanies these deficits, and these characteristics can range from mild to severe. The past several decades have seen a significant rise in the prevalence of ASD. The etiology of ASD remains unknown; however, genetic and environmental risk factors play a role. Multiple hypotheses converge to suggest that neuroinflammation, or at least the interaction between immune and neural systems, may be involved in the etiology of some ASD cases or groups. Repeated evidence of innate immune dysfunction has been seen in ASD, often associated with worsening behaviors. This evidence includes data from circulating myeloid cells and brain resident macrophages/microglia in both human and animal models. This comprehensive review presents recent findings of innate immune dysfunction in ASD, including aberrant innate cellular function, evidence of neuroinflammation, and microglia activation.

1. Introduction

Typically diagnosed in early childhood, autism spectrum disorder (ASD) is a complex heterogeneous developmental disorder involving the early onset of behavioral abnormalities, social impairments, and communication deficits. ASD can present at various levels of severity from mild to severe and has a male preponderance, with as many as four males diagnosed per female; however, this may be changing due to our shifting understanding of the differences between male and female presentation of ASD. The prevalence of ASD has risen substantially over the past several decades to 1 in 44 children (Maenner, 2021). While the etiology of ASD remains unknown, several risk factors are associated with the development of ASD, including genetic and environmental risk factors.

Twin studies support a strong role of genetics, with differences in concordance rates among monozygotic versus dizygotic twins pointing to a heritable component of ASD. However, concordance rates from twin studies often vary, and shared environmental factors may be contributing to susceptibility (Hallmayer, 2011; Castelbaum, 2020; Pugsley, 2021). Many genes identified as contributing to ASD in large-scale genetic studies are common variants that carry low risk individually;

however, additively, these variants increase the risk of ASD (Gaugler, 2014). Several high-confidence ASD variants converge onto inflammatory pathways, including the *MTOR* and *PTEN* genes. Rare inherited or de novo variants have also been identified and carry a substantially higher risk; however, these can only account for a small percentage of ASD cases (Iossifov, 2014) as do monogenic disorders such as Fragile X syndrome (FXS). Therefore, no single gene or common set of genes has been identified in the majority of ASD cases, and recent studies have identified environmental exposures implicated in ASD risk. These exposures may be exacerbated by gene-by-environment interactions and epigenetic mechanisms (Pugsley, 2021; Tordjman, 2014).

Several environmental risk factors have been clinically identified, with the majority occurring during gestation. Maternal autoimmunity confers a significantly increased risk for neurodevelopmental disorders (NDD) in offspring, including ASD (Chen, 2016). Maternal obesity, diabetes, and immune-mediated diseases such as asthma, also substantially increase the risk of NDD, as do exposure to toxicants, pesticides, and air pollution. These disorders and exposures share a commonality of immune activation and increased inflammation (Han, 2021). Notably, inflammation during gestation has been identified as a significant risk factor for ASD and other neurological disorders, although a recent

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population study in Sweden only identified an association, not a causal link, of maternal infection with ASD (Atladdottir, 2010; Brown, 2014; Brynge, 2022). In support of these findings, animal models of maternal immune activation (MIA) with various immune initiators have revealed ASD-relevant behaviors and have provided evidence of innate immune activation in MIA offspring, suggesting that early exposure to maternal inflammation may be inappropriately priming the fetal immune response and may lead to future dysfunction of this arm of the immune system (Meyer, 2014; Patterson, 2011; Careaga et al., 2017).

The network of cellular and chemical components that make up the immune system confers protection from invaders through an intricate system designed to identify “self” from “other”. Sentinel cells of the innate immune system include neutrophils, monocytes, and their tissue counterparts – macrophages. They are activated when broadly-recognizing pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) recognize conserved pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009). Activation through these receptors results in a cascade of inflammatory responses that lead to removal of the invader and activation of the adaptive arm of the immune system, which later confers specific and prolonged protection to future invasion by the same pathogen. Associated with activation of the innate arm of the immune system, inflammatory cytokines such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , and IL-6 are acute phase response cytokines produced and released during the early stages of a response to a pathogen. Elevations in the innate cytokines may indicate chronic inflammation or innate immune dysfunction.

2. Innate immune findings in ASD

2.1. Inflammatory cytokines

Cytokines are key chemical mediators of the immune response produced by cells when activated by exposure to an antigen or immunogen. They are fast-acting cell-signaling molecules that require appropriate regulation to maintain a balance between pro- and anti-inflammatory responses. This balance is especially critical during neurodevelopment as cytokines and their receptors are also expressed by neurons, and they are intricately involved in the proper development of the central nervous system (CNS). For example, these chemical messengers play essential roles in regulating synapse formation, maturation and elimination, cell renewal processes, and survival and apoptosis of cells of the CNS. Disrupted cytokine and chemokine expression during critical neurodevelopmental periods show the substantial influence of these signaling molecules on cells within the CNS, including radial glial cells and neural progenitors, neurons, microglia, astrocytes, and oligodendrocytes (reviewed in (Deverman and Patterson, 2009; Zengeler and Lukens, 2021).

Increases in these inflammatory cytokines have been identified repeatedly in ASD, providing evidence of peripheral innate activation and dysregulation compared to typically developing children (Table 1). Early studies in ASD cytokine research identified increases in inflammatory cytokines in whole blood cultures and TLR4-stimulated peripheral blood mononuclear cells (PBMC) (Croonenberghs, 2002; Jyonouchi et al., 2001). Activation of TLR4 on innate immune cells, including monocytes, initiates a proinflammatory cascade and production of cytokines including TNF- α , IL-1 β , and IL-6. In adults with severe autism, serum IL-1 β and IL-6 were significantly higher than healthy controls (Emanuele, 2010). In a large cohort of ASD children ages 2–5, significant elevations of IL-1 β , IL-6, and IL-12p40 were seen compared to typically developing children. Moreover, these elevated inflammatory cytokines were associated with worsening behaviors (Ashwood, 2011a). Similar increases in IL-1 β and IL-12(p70) were also seen in a cohort of high-functioning ASD males, along with increases in IL-1RA and T helper (T_H) cell-associated cytokines IL-5, IL-13, and IL-17 (Suzuki, 2011). In a recent study, individuals with ASD had increased inflammatory cytokines in serum, including IL-1 β , IL-6, IL-12, IL-23, and TNF- α , providing

Table 1
Altered cytokines and chemokines in ASD.

