The Reproductive Origin of the Autism Spectrum Disorder: An Immunological Point of View

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder featured with dysregulations in social interaction, communication and repetitive behavior. Prenatal exposure to some factors is associated with increased ASD incidences in human studies, such as endocrine-disrupting chemicals, infections etc. In this review, we try to integrate the prenatal risk factors and investigate their contribution to the pathogenesis of ASD. These factors can elicit maternal immune activation (MIA) and increase the circulating cytokines in the fetus, which primes the fetal microglia for enhanced inflammatory responses, thereby more susceptible to a second challenge later. The heightened inflammatory responses induced by MIA are termed innate immune memory (IIM), implicated in microglia. Treatment targeting the IIM such as colony-stimulating factor 1 receptor (CSF1R) inhibitors can correct the ASD-like behaviors in rodent MIA offspring. Taken together, the paper suggests that MIA mediates the relationship between some prenatal risk factors and the pathogenesis of ASD. The framework offers a unified understanding of the reproductive origin of ASD. The high risk of MIA during the current 2019-novel coronavirus (2019-nCoV) outbreak might provide an opportunity to initiate clinical trials of CSF1R inhibitor drugs in the MIA offspring. It would be important to develop a biomarker in rodent models that indicates IIM in microglia is sufficiently induced to offer selective treatment to at-risk infants. Future animal studies should also focus on combining MIA and a second challenge with different nature, timing and severity (two-hit model) to determine a sufficient combination for the development of ASD and other neurodevelopmental disorders.

Keywords: ASD; Prenatal Risk Factors; MIA; IIM in Microglia; CSF1R; Two-hit Model of ASD

Table of contents

The Reproductive Origin of the Autism Spectrum Disorder: An Immunological Point of View

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by three core features: deficits in social communication, impaired social interaction and the presence of repetitive and stereotyped behaviors, interests or activities during early periods of development that negatively impact social, occupational or other domains (American Psychiatric Association, 2013). Approximately 1.85% of the 8-year-olds (one in 56) from the USA has ASD and it is 4.3 times as prevalent among boys as among girls (Maenner et al., 2020). Children from different racial, ethnic, and socioeconomic backgrounds are all affected (Durkin et al., 2017). In the Netherlands, the disorder influences roughly 0.6% of the whole population (Versteegde et al., 2016).

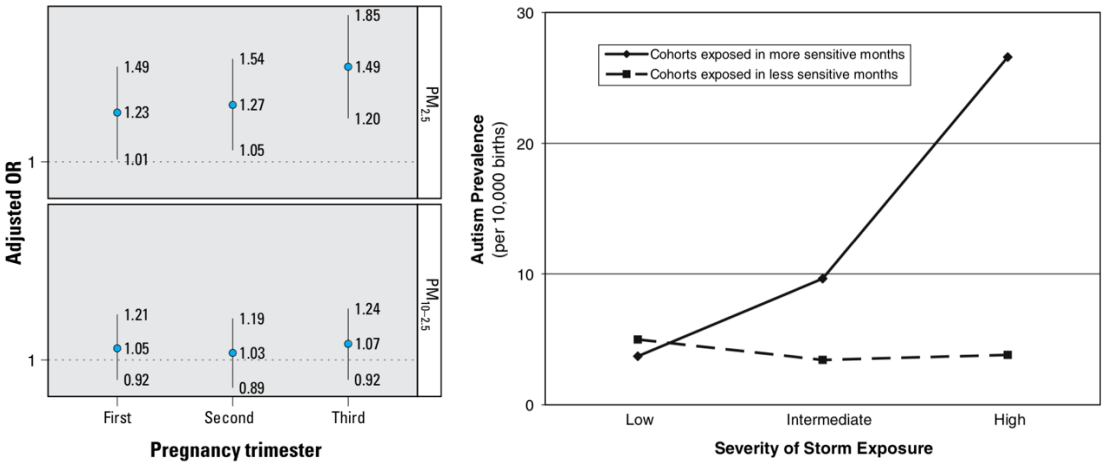
Progress has been continuously made towards gaining an understanding of the mechanisms behind the disorder. Genetics is an acknowledged risk factor with an estimated heritability of 83% based on Swedish twin studies (Sandin et al., 2017). To illustrate, an increased *de novo* mutation load was found in the regulatory elements of ASD risk genes, such as chromodomain helicase DNA binding protein 8 (CHD8) and fragile X mental retardation protein (FMRP; Short et al., 2018), which could lead to widespread RNA editing dysregulations in ASD brains (Tran et al., 2019). Meanwhile, the importance of non-genetic risk factors is also being increasingly recognized (Bolte, Girdler, & Marschik, 2019).Various prenatal risk factors such as maternal stress, malnutrition, endocrine disrupting chemicals and infections have all been proposed to play a role in the pathogenesis of ASD (Afroz and Alvina, 2019; Lee et al., 2015; Long et al., 2019; Robert et al., 2013). Therefore, in addition to the genetic factors, understanding mechanisms underlying prenatal risk factors of ASD can be pivotal for prevention or intervention of the disorder in an early stage.