Author/Year	Subjects (M/F)	Methods	Innate Immune Findings in ASD
Jyonouchi et al. 2001	71 ASD (56/15) 23 SIB (16/7) 17 TD (7/10) 1–16 yrs	PBMC culture supernatant ELISA	↑ TNF- α , IL-1 β , and/or IL-6 after activation of TLR4 in some ASD, high variability among subjects
Croonenberghs 2002	13 ASD (13/0) 13 TD (13/0) 12–18 yrs	Serum and whole blood culture supernatant ELISA	↑ IFN- γ and IL-1RA in whole blood cultures suggest ↑ activation of peripheral monocytes
Okada 2007	19 ASD (19/0) 21 TD (21/0) 18–28 yrs	Serum ELISA	↓ TGF- β 1
Ashwood 2008	75 ASD (68/7) 36 TD (24/12) 32 DD (28/4) Median age 3–4 yrs	Plasma ELISA	↓ TGF- β 1, associated with worse behavioral scores
Ashwood 2011a	97 ASD (84/13) 87 TD (71/16) 39 DD (28/11) 2–5 yrs	Plasma multi-plex assay	↑ IL-1 β , IL-6, IL-8, IL-12p40, increased cytokines were associated with worse behavioral scores
Suzuki 2011	28 ASD (28/0) 28 TD (28/0) 7–15 yrs	Plasma multi-plex assay	↑ IL-1 β , IL-12(p70), GRO- α (CXCL1) (Also saw ↑ IL-1RA, IL-5, IL-8, IL-13, IL-17)
Abdallah 2012a	359 ASD (291/68) 741 CTL (595/146)	Dried neonate blood spot multi-plex assay	↓ RANTES, no differences in chemokines after adjustment (measured MCP-1, MIP-1 α and RANTES)
Manzardo 2012	99 ASD (74/25) 40 SIB (28/12) 5–10 yrs	Plasma multi-plex assay	↓ cytokines/chemokines
Ricci 2013	29 ASD (27/2) 29 TD (27/2) 2–21 yrs	Serum ELISA	↑ IL-1 β , IL-6, IL-23, IL-12 and TNF- α
Hashim 2013	50 ASD (27/23) 50 TD (30/20) 50 DD	Serum ELISA	↓ TGF- β 1 and IL-23. Levels of TGF- β 1 and IL-23 negatively correlated with severity of autism

(continued on next page)

Table 1 (continued)

Author/Year	Subjects (M/F)	Methods	Innate Immune Findings in ASD
	(28/22) 6–12 yrs		
Zerbo 2014	84 ASD (73/11) 159 GP (139/20) 49 DD (29/20)	Dried neonate blood spot multi-plex assay	↑ MCP-1, ↓ RANTES
Mostafa and Al-Ayadhi 2015	62 ASD (48/14) 62 TD (47/15) 4–12 yrs	Serum ELISA	↑ ENA-78 (CXCL5)
Masi 2015	17 studies	Systematic review and meta-analysis	↑ IL-1β, IL-6, IFN-γ, eotaxin, IL-8 and MCP-1. ↓ TGF-β1
Shen 2016	42 ASD (38/4) 35 TD (19/16) 3–6 yrs	Plasma multi-plex assay	↑ RANTES, MIP-1α, and MIP-1β, ↓ IP-10 and MIG Levels of MIP-1α, MIP-1β and IP-10 associated with impairments in social behaviors
Saghazadeh 2019	38 studies	Systematic review and meta-analysis	↑ IL-1β, IL-6, TNF-α and IFN-γ

ASD = autism spectrum disorder; SIB: non-ASD sibling; TD = typically developing; PBMC = peripheral blood mononuclear cells; TLR = Toll-like receptor; DD = developmental disability/delay; CTL = control; GP = general population control.

additional evidence of peripheral innate immune activation (Ricci, 2013). We recently characterized a cohort of ASD children based on PBMC responses to lipopolysaccharide (LPS) stimulation and found those with higher innate inflammatory responses or increased T cell activation had more behavioral impairments (Careaga, 2017). A subset (endophenotype) of children with ASD that exhibited increased innate immune response and severe behavioral and cognitive problems emerged, accounting for at least a third of the subjects tested. When considering the balance of pro- and anti-inflammatory mediators, the regulatory cytokine TGF-β is often decreased in ASD and associated with worsening behaviors (Ashwood, 2008; Okada, 2007; Hashim, 2013). This cytokine is critical for dampening the inflammatory responses of monocytes and macrophages during immune response resolution, and deficiency may contribute to prolonged or dysfunctional activation of these cells.

Chemokines are cytokines that mediate chemotaxis and recruitment of immune cells to sites of inflammation. Several chemokines associated with innate immune activation are elevated in ASD. Increases in monocyte chemoattractant protein (MCP)-1, regulated on activation, normal T cell expressed and secreted (RANTES), eotaxin, IL-8, and C-X-C motif chemokine 1 (CXCL1, previously known as Gro-α) have been identified in children with ASD (Ashwood, 2011a; Suzuki, 2011). Supporting these findings, Shen et al. recently found elevations of RANTES, macrophage inflammatory protein (MIP)-1α, and MIP-1β and decreases in interferon gamma-induced protein (IP)-10 (also known as CXCL10) and monokine induced by gamma interferon (MIG, also known as CXCL9) in children with ASD. Furthermore, MIP-1α, MIP-1β, and IP-10 concentrations were associated with impairments in social behaviors (Shen, 2016). Increases in CXCL5 have also been seen in ASD (Mostafa and Al-Ayadhi, 2015). Additionally, neonatal blood spots from ASD children were found to have increased MCP-1 (Zerbo, 2014). Elevated

MCP-1 in amniotic fluid was also associated with an increased risk of having a child with ASD (Abdallah, 2012b). These data suggest that chemotaxis and migration of monocytes/macrophages may be impacted in autism after diagnosis, at birth, and potentially in the womb during pregnancy.

Although the majority of ASD studies have identified increases in inflammatory cytokines and chemokines, findings are not always consistent. Reduced cytokines and chemokines were found in neonatal blood spots from ASD females, with no differences in ASD compared to typically developing (TD) in adjusted regression model estimates (Abdallah, 2012a). In one study utilizing multi-plex analysis of 29 cytokines in plasma, the concentration of eight cytokines/chemokines was consistently lower in ASD. However, this study used siblings of ASD as controls (Manzardo, 2012). ASD is a highly heritable disorder, and families may broadly display immune dysfunction across members; therefore, using siblings as controls can be problematic. Other contradictory findings can be due in part to differences in patient populations and mismatched controls, variations in sample type and processing, and different immunological assays and technologies used for research. Systematic reviews and meta-analyses can help identify consistencies and eliminate some of the noise produced by differences in research. One such meta-analysis looked across 17 studies and concluded that there are consistent elevations in IL-1β, IL-6, and IFN-γ found in individuals with ASD. The chemokines eotaxin, IL-8, and MCP-1 are also frequently elevated, and concentrations of the critical regulatory cytokine active- TGF-β1 are decreased in ASD (Masi, 2015). Results from another recent meta-analysis support these findings, with elevations in IL-1β, IL-6, TNF-α, and IFN-γ seen across 38 studies (Saghazadeh, 2019).

2.2. Innate immune cells

Cytokines seen repeatedly in ASD, such as IL-1β, IL-6, and TNF-α, are produced during activation of the innate arm of the immune system. This arm is responsible for the first line of defense against foreign antigens. When physical or chemical barriers of the host are breached, cells of the innate arm called monocytes act as sentinels, which are recruited to assaulted tissues by chemokines released at sites of infection. Here, these cells join their tissue-resident counterparts to become macrophages. They are critical during the early immune response for producing inflammatory cytokines to initiate an acute phase response in an attempt to control the infection directly (Dey et al., 2015). Monocyte and macrophage mediators produced as a result of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) binding to cytokine gene promoters and inflammasome activation influence downstream adaptive responses and later, under optimal conditions, lead to the resolution of inflammation. Conversely, dysfunction in the activity of these cells can lead to excessive and chronic inflammation, inappropriate adaptive immune responses, autoimmunity, and other immune-mediated conditions – many of which have been seen in ASD (Hughes, 2018). A hallmark of macrophages is their plasticity. These cells skew to a spectrum of phenotypes depending on microenvironment exposures. In vitro, they polarize to a classical inflammatory “M1” phenotype through stimulation with LPS plus IFN-γ or TNF-α. This polarization leads to production of the canonical proinflammatory cytokines IL-1β, IL-6, and TNF-α and helps to drive T helper cell (T_H)-1 differentiation. Conversely, macrophages can be alternatively activated to an “M2” phenotype when exposed to IL-4 and IL-13 in vitro, leading to production of IL-10 and suppression of the “M1” phenotype (Italiani and Boraschi, 2014).

Immune research has revealed increased numbers of innate immune circulating cells, including monocytes, in children with ASD (Sweeten et al., 2003; Tural Hesapcioglu, 2019)(Fig. 1). In some studies, monocyte numbers do not differ; however, monocyte activation is dysregulated. For example, Enstrom et al. found altered cytokine production after activation of TLRs on isolated peripheral CD14⁺ monocytes from ASD children, with increases in the canonical innate inflammatory cytokines