In this review, we will try to integrate the prenatal risk factors and investigate whether they share some common pathways regarding the pathogenesis of ASD. Subsequently, we try to delineate what these common pathways could be from cytokine signaling and the innate immune memory of microglia perspectives. Targeting the common pathways could help with the advancement of a more generic but effective treatment. Most importantly, this paper may provide some insight into the preventive strategies of a potential surge of ASD incidences among the offspring of those pregnant women who experience an increased risk of psychological stress, dietary imbalance and infection during the 2019-novel coronavirus (2019-nCoV) outbreak.

Association between Prenatal Risk Factors and ASD

The developmental origins of health and disease (DOHaD) hypothesis postulates that exposure to certain environmental factors in utero and during critical periods of development may have a significant impact on one's health (Barker et al. 1990; Gage et al., 2016). A number of these prenatal environmental factors have been recognized to be associated with an increased incidence of ASD in human studies. For instance, there was a significant positive relationship between maternal exposure to air pollutants ($PM_{2.5}$) and odds of a child having ASD (Raz et al., 2015; see figure 1a). Also, based on records of hurricanes and tropical storms in Louisiana, the USA, prevalence of ASD was also associated with maternal exposure to stressful events during critical periods (Kinney et al., 2008; see figure 1b). Other factors

include dietary imbalance (Afroz and Alvina, 2019), endocrine-disturbing chemicals (Hamra et al., 2019; Long et al., 2019), and infections, virus and parasites (Lee et al., 2015).



(a) (b)

Figure 1. (a) exposure to particulate matter during pregnancy trimesters and odd ratio for ASD, which is adjusted for child sex, year and month of birth, paternal age at birth etc. ASD cases: n = 160; Controls: n = 986 (Raz et al., 2015); (b) a dose-response curve of the severity of storm exposure and the prevalence of autism during sensitive periods of pregnancy. More sensitive months (5-6, 9-10); Less sensitive months (1-4, 7-8) (Kinney et al., 2008).

Furthermore, these prenatal environment factors may interact with the genes such as CHD8 or FMRP to increase the risk of ASD in the offspring (Chaste & Leboyer, 2012). On that account, when the genetic risk factors are known, it is feasible to protect individuals against ASD risks by targeting these environmental risks (Beverdorf et al., 2019; see figure 2a). In particular, compared to ASD risk genes, the environmental factors are more accessible.

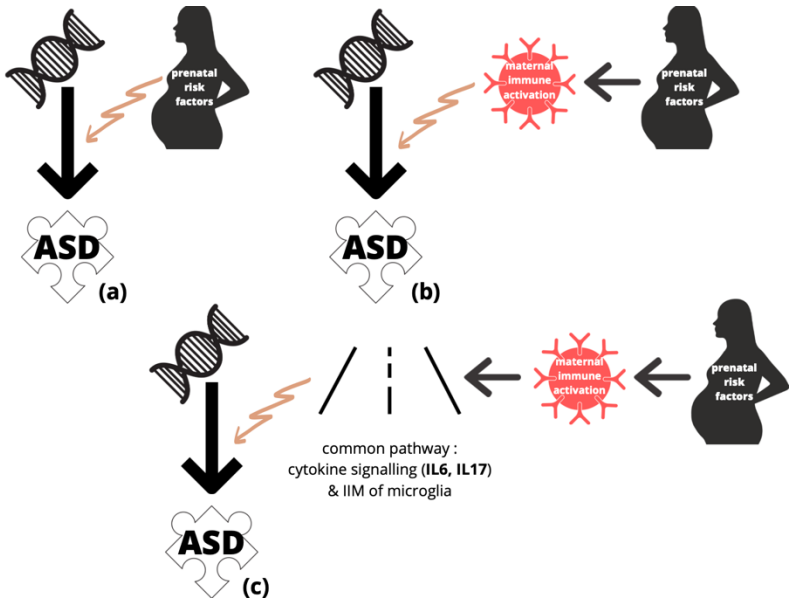


Figure 2. (a) The link between some prenatal risk factors and ASD. As shown, prenatal risk factors interact with genetic factors, contributing to the pathogenesis of ASD (modified from Beverdorf et al., 2019); (b) Maternal immune activation is proposed as a mediator between the prenatal risk factors and the development of ASD; (c) Cytokine signaling and the innate immune memory of microglia may underlie the effect of maternal immune activation on ASD risk in the offspring.

The exact mechanisms of how these prenatal environmental factors may contribute to the development of ASD have long been under investigation. The prenatal risk factors have been proposed to induce divergent effects. For instance, air pollutants are more likely to be associated with an increased risk of ASD in participants with a functional promotor variant (rs1858830) in the MET receptor tyrosine kinase (MET) gene (Volk et al., 2014). Another case in point is the serotonin transporter (SERT), which plays a role in stress reactivity. Genetic variations in the SLC6A4 gene which encodes SERT can alter aspects of its function and are associated with compulsive and rigid behaviors in ASD (McCauley et al., 2004). Offspring of the dams lacking a SERT gene and exposed to prenatal stress had decreased social interaction (Jones et al., 2010). Recently, increasing evidence points to the role of the immune system in ASD. Transcriptomic analysis of post-mortem brain samples from ASD patients reveals upregulation of immune response genes (Gupta et al., 2014). During the 2015-2016 Zika virus pandemic, the maternal infection of Zika virus posed a risk of fetal microcephaly and abnormalities of the central nervous system in the offspring (Brasil et al., 2016). Therefore, different prenatal risk factors such as air pollutant, stress and infection, may contribute to the pathogenesis of ASD via different mechanisms.

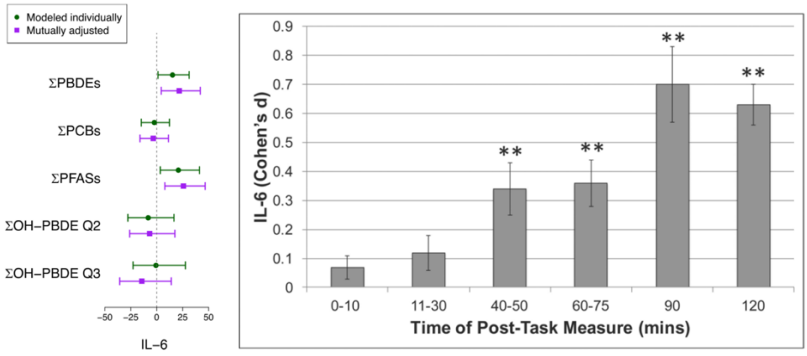
Although all these mechanisms may reveal some part of the etiology underlying ASD, it also brings uncertainty as to what treatment would be the most effective under certain conditions. In addition, the choice of treatment is hard to determine as the cause is highly divergent. Thus, it would be more promising to find some common ground between the prenatal risk factors so that the treatment can be more generic but still effective.

MIA Shared by Prenatal Risk Factors

Maternal immune activation (MIA) may be a critical link between these prenatal environmental factors and neurodevelopmental challenges in children. Maternal immune activation refers to an excessive circulation of inflammatory markers beyond a normal range (Boulanger-Bertolus et al., 2018). Fetuses *in utero* are normally under a noninflammatory immune environment (Chaouat, 2007; Jones & Saporita, 2016). When the immune factors are disrupted during gestion, fetuses can experience adverse developmental consequences (Jones et al., 2017).

One can argue that the relevance of maternal infection to a greater risk of ASD may not be associated with the presence of a specific virus or bacteria per se such as Zika (e.g. Brasil et al., 2016), but in the immune response they invoke (Boulanger-Bertolus et al., 2018). It has been found that women with autistic offspring had elevated inflammatory markers and antibodies during pregnancy (Brown et al., 2014). Most importantly, the mentioned prenatal environmental risk factors have all been linked to increased systemic inflammation in either pregnant women or in a general population. For example, exposure to endocrine disrupting chemicals such as polybrominated diphenyl ethers (PBDE) is positively associated with proinflammatory factors (e.g. Interleukin 6) in pregnant women (Zota et al., 2018; see figure 3a). A short-term exposure to ambient air pollution was also found to increase systemic inflammation in a general population (Li et al., 2017). Similarly, psychological stress (Marsland et al., 2017; see figure 3b), diets high in salt (Yi et al., 2015) can also induce reliable increase in the circulating inflammatory factors such as IL6 in human participants.

Therefore, maternal immune activation may bridge the impact of these prenatal risk factors and the developmental of ASD (see figure 2b in the previous section).



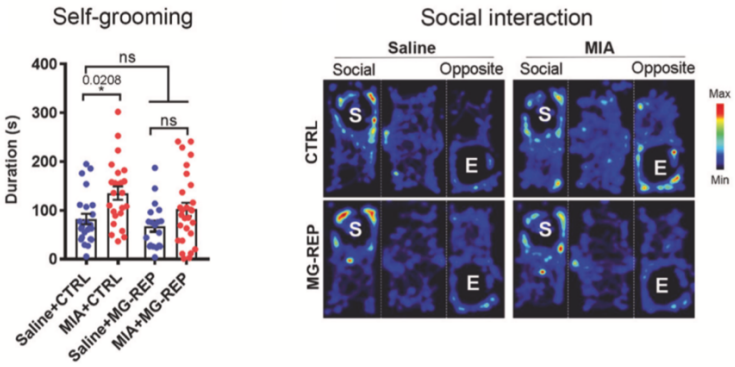
(a) (b)

Figure 3. (a) Percent difference in interleukin 6 associated with endocrine-disrupting chemicals sums, modeled individually and adjusted for other categories. As shown, a doubling in ΣPBED was associated with a 15.26% increase in IL-6. All models were adjusted for age, gestational weeks, education etc. (Zota et al., 2018); (b) Standardized mean difference (Cohen's d) in Il-6 before and after artificial laboratory challenges. There was a significant increase in IL-6 among participants after exposure to the challenge (Marsland et al., 2017).