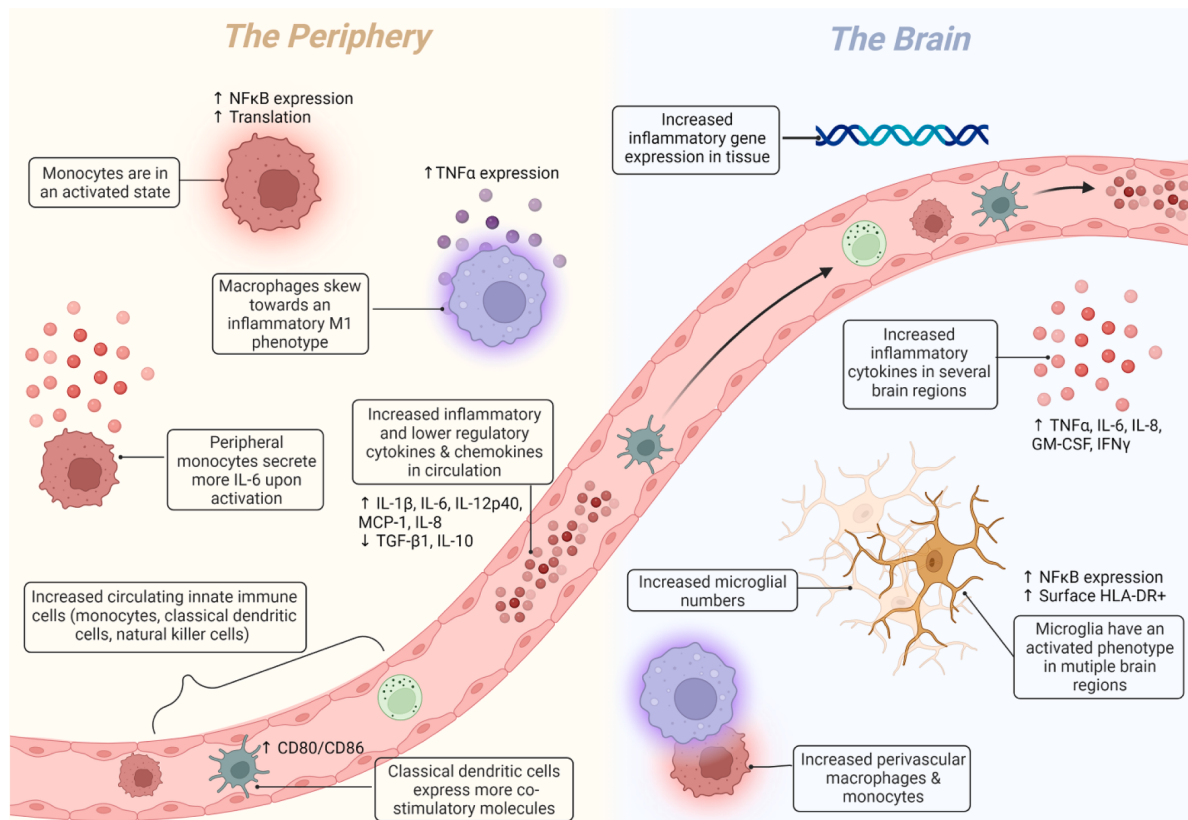


Fig. 1. Cellular and cytokine dysregulation of the innate immune system in both the periphery and brain of ASD individuals. Inflammatory cytokines produced mainly by cells of the innate immune system are elevated in the periphery of ASD individuals. Increased frequencies and altered activation of innate cells such as monocytes, macrophages, and dendritic cells are found, contributing to the establishment and perpetuation of inflammation in ASD. Notably, inflammatory cells and cytokines of the innate immune system can have consequences on brain homeostasis. Peripheral cells and cytokines can breach barriers in the brain and upregulate inflammatory gene expression. Inflammatory conditions in the brain may also alter tissue-resident immune cells, as altered microglial activation and cellular density have been documented in several regions of the ASD brain. CD80, cluster of differentiation 80; CD86, cluster of differentiation 86; GM-CSF, granulocyte macrophage colony-stimulating factor; HLA-DR, human leukocyte antigen-DR isotype; IFN γ , interferon-gamma; IL-1 β , interleukin-1beta; IL-6, interleukine-6; IL-8, interleukine-8; IL-10, interleukine-10; IL-12p40, interleukin-12subunit40; MCP-1, monocyte chemoattractant protein-1; NF κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; TGF- β , transforming growth factor-beta; TNF α , tumor necrosis factor-alpha.

TNF- α , IL-1 β , and IL-6 following activation of TLR2. IL-1 β was also increased after TLR4 activation with LPS, and concentrations of IL-1 β positively correlated with ASD-associated behaviors, including impaired social interactions and non-verbal communication (Enstrom, 2010). Monocytes from ASD children also had increased expression of CD95, which may reflect increased activation (Ashwood, 2011b). Whole peripheral blood cultures from ASD children also showed evidence of increased activation of monocytes through elevated production of innate-associated cytokines (Croonenberghs, 2002). Isolated PBMC showed elevated gene expression of caspases, including caspase-1 (also known as IL-1 converting enzyme). Caspase-1 is a proteolytic cleavage enzyme with critical involvement in inflammasome activity, leading to activation of IL-1 β (Siniscalco, 2012).

More recently, Jyonouchi et al. grouped ASD based on monocyte production ratios of IL-1 β and IL-10 after stimulation of several TLRs (high, moderate, and low ratios). The IL-1 β /IL-10 ratio groups differed significantly in monocyte expression of miRNAs and mitochondrial respiration. When probed with β -glucan, monocytes from the high-ratio group responded with increased pro-inflammatory responses (IL-1 β and TNF- α), while the low-ratio group responded with increased anti-inflammatory responses (Jyonouchi and Geng, 2019; Jyonouchi, 2017; Jyonouchi, 2019). Overexpression of the IL-17A receptor on ASD monocytes has recently been identified, along with increased expression

of NF- κ B and inducible nitric oxide synthase (iNOS), markers of inflammatory activity (Nadeem, 2018). These markers further increased expression after treatment with IL-17A, the ligand for the IL-17A receptor. IL-17 is associated with autoimmune disorders, including rheumatoid arthritis and multiple sclerosis, and IL-17A was recently identified to have substantial involvement in the MIA rodent model of ASD (Choi, 2016). The transcription factor NF- κ B plays a significant role in mediating innate inflammatory immune responses (Liu, 2017). Evidence supports increased NF- κ B transcriptional activity, as elevated NF- κ B mRNA and protein are observed in ASD peripheral blood and ASD lymphoblastic cell lines. However, some inconsistencies exist across studies, potentially due to differing methodologies (reviewed in (Liao and Li, 2020)). We recently investigated gene expression in TLR-activated monocytes and found increased gene expression of NFKB1, a subunit of NF- κ B, in TLR-activated monocytes from autistic children. We also found dysregulated gene expression of translational machinery in monocytes from ASD children compared to typically developing controls, which may be contributing to increased inflammatory responses in a subset of these children as a failure to dampen a prolonged immune response through regulation of translation (Hughes, 2022a). We further identified increased IL-6 production in LPS-stimulated monocytes from ASD children. This increase was associated with restricted and repetitive behaviors, supporting the role of excess inflammation associated with

Table 2
Altered peripheral innate cell numbers and function.