Evidence of MIA in the Pathogenesis of ASD

Nevertheless, epidemiology studies in human participants alone cannot establish a causal relationship between MIA and ASD risk (Estes & McAllister, 2016). Clinical research has also its shortcomings in identifying the molecular pathways downstream of MIA due to the limitations of experimental invasiveness (Estes & McAllister, 2016). On that account, the potential mechanisms for the MIA model of ASD will be mainly discussed with rodent models.

In a prototypical MIA regimen, pregnant rodents receive a treatment of polyinosinic:polycytidylic acid (Poly I:C; a synthetic double stranded RNA molecule) on embryonic day 12.5 (Smith et al., 2007). Poly I:C agonizes the toll-like receptor-3 and induces an unspecific innate response to viral infection (Smith et al., 2007). The offspring will then be tested in various tasks to determine behavioral changes pertaining to ASD. In general, immune activation during pregnancy can result in three core behavioral features of ASD in the offspring, including decreased social interaction, aberrant communication, and increased repetitive or focused behavior (Smith et al., 2007; Ikezu et al., 2020).



(a) (b)

Figure 4. (a) MIA male offspring showed increased repetitive behavior measured as time spent grooming, which could be corrected by microglia depletion treatment (MG-REP) (Ikezu et al., 2020); (b) Heatmaps of Three

Chamber Social Interaction Test showed that MIA male offspring did not have a preference for the tube containing social mouse over the empty tube, which could also be corrected by microglia depletion treatment (Ikezu et al., 2020).

The Effect of MIA on Cytokine Signaling

In response to the injection of Poly I:C, cytokine levels of both IL-6 (interleukin 6) and IL-17a (interleukin 17a) increase in pregnant rodents, which has an impact on the behavioral abnormalities in MIA offspring (Bergdolt and Dunaesky, 2019; see figure 2c). IL-6 is an interleukin which functions as a proinflammatory cytokine. A single injection of recombinant IL-6 in healthy pregnant rodents contributed to the underperformance in prepulse inhibition and latent inhibition in adult offspring (Smith et al., 2007). Nonetheless, coadministration of an antibody against IL-6 in the MIA mice reversed the behavioral abnormalities in the offspring (Smith et al., 2007).

Elevated IL-6 level appears to be important due to the activation of the IL-6 pathway in the placenta (Bergdolt and Dunaesky, 2019). Maternally derived IL-6 activated the JAK/STAT3 pathway in the spongiotrophoblast of the placenta, which could influence the production of critical placental factors during fetal developmental (Hsiao and Patterson, 2011). The behavioral impairments in the offspring could, however, be prevented by deletion of the IL-6 receptor IL-6Ra in placental trophoblasts (Wu et al., 2017).

Likewise, maternal IL-17a is also fundamental for the ASD-like phenotype in the offspring (Bergdolt and Dunaesky, 2019). IL-17a is downstream of IL-6 and also a proinflammatory cytokine. The level of IL-17a increased in the placenta and maternal serum in response to immune activation (Choi et al., 2016). Subsequently, an increase in the IL-17a receptor subunit A (IL-17Ra) was observed in the fetal brain (Choi et al., 2016). The upregulation of IL-17Ra in the fetal brain and its accompanying behavioral abnormalities could be prevented by blocking the receptor with IL-17a antibodies (Choi et al., 2016).

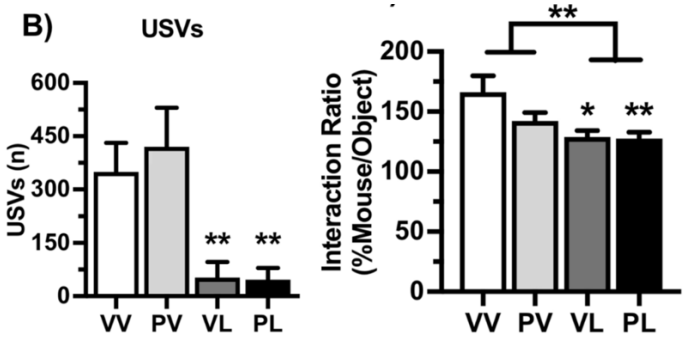
The role of IL-17a in MIA later inspired the finding that the maternal gut microbiome plays a role in aberrant behavior and the maldevelopment of brain in MIA offspring (Kim et al., 2017). The elevation of IL-17a in the maternal serum was accompanied by the presence of segmented filamentous bacteria (SFB) in the maternal microbiota. These bacteria help Th17 cells to differentiate, which produce IL-17a. MIA offspring of pregnant rodents lacking SFB did not exhibit behavioral abnormalities or pathology in the brain (Kim et al., 2017). Yet, after the transplantation of fecal microbiota containing SFB, offspring of pregnant rodents originally lacking SFB developed ASD-related phenotypes (Lammert et al., 2018). Therefore, the interaction between microbiota and gestational IL-17a is also important for the pathogenesis of MIA-induced behavioral abnormalities.

In general, the maternal cytokine dysregulation is thought to exert an influence on fetal cytokine expression and the effect can be long-lasting. The initial dysregulation took place approximately three hours after Poly I:C injection, reflected by enhanced levels of IL6 in the fetal brain and its downstream activation in the hindbrain (Wu et al., 2017). A pattern of cytokine dysregulation existed in the frontal and cingulate cortices in the MIA offspring (Garay et al., 2013). There was an increase in proinflammatory cytokines at birth, reduced levels during neurodevelopment, and a return to overexpression in the adult brain (Garay et

al., 2013). Indeed, dysregulation of the inflammatory markers in the amygdala was also maintained from the prenatal environment to adulthood (O'Loughlin et al., 2017). Therefore, the maternal cytokine dysregulation may induce a persistent effect on the cytokine expression in offspring brain.

From MIA to the Two-hit Model of ASD

Meanwhile, other studies challenged the idea that MIA alone would be enough to induce an ASD phenotype in the offspring. Some studies found that using either a full dose of Poly I:C or a subthreshold but physiologically relevant dose of Poly I:C could not produce constant result in ASD-like phenotypes among the MIA offspring (Carlezon et al., 2019). On the one hand, it makes sense since in human beings most of the maternal infections would not lead to neuropsychiatric disorders in the offspring (Custodio et al., 2018); on the other hand, when the offspring were exposed to a second immune challenge at a later stage, they would exhibit behavioral abnormalities (Carlezon et al., 2019; see figure 5). Thus, MIA may function as a disease primer which increases susceptibility of ASD in individuals (Estes & McAllister, 2016).



(a) (b)

Figure 5. (a) Ultrasonic vocalization analysis showed that prenatal Poly I:C alone was not sufficient to induce deficits in social communication, unless a second immune challenge was also administered. V: vehicle; P: Poly I:C; L: LPS. * $p < .05$, ** $p < .01$ (Carlezon et al., 2019); (b) similar result was found in social interaction.

To further explore the possibility of accumulating effects, studies have developed a "two-hit" immune activation regimen in rodents involving MIA (using Poly I:C) followed by PIA (using LPS; e.g. Carlezon et al., 2019). To illustrate, lipopolysaccharide (LPS) was administered in mice during early neurodevelopmental stages as postnatal immune activation (Carlezon et al., 2019). LPS agonizes the toll-like receptor 4 and induces an innate response to bacterial infection. The treatment of LPS alone during early development may also contribute to ASD-like phenotypes in mice (Carlezon et al., 2019; see figure 5).

In general, the two-hit paradigm is consistent with the understanding that the cause of neuropsychiatric disorders is multifactorial (Clarke et al., 2009). It helps to investigate whether MIA can act as a disease primer and increase the susceptibility in offspring to a second immune challenge early during development (Carlezon et al., 2019). Indeed, it was found earlier that peripubertal stress unmasked MIA-induced ASD phenotypes in mice (Giovanoli et al., 2013). Moreover, the two-hit paradigm has a good construct validity. An alteration was found in the excitatory/inhibitory balance in projection from the medial prefrontal cortex to basolateral amygdala under the two-hit regimen, which is often implicated in ASD patients (Li et al., 2018). Male mice also experienced changes in sleep and

epileptiform activities, which is common comorbid medical conditions in human ASD (Missig et al., 2018). Therefore, the two-hit paradigm in mice involving both MIA and PIA is a valid model to investigate the pathogenesis of ASD in humans.

Microglia: Candidate for Fetal Cytokine Dysfunction

There are different theories as to how maternal cytokines influence the cytokine dysregulations in the offspring. One proposal suggested that the maternal inflammatory cytokines may pass the placenta, thereby having an impact on the fetal peripheral nervous system (Abdallah et al., 2013). Yet, it has been found the peripheral serum cytokine had no significant relationship with MIA-induced alterations in brain cytokines in the same animal (Garay et al., 2013). Since the blood brain barrier was intact and no infiltration of peripheral immune cells was detected in the brain of MIA offspring, the alteration in the fetal brain was less likely be the result of infiltration of circulating cytokines (Garay et al., 2013). Instead, one may argue that MIA directly influences the central nervous system of the fetus brain during development and exerts its long-lasting effect via the immune memory of microglia.

In brain disorders, microglia serve as the immune cells of the brain. They stem from myeloid precursors which migrate into the brain during early embryonic development and remain as the resident macrophage in the central nervous system as the blood brain barrier becomes more intact (CNS; Ginhoux et al., 2010; Prinz and Mildner, 2011). They are highly ramified, composed of long-branching processes and a small cell body, which perform constant screening of the microenvironment. When activated by a stimulus, microglia function as effective immune cells, thereby mediating the innate immune responses to coordinate different activities against CNS injuries or pathologies (Eggen et al. 2013).