Author/Year	Subjects (M/F)	Methods	Innate Immune Findings in ASD
Sweeten et al. 2003	31 ASD (27/4) 24 TD (24/4) 2–12 yrs	Whole blood flow cytometry, plasma ELISA	↑ numbers of monocyte, ↑ plasma neopterin indicative of monocyte/macrophage activation
Enstrom 2009	17 ASD (14/3) 16 TD (13/3) 2–5 yrs	CD56 + NK cells isolated from PBMC, co-cultured with MHC-devoid K562 cells to assess cytotoxicity via flow cytometry	↑ CD56 + NK cell frequency and dysfunctional cytotoxic activity under both resting and stimulation conditions. Increased expression of inhibitory killer cell immunoglobulin receptors (KIR) and cytolytic genes (N for gene expression studies: ASD 35, TD 11)
Enstrom 2010	17 ASD (14/3) 16 TD (13/3) 2–5 yrs	Supernatant from TLR-stimulated monocytes isolated from PBMC – multi-plex assay	↑ IL-1β, IL-6, TNF-α after TLR2 activation, ↑ IL-1β after TLR4 activation, ↑ IL-1β, IL-6, GM-CSF and MCP-1 after TLR9 activation
Ashwood 2011b	35 HFA (29/6) 35 LFA (29/6) 35 TD (29/6) 4–6 yrs	Whole blood immune cell quantification and phenotyping via flow cytometry	40 % ↑ in NK cells, increased CD14 ⁺ CD95 ⁺ monocytes (increases also seen in activated B and T cells)
Siniscalco 2012	15 ASD 10 TD	PBMC RT-PCR, Western blot, immunocytochemistry	Over-expression of caspases at gene and protein level in PBMC, suggesting innate activation
Onore 2012	49 (42/7) ASD 31 (20/11) TD Median age: 2.91, 3.13	Plasma adhesion molecules assessed by ELISA	↓ sPECAM-1 and sP-selectin sPECAM-levels negatively correlated with repetitive behavior
Jyonouchi 2017	69 (52/16 [sic]) ASD 27 (16/11) TD 2.8–27 yrs	microRNA sequencing of monocytes isolated from PBMC, supernatant from TLR-stimulated monocytes – ELISA,	When grouped based on monocyte responses to TLR stimulation, “high” IL-1β/IL-10 ratio responders had increased expression of miRNAs associated with immune signaling pathways.
Nadeem 2018	40 (32/8) ASD 35 (30/5) TD 3–11 yrs	Flow cytometry on isolated CD14 ⁺ isolated monocytes, real-time PCR, Western blot, ELISA	↑ IL-17A receptor, NF-κB and iNOS on ASD monocytes Activation of the IL-17A receptor increased iNOS expression through NF-κB, which was reversed with IL-17RA antibody treatment.
Tural Hesapcioglu 2019	45 ASD (36/9) 43 TD (33/10) Mean age: 13.5, 11.9	Complete blood count	↑ monocytes, ↓ lymphocyte-to-monocyte ratio,
Jyonouchi 2019	152 (130/22) ASD 41 (27/17) Non-ASD 1.9–29.6 yrs	Monocytes isolated from PBMC and stimulated with TLR-agonists and β-glucan, supernatant measured via ELISA.	↑ IL-1β, TNF-α by β-glucan stimulated monocytes in “high” IL-1β/IL-10 ratio producing monocytes from ASD (previously identified after stimulation with TLR-agonists)
Yamauchi 2021	29 ASD (24/5) 30 TD (25/5) Mean age: 28.0, 27.2	CD14 ⁺ monocytes isolated from PBMC and cultured to differentiate into M1/M2 mRNA analyzed for gene expression using qRT-PCR.	↑ TNF-α and IL-1β mRNA expression in monocyte-derived M1 macrophages ↑ TNF-α M1/M2 ratio
Hughes 2022a	17 AD (13/4) 9 PDDNOS (7/2) 22 TD (18/4) 4–9 yrs	RNA seq analysis of mRNA from CD14 ⁺ monocytes cultured with TLR-2/4 agonists	↑ gene expression associated with inflammatory pathways in AD compared to PDDNOS and TD.AD and PDDNOS monocytes also lacked the significant downregulation of genes involved in translation seen in TD monocytes 24 h after stimulation.
Hughes 2022b	25 ASD (19/6) 20 TD (16/4) 4–9 yrs	CD14 ⁺ monocytes isolated from PBMC and cultured with TLR-2/4 agonists Multi-plex analysis of supernatant	↑ IL-6 after TLR4 activation IL-6 concentrations associated with worse scores on the Repetitive Behavior Scale.

ASD = autism spectrum disorder; TD = typically developing; NK cells = Natural Killer cells; PBMC = peripheral blood mononuclear cells; MHC = major histocompatibility complex; TLR = Toll-like receptor; NF-κB = nuclear factor kappa B; iNOS = inducible nitric oxide synthase; AD = autistic disorder; PDDNOS = pervasive development disorder not otherwise specified.

innate immune responses in aberrant behavior. Differences in gene expression and cytokine production were only seen after cells were activated; there were no differences at baseline (Hughes, 2022a; Hughes, 2022b). Similarly, Yamauchi et al. found increased gene expression of TNF- α in monocyte-derived macrophages from ASD individuals; however, this was only after differentiation to M1-macrophages. Neither baseline monocytes nor differentiated M2-macrophages showed this elevation (Yamauchi, 2021).

Aberrant numbers or activation of other innate immune cells are also present in ASD, including significantly increased numbers of peripheral myeloid dendritic cells (mDCs) (Breece, 2013)(Fig. 1). Changes in the mDC population may be due to increased differentiation of monocytes into dendritic cells. Further work has shown that peripheral mDCs from ASD children express more surface CD80/CD86 co-stimulatory molecules than TD children (Saad, 2017). Differences in DC correlated with amygdala volume, gastrointestinal issues, and impaired behaviors (Breece, 2013). In further studies, ASD children with gastrointestinal issues had significantly increased monocyte, eosinophil, and neutrophil infiltration in duodenum, colon, and terminal ileum biopsies (Ashwood, 2003; Furlano, 2001). In addition, significantly increased numbers of natural killer (NK) cells are seen in ASD children compared to controls (Ashwood, 2011b). Transcriptional profiling of whole blood from children with ASD also revealed increased expression of genes belonging to the NK cell KEGG pathway (Gregg, 2008). Later examination confirmed abnormalities in gene expression associated with NK cells, including increased expression of inhibitory killer cell immunoglobulin receptors (KIR) and cytolytic genes. Furthermore, ASD children had increased CD56⁺ NK cell frequency and dysfunctional activity in isolated NK cells under both resting and stimulative conditions (Enstrom, 2009). Function of NK cells was increased under basal conditions, but there was a failure to respond to stimulus in the form of tumor cells.

Decreased soluble adhesion molecules were also seen in ASD plasma, including soluble platelet endothelial cell adhesion molecule-1 (sPECAM-1), which plays an important role in mediating innate immune cell adhesion to and migration across the endothelial wall to facilitate recruitment to inflamed tissues (Onore, 2012). In mouse models, decreased sPECAM-1 is associated with decreased vascular integrity and increased blood-brain barrier permeability, and deficient mice are more prone to autoimmunity (Woodfin et al., 2007). See Table 2 for a comprehensive list of aberrant cellular findings associated with innate peripheral immune dysfunction.