Most importantly, microglia are a stable population and have a relatively long lifespan (Réu et al., 2017). They can live more than 15 months in the mouse cortex and up to 50% of these cells could sustain themselves the entire mouse lifespan (Füger et al., 2017). In humans, microglia also have an average lifespan of 4.2 years and renew slowly at a median rate of 28% per year, some of which could even survive over 20 years (Réu et al., 2017). Therefore, before microglia migrates to the central nervous system, they can already be influenced by the effect of MIA and its low turnover rate can make them a good candidate for remembering and maintaining the cytokine dysregulation in the fetus brain (Neher & Cunningham, 2019).

Novel Discovery of IIM in Microglia

In vertebrates, it has been long thought that only adaptive/acquired immunity is capable for the immunological memory responses. After encountering a pathogen, naïve B and T lymphocytes proliferate and develop antigen-specific, long-lasting memory cells, providing effective and specific protection against reinfection by the same microorganism (McHeyzer-Williams & McHeyzer-Williams, 2005). However, in recent years, the classical paradigm has been challenged by research revealing that cells of the innate immune system, including monocytes, macrophages and natural killer cells, also have the ability to develop immunological memory (Netea et al., 2016). An initial immune stimulus triggers such extensive cell reprogramming that the same innate immune cells present enhanced or reduced responsiveness even after a few months in both mouse models and humans (Arts et al., 2018; Schaafsma et al., 2015; Wendeln et al., 2018).

Innate immune memory (IIM) includes two concepts: immune training and immune tolerance, referring to elevated or diminished immune responses upon secondary stimulation, respectively (Neher & Cunninghan, 2019). The formation of the innate immune memory lies in epigenetic reprogramming of the murine macrophages, such as with DNA methylation and chromatin reorganization (see review Netea et al., 2016; Netea et al., 2020). The reprogramming process can also be regulated by metabolic rewiring of the immune cells or their progenitors (Dominguez-Andres et al., 2019). In general, these changes have an impact on the transcriptomic profile and functional phenotype of microglia, which mediates inflammatory response in pathologies.

IIM of Microglia Under the Two-hit ASD model

Existing research indicates long-standing alteration in microglial function following immune stimulation. For instance, administration of inflammatory stimuli such as the viral mimetic poly I:C to pregnant mice can lead to modifications in microglial responses and function in the offspring when growing up (Mattei et al., 2017). These offspring during adulthood displayed deficits in novel object recognition, sociability and behavioral inhibition. Transcriptomics analysis revealed that the offspring displayed an altered transcriptome signature, pertaining to reduced gene expression in cell migration, phagocytosis and upregulation in long-term neuronal synaptic plasticity etc (Mattei et al., 2017). Therefore, early-life events could leave a distinctive footprint in the development and function of the brain via the long-term microglial memory of a previous inflammatory episode (Neher & Cunninghan, 2019).

Recently, Carlezon and colleagues (2019) coupled a postnatal immune activation at day 9 with the MIA at gestational day 12.5 to study the effect of the two-hit model on the development of ASD-like behavior in mice offspring. This combined method resulted in abnormalities in social and repetitive behaviors more prominent in males than in females, which was consistent with the human epidemiological result (Maenner et al., 2020). The two-hit model contributed to sex-specific disruptions in the expression of CNS proinflammatory genes (Carlezon et al., 2019). Even though both male and female mice experienced increases in proinflammatory genes, the expression of anti-inflammatory factors was only upregulated in female mice. It was suggested the equilibrium between the pro- and anti-inflammatory gene expression might explain why ASD-like behaviors were less evident in female mice (Carlezon et al., 2019).

Of note, whereas the researchers only interpreted the result as the validity of the two-hit model of ASD, their study also shed some light into how innate immune memory may have played a role in the development of ASD-like behavior. PIA male mice in combination with MIA (preconditioned group) compared to PIA only (naïve group) showed a heighted response of proinflammatory gene expression of IL-6 and IL-1ß in male brain areas such as medial prefrontal cortex and amygdala (Carlezon et al., 2019; see figure 6a & 6b), suggesting a potential role of immune training of pro-inflammatory genes in the male mice brain. Meanwhile, anti-inflammatory genes such as IL-10 and TGF-ß1 in male mice brain was reduced in the preconditioned group compared to the naïve group, suggesting a potential role of immune tolerance of anti-inflammatory genes (see figure 6c & 6d). The innate immune

memory may explain why MIA could act as a primer for ASD and make individuals more susceptible to develop ASD when encountering pubertal stress or other postnatal environmental factors. Therefore, in addition to the original conclusion that the two-hit model may produce sex-specific effect on the vulnerability of ASD-like behavior, MIA exposure could also render male mice more susceptible to a second hit.

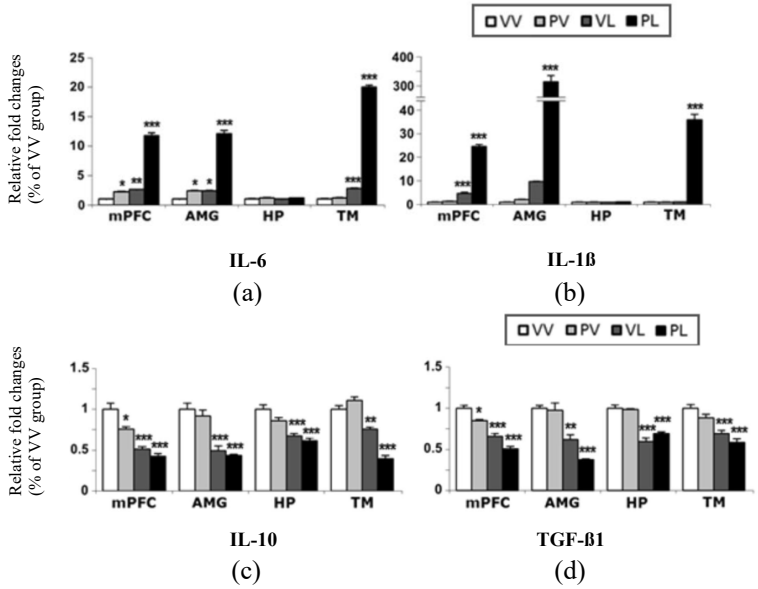


Figure 6. (a) & (b) Immune training of the proinflammatory genes in several brain areas, reflected by heightened responses in the PL group compared to the VL group. V: vehicle; P: Poly I:C; L: LPS (Carlezon et al., 2019); (c) & (d) Immune tolerance of the anti-inflammatory genes in some brain areas, reflected by reduced responses in the PL group compared to the VL group (Carlezon et al., 2019). *Note: the authors did not provide statistical significance of the difference between VL and PL groups as it was not their research focus. The conclusion drawn in this paper is based on the visual inspection of their graphs and the difference is rather evident.

Microglia Depletion as a Treatment

As innate immune memory may play a role in the effect of MIA on ASD-like behavior, increasing studies have tried to reset microglia to correct this memory. For instance, colony stimulating factor 1 receptor (CSFR1) inhibitors can be a novel treatment to reduce the susceptibility to ASD-like behavior (Ikezu et al., 2020). This treatment led to microglial depletion and regulation in the MIA offspring. It corrected the transcriptomic profile of microglia, which was associated with synaptic, neurophysiological and behavioral alterations (Ikezu et al., 2020; see figure 4 for the behavioral effect of CSFR1 inhibitors). Therefore, microglia depletion can be a novel prevention therapy for ASD-like behaviors due to its effect on resetting the transcriptomic and functional properties of the resident immune cell in the brain.

Currently, there are already ongoing human clinical trials to investigate the effect and safety of some CSF1R inhibitor drugs in patients with Amyotrophic Lateral Sclerosis (ClinicalTrials.gov Identifier: NCT04066244) and Alzheimer's Disease (NCT04121208). In the study of Ikezu et al. (2020), it was shown that microglial depletion in healthy mice had no adverse effect on behavioral phenotype (see figure 4). Therefore, taken safety into consideration, in response to a potential surge of ASD incidences due to a greater risk of maternal immune activation during the 2019-nCoV outbreak, similar clinical trials should be conducted in the future if possible.

Discussion and Future Perspectives

MIA a Universal Primer?

So far, we have demonstrated that maternal immune activation could mediate the relationship between prenatal risk factors and the pathogenesis of autism spectrum disorders. Yet, some of the core symptoms of ASD revealed in animal models, such as decreased social interaction, aberrant communication, and increased repetitive or focused behavior, are commonly shared by schizophrenia and other neurodevelopmental disorders (see review De Crescenzo et al., 2019). Therefore, it is currently unclear whether MIA functions as a unique primer for the development of ASD or a universal primer for neurodevelopmental disorders in general.

ASD and schizophrenia share genetic roots and there are considerable etiological overlaps between these two disorders (Kushima et al., 2018), which may be considered as a single spectrum disorder with ASD and schizophrenia at two ends (Byars et al., 2014). Patients with ASD compared to schizophrenia exhibited a higher density of the cortex, extreme brain growth and a lack of gray matter loss (Courchesne et al., 2007). Indeed, MIA not only led to the core aberrant behaviors, it also increased basal dendritic spine density and enhanced microglia-neuron interaction (Ikezu et al., 2020). This finding indirectly lends some evidence to the assumption that MIA is a unique primer for ASD due to the excessive brain growth, which is rather unique to ASD compared to schizophrenia.

However, systematic and more specific assays are needed to assess the brain anatomical difference in MIA offspring to draw a clearer conclusion. For instance, future studies can use magnetic resonance imaging (MRI) to compare the relative thickness of the cortex and gray and white matter volume in the MIA and control rodent offspring.