2.3. Neuroinflammation and innate immune cells in the brain

Microglia are specialized macrophages that reside in the brain and CNS. They play a critical role not only in neurodevelopment but also in CNS homeostasis throughout life. During neurodevelopment, microglia are responsible for phagocytosing excess neuronal precursor cells to regulate neurogenesis by restraining cell production (Cunningham et al., 2013). They also play a role in supporting neuronal survival while also limiting axon outgrowth (Li and Barres, 2018). Production of brain-derived neurotrophic factor (BDNF) by microglia promotes survival and differentiation of neurons, regulation of synaptic formation and transmission, and synaptic plasticity critical for memory and learning (Miranda, 2019; Parkhurst, 2013). Similar to peripheral monocytes, they are responsible for surveilling their local environment and can become activated when exposed to a foreign antigen or under inflammatory conditions following infection or injury. Under these conditions, microglia release pro-inflammatory cytokines and can experience aberrant activation, including excessive proliferation. If occurring during developmentally-delicate periods, prolonged inflammatory activity of microglia can alter neurodevelopment (Li and Barres, 2018). It was originally proposed that these cells polarized in a similar fashion to peripheral macrophages. However, more recent evidence suggests that microglia are not fully committed to one phenotype or activation state (Ransohoff, 2016).

Table 3
Innate immune findings in postmortem ASD Brain.

Author/ Year	Subjects (M/F)	Methods	Main Findings
Vargas 2005	Post-mortem brain: 15* ASD (12/3) 12* Non-ASD CTL (9/3) 5–50 yrs CSF: 6 ASD (4/2) 9 Non-ASD CTL (3/6) 2–45 yrs *n differed depending on fresh frozen vs fixed samples	ICC staining on fixed brain samples from MFG & ACG from cortex, and GCL and white matter from cerebellum. Cytokine protein array and ELISA on homogenized samples from fresh frozen MFG, ACG, and cerebellum. Cytokine protein array on CSF.	Loss of neurons in the Purkinje and GCL. ↑ staining of GFAP and HLA-DR in various regions including near Purkinje cells and GCL in cerebellum, indicating activation of astroglia and microglia. ↑ perivascular macrophages and monocytes in 4 of 10 ASD brains. Complement membrane attack complexes found in Purkinje cells, with accumulation of suspected microglia nearby. ↑ IL-6 (31.4 fold) in the ACG, other cytokines/chemokines increased depending on region. Multiple cytokines/chemokines ↑ including MCP-1 in CSF
Li 2009	8 ASD (5/3) 8 TD (5/3) 4–37 yrs	Multiplex analysis of cerebral cortex homogenized extracts	↑ TNF α , IL-6, IL-8, GM- CSF ↑ IFN- γ ; ↑ IFN- γ :IL-10 ratio
Morgan 2010	13 ASD (13/0) 9 TD (9/0) 3–44 yrs	IHC staining of formalin-fixed dorsolateral prefrontal cortex	Evidence of Iba-1 ⁺ microglia activation including amoeboid morphology, ↑ microglia somal volume in white matter (trend in gray matter), and ↑ microglia density in gray matter. Lack of Iba-1 colocalization with IL- 1R1 suggests long- standing rather than acute inflammation.
Wei 2011	6 ASD (4/2) 6 TD (4/2) 4–14 yrs	IHC staining of formalin-fixed cerebellum CGC derived from C57BL/6J neonatal pups, cells were transfected with IL-6 GFP Adenovirus or GFP Adenovirus for control	↑ IL-6 in cerebellum of ASD brain IL-6 stimulated formation of excitatory neurons and altered adhesion and migration of cerebellar granule cells in mice
Morgan 2012	13 ASD (13/0) 9 TD (9/0) 3–44 yrs	IHC staining and hematoxylin/ eosin counter-staining of formalin-fixed dorsolateral prefrontal cortex tissue slices	↑ spatial proximity of Iba-1 ⁺ microglia to neurons ↑ contact of microglia with neurons, including microglial processes encircling neuronal somal bodies
Tetreault 2012	11 ASD (9/2) 12 TD (11/1) 2–23 yrs	ICC staining of formaldehyde-fixed fronto-insular (FI) and visual cortex (VC)	↑ Iba-1 ⁺ microglia density in both regions of cortex examined

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Table 3 (continued)

Author/ Year	Subjects (M/F)	Methods	Main Findings
Young 2011	9 ASD** (5/4) 9 TD (5/4) 5–79 yrs **Rett syndrome n = 1	IHC and WB of formalin-fixed orbitofrontal cortex or tissue microarray, depending on source	↑ NF-κB in astrocytes and microglia ↑ CD11b ⁺ and CD11c ⁺ microglia with significantly increased p65 nuclear translocation in ASD cells compared to TD.
Malik 2011	7 ASD (5/2) 7 TD (4/3) 4–14 yrs	WB and ELISA on fresh frozen cerebellum and frontal cortex homogenates. IHC on formalin-fixed cerebellar cortex.	↑ IKKα expression in ASD cerebellum. IKKα phosphorylates the inhibitory subunit IκBα of NF-κB. No increases in protein expression or phosphorylation of IκBα and NF-κB p65 were seen in ASD brain.

ASD = autism spectrum disorder; CTL = control; CSF = cerebrospinal fluid; ICC = immunocytochemistry; MFG = middle frontal gyrus; ACG = anterior cingulate gyrus; GCL = granular cell layer; TD = typically developing; CGC = cerebellar granular cells; GFP = green fluorescent protein; IHC = immunohistochemistry.

Investigations of postmortem brain tissue from ASD individuals have revealed evidence of neuroinflammation in the brain, including microglia activation (detailed in Table 3)(Fig. 1). One of the first studies to identify the presence of neuroinflammation examined tissues from 11 ASD subjects and found regional neuronal loss and evidence of neuroinflammation in the cerebral cortex, white matter, and cerebellum. Increased staining of GFAP and HLA-DR in multiple brain regions suggested marked activation of microglia and astroglia. Increased MCP-1 was also seen in cerebrospinal fluid (CSF) (Vargas, 2005). A later study found significantly elevated cytokines associated with innate activation, including TNF-α, IL-6, and GM-CSF, and increased IFN-γ and IL-8 in postmortem tissue from the frontal cortex of 8 ASD subjects (Li, 2009). Microglia were then characterized using immunostaining to identify differences in activation phenotypes; ASD tissues showed evidence of increased microglial activation, including amoeboid presentation. Additionally, density and somal volume of microglia were increased in ASD. These researchers also found no evidence of acute inflammation, suggesting microglia have long-standing activation (Morgan, 2010). Furthermore, the same group later found increased spatial proximity of microglia to neurons in the dorsolateral prefrontal cortex of ASD brain, with microglia processes frequently encircling the somal bodies of neurons in ASD samples (Morgan, 2012). Increased density of microglia has also been identified in two separate regions of cortex in the ASD brain compared to controls (Tetreault, 2012). NF-κB is increased in the postmortem ASD brain, with localization to glial and neuronal cells (Young, 2011), and an enzyme complex that activates NF-κB is also elevated in the ASD brain (Malik, 2011). More recently, it was shown that cells of the adaptive arm of the immune system are increased in postmortem ASD brain and seen accumulating near blood vessels, further supporting evidence of neuroinflammation in ASD (DiStasio, 2019).

While the postmortem evaluation of ASD brain tissue has consistently shown neuroinflammation and microglial activation, ASD studies using positron emission tomography (PET) to study microglia - in “real-time” - have provided mixed results. To date, PET evaluating microglia activation in ASD has relied on the radiolabeling of ligands that bind to the mitochondrial translocator protein (TSPO), a protein expressed on the outer mitochondrial membrane by activated microglia (Cosenza-Nashat, 2009). The first study to evaluate microglial activity in this fashion used the first generation [¹¹C]-PK11195 radiotracer to measure TSPO expression in 20 high-functioning male ASD and typically developing control subjects (Suzuki, 2013). Using the binding potential as the

output PET parameter, [¹¹C]-PC11195 binding was higher in the cerebellum, frontal, temporal parietal, limbic and subcortical regions. Higher radiotracer binding was interpreted as higher TSPO expression and activated microglia phenotypes in high functioning ASD subjects. However, recent studies do not corroborate increased TSPO expression. In one study evaluating the binding potential of the second generation radiotracer [¹¹C]-PBR28 in 15 ASD males, TSPO expression was seen to be lower in several brain regions (Zürcher, 2021). Additionally, TSPO expression did not differ in low or high-functioning ASD. In another study using the total distribution volume as the primary PET output parameter and the second generation [¹⁸F]-FEPPA radiotracer, TSPO expression was also lower in both males and females with ASD (Simpson, 2022). Mixed findings between studies may be due to several reasons. The radiotracer used since the first-generation [¹¹C]-PK11195 has a high non-specific binding affinity. The PET output parameter (binding potential vs total distribution volume) can influence findings, as previously documented in a meta-analysis of patients with psychiatric disorders (Marques, 2019). Additionally, the measurement of TSPO for microglial activation may also introduce variability. While TSPO is expressed by activated microglia, endothelial cells during homeostasis and astrocytes in a disease context can also express TSPO (Cosenza-Nashat, 2009). Lastly, Suzuki and colleagues did not identify if their research subjects had TSPO gene polymorphisms, as polymorphisms can influence radiotracer binding. Additional PET studies using microglial-specific proteins, correctly identifying subjects with high and low-TSPO binding affinity, and analyzing both the binding affinity and total volume distribution will aid in clarifying the abovementioned findings.

Transcriptomic analyses of various brain regions support findings of increased immune responses in the ASD brain, with enrichment in immune-specific genes seen in several studies (Table 4). Increased expression of immune system-related transcripts was first seen in the temporal cortex of postmortem ASD brains. Pathway analysis resulted in 31 BioCarta gene sets that differed from controls, of which 19 involved immune pathways. Several of these belonged to innate inflammatory pathways, including NFKB, IL1R1, and IL6 (Garbett, 2008). Voineagu et al. found dysregulated genes in the ASD brain, with decreased expression of neuronal genes involved in synaptic function and upregulated genes associated with inflammatory responses and microglial activation. Using weighted-gene co-expression network analysis (WGCNA), they further identified two network modules associated with ASD, with the first involving under-expressed neuronal genes and overlapping with several autism susceptibility genes. The second module associated with ASD was enriched in overexpressed immune genes, and later work by this same group supported these findings (Voineagu, 2011; Parikshak, 2016). Similar results were also seen in a later study across several neuropsychiatric disorders, including ASD, schizophrenia, and bipolar disorder (Gandal, 2018). One study looked for enrichment of the immune module in a larger ASD data set and specifically identified M2 activation of microglia and type I interferon responses in the ASD brain. The authors argued that the increased expression of genes associated with M2 activation could be an effort to mediate M1 inflammatory skewing that occurs during type I IFN responses (Gupta, 2014). Meta-analysis of previous ASD cortex RNA-Seq studies utilizing datasets from GEO (Gene Expression Omnibus) unearthed newly identified differentially expressed genes in the ASD brain, including TNF signaling and complement cascade pathways (Rahman, 2020). Methylation studies have also reported hypomethylation of genes associated with the immune response, leading to increased expression of inflammatory genes (Nardone, 2014). Taken together, these studies provide evidence that neuroinflammation may play a role in ASD. However, sample sizes for postmortem ASD studies are limited and more research is needed in this area to elucidate potential mechanisms of neuroinflammation that contribute to ASD and other NDD.

Table 4
Brain transcriptome immune findings.

Author/ Year	Subjects (M/F)	Methods	Main Findings
Garbett 2008	Frozen samples of superior temporal gyrus from 6 ASD and 6 TD	GeneChip microarrays on RNA isolated from frozen samples of superior temporal gyrus	152 differentially expressed genes in ASD brain (↑ 130, ↓ 22). 19 BioCarta pathways involving immune function were altered in ASD including NFKB, IL1R, TOLL, INFLAM and IL6. Authors suggest an expression pattern associated with autoimmune rather than acute or non-specific immune activation.
Voineagu 2011	Frontal and temporal cortex, and cerebellum from 19 ASD and 17 TD. Further analysis (after filtering microarray data for quality) on 58 cortex samples from 29 ASD, 29 TD; and 21 cerebellum samples from 11 ASD, 10 TD.	Illumina microarrays on frontal and temporal cortex, cerebellum.	444 DEGs in cortex, but only 2 DEGs in cerebellum. Of the DEGs in the cortex, GO enrichment analysis showed that ASD had 209 downregulated genes associated with synaptic function, and 235 upregulated genes involved in immune/inflammatory responses
Gupta 2014	Cortex samples from 32 ASD and 40 TD	RNA Seq for differential gene expression	Enrichment of M2 microglia markers and Type I interferon signaling in ASD cortex, downregulation of genes associates with neuronal transmission and GABAergic signaling.
Parikshak 2016	251 samples of frontal cortex, temporal cortex and cerebellum from 48 ASD and 49 TD	RNA Seq for differential gene expression, long non-coding RNA (lncRNA) expression, and alternative splicing.	Pathway analysis of ASD DEGs in cortex showed decreased expression of genes associated with neuronal pathways, and upregulated genes enriched for microglia and astrocytes. Two brain-enriched lncRNAs, LINC00693 and LINC00689, were upregulated in ASD, while alternative splicing events for neuronal exons were downregulated.
Gandal 2018	Data from N = 1695 samples that included ASD, SCZ and BD	Integration of gene expression and genotype data generated from 1695 frontal/temporal cortex samples from ASD, SCZ and BD	WGCNA identified four neural-immune modules significantly upregulated depending on disorder: NF-κB signaling upregulated across all three disorders, astrocyte and IFN-response modules upregulated

Table 4 (continued)

Author/ Year	Subjects (M/F)	Methods	Main Findings
			in ASD and SCZ, and microglia module upregulated in ASD but downregulated in SCZ and BD.
Rahman 2020	Data from 15 ASD and 15 TD cortex samples	Meta analysis of previous RNA Seq data	235 new DEGs not detected in previous studies. GO enrichment analysis identified upregulation of genes involved in inflammatory immune response pathways, including TNF signaling.

ASD = autism spectrum disorder; TD = typically developing; DEGs = differentially expressed genes; GO = Gene Ontology; SCZ = schizophrenia; BD = bipolar disorder; MDD = depression; AAD = alcoholism; WGCNA = weighted gene correlation network analysis.

3. Preclinical evidence of innate immune dysfunction

In support of clinical findings, preclinical studies relevant to ASD have also identified dysregulation of innate inflammatory responses and activation of macrophages and microglia. For example, in the poly(I:C)-induced MIA model, MIA drove increased inflammatory cytokine production by bone marrow-derived macrophages obtained from offspring (Onore, 2014). In an LPS-based MIA study, MIA offspring exhibited increased peripheral IL-1β and IL-6 throughout growth. Peripheral inflammation increased further after LPS re-exposure at 8 weeks, along with substantial increases in inflammatory cytokines, chemokines, and cell adhesion molecules in the brain (Hsueh, 2018). Other studies have revealed that MIA offspring exhibit altered microglia morphology and increased motility of microglia processes (Ikezu, 2021; Pratt, 2013; Ozaki, 2020). In an asthma model of MIA, offspring with increased gene expression and methylation patterns in multiple autism susceptibility genes exhibited increased microglial activation (Vogel Ciernia, 2018). A recent study using a two-hit model of MIA plus prenatal hypoxia found infiltrating monocytes and neuroinflammation in the brain. When monocytes were blocked from entering, neuroinflammation was ameliorated (Chen, 2020). Supporting the hypothesis that inflammation plays a significant role in aberrant behavior, in the MIA model, increased innate inflammatory cytokines in plasma at adulthood were associated with significantly impaired social behavior compared to those that did not exhibit this inflammatory response (Mueller, 2021). Other animal models have also documented associations between innate inflammatory cytokines and impaired behavior. In a non-human primate MIA model, resting and stimulated PBMCs from MIA offspring produced more innate inflammatory cytokines at one and four years post-birth, which was also associated with more stereotyped behaviors (Rose, 2017).

Hsiao et al. identified gastrointestinal dysfunction in poly(I:C) offspring, with increased inflammatory IL-6 in the colon coinciding with decreases in tight junction proteins. These offspring also had shifts in gut microbiota and alterations in microbial metabolites produced by the microbiota that were associated with worse behaviors. These behaviors were corrected with the addition of the commensal *Bacteroides fragilis*, suggesting that MIA-induced changes in the gut microbiota may be contributing to behaviors (Hsiao, 2013). The composition of maternal microbiota was also found to be a critical component for pathological outcomes in MIA – if dams lacked a certain commensal bacteria responsible for driving IL-17 responses, the offspring did not exhibit the typical MIA behavioral manifestations (Choi, 2016). Altered myeloid cell numbers were also observed in this model. Gut dysfunction is

prevalent in ASD, and the microbiota composition are often altered (reviewed in (Hughes et al., 2018)). Altered microbiota may be contributing to immune dysfunction, as these commensal microbes produce metabolites including short-chain fatty acids that are critical for gut-immune homeostasis (Parada Venegas, 2019), and have been shown to influence the behavior of monocytes and macrophages in culture (Wenzel, 2020; Ji, 2016).

4. Conclusion

There is growing evidence that innate immune dysfunction plays a role in ASD. Immune abnormalities involving excess inflammation and activation of innate immune cells are seen consistently across ASD studies. Aberrant inflammatory activation is also reflected in studies of ASD brain, suggesting a role for neuroinflammation. Maternal inflammation is risk factor for ASD, with maternal immune system activation during gestation resulting in ASD-like behavior and altered innate immune function in offspring in animal models. It is well understood that components of immune system, including microglia and the cytokines they produce, play an essential role in neurodevelopment. However, the underlying cause(s) of aberrant neurodevelopment and immune dysfunction in ASD remains elusive. The seemingly chronic immune dysregulation in ASD is likely influenced by mechanisms that govern long-term cellular phenotype and behavior. For instance, epigenetic modifications involved in trained immunity may be skewing myeloid cells towards an activated state, or alterations in the development of hematopoietic stem cells into myeloid cells may be skewed. Future studies will need to take into consideration the heterogeneity of ASD to fully elucidate etiology and the role of the immune system. Some studies have been successful in identifying subgroups of ASD children based on particular innate immune signatures. Further research and the use of unbiased technological approaches will aid in our understanding of how the innate immune system can be used to characterize ASD heterogeneity, ultimately improving potential personalized therapeutic strategies and the quality of life for autistic people and their families.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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