Furthermore, the timing of MIA can be a critical factor contributing to different neurodevelopmental disorders (Minakova & Warner, 2018). The typical protocol in experimental animals to induce MIA is to administer Poly I:C at embryonic day 12.5. Contingent on the timing of MIA, its effect on offspring is distinct. Whereas exposure on gestational day 9 inhibited spatial exploration in adulthood, exposure on day 17 altered the capability in learning tasks (Meyer et al., 2006). Apoptosis was also upregulated in the dentate gyrus and there was reduced hippocampal neurogenesis among the adult offspring when MIA was administered on gestational day 9 compared to day 17 (Meyer et al., 2006), indirectly supporting the notion that MIA at gestational day 9 may be associated with increased risk of schizophrenia specifically. Therefore, MIA can also be a specific primer for different neurodevelopmental disorders contingent on the timing of exposure. Future studies should focus on providing a systematic overview of the behavioral alterations induced by MIA at different timepoints prenatally.

On the contrary, if MIA is a universal primer, the neuroinflammation experienced in the developing fetus may be responsible for the induction of pathological hallmarks shared by both ASD and schizophrenia (Meyer et al., 2011), whereas the nature, timing, severity of the second hit at a later stage determines a specific disorder. Much is unknown in this field as most animal studies focus exclusively on the maternal immune activation (the first hit). Future studies in rodent models should combine a variety of second hits (PIA) with MIA to

demonstrate whether different combinations can lead to different neurodevelopmental disorders. This line of translational research of determining the feature of MIA, PIA and a combination of both challenges may be of great importance. In our daily life, women during pregnancy can be exposed to a variety of immune stimulants in all trimesters and their children at a later stage (e.g. during adolescence) would also encounter many sources of stresses, which can be translated into a second immunological hit.

IIM of Microglia: Pathogenesis or Prevention of ASD?

Based on the discussion above, it is tempting to draw the conclusion that the first immune challenge (MIA) exposes the offspring to a higher risk of a second hit (PIA) later. This was evidenced by the finding that the two-hit offspring experienced heightened pro-inflammatory responses (trained immunity) compared to the non-primed offspring and the two-hit offspring exhibited ASD-like behavior (Carlezon et al., 2019). Nonetheless, the same type of trained immunity, for example, has also a cross protective effect of a lethal *Candida albicans* infection in animal models (Kleinnijenhuis et al., 2012) and a yellow fever virus vaccination strain in human volunteers (Arts et al., 2018) and after bacillus Calmette-Guerin (BCG) administration. Therefore, we caution against overinterpreting that the MIA primes microglia in the offspring for an increased risk of ASD.

It is conceivable that the heighted response elicited by the second hit can be beneficial in the beginning and only when the second hit elicits a long-lasting disturbance in the brain homeostasis, would it lead to the pathogenesis of neurodevelopmental disorders. On that account, although current evidence suggests that MIA leads to a greater risk for ASD in the offspring, it may also offer a protective effect on the pathogenesis of ASD upon the second hit initially. It would be important for future studies to investigate under what circumstances MIA reduces or increases the risk of ASD when encountering a second hit.

If it is only possible to determine the effect MIA when unmasked by PIA and given the ample chance that humans will be exposed to a second immune challenge, it may be hard to manipulate the second hit for preventive treatments. Instead, MIA as the primer can be a better candidate for treatment options. A case in point is to identify a biomarker indicating that MIA induces a conformational change in the epigenome of fetal microglial cells and selectively administer anti-inflammatory drugs to newborns with the biomarkers.

What might also be feasible in the future is to correct the transcriptomic changes of microglia in every newborn to eliminate the effect of MIA, such as by using CSF1R inhibitors as in the study of Ikezu et al. (2020). Most importantly, administration of CSF1R to healthy offspring also did not cause behavioral abnormalities, which means even if in the absence of a reliable biomarker, it could be safe to administer the CSF1R inhibitor to deplete the potential primed microglia and allow new non-affected microglia to replenish in the offspring brain.

Nonetheless, although CSF1R inhibitor did not lead to obvious aberrant behavior in healthy individuals, it should be noted that the replenished microglia were different in the MIA offspring (same transcriptomic profile as the healthy offspring without MIA) and in the healthy offspring (a unique profile). Since the behavioral test was only conducted at one end point, it is not certain whether the unique transcriptomic profile in the CSF1R-inhibitor-treated healthy rodent offspring will lead to aberrant behavioral phenotypes at a much later

stage. Therefore, before applying the CSF1R inhibitor treatment in clinical trials, it is necessary to investigate its long-term effect on healthy offspring in rodent models.

Heritability of IIM in microglia

In general, the key evidence of the reproductive origin of ASD from an immunological point of view is built on the innate immune memory of microglia. As shown before, the average lifespan of microglia is 15 months in the mouse cortex (50% of their life; Füger et al., 2017) and 4.2 years in humans (0.05% of our life, estimated with an average life expectancy of 80; Réu et al., 2017), one key question is how long the effect of MIA can last? In other words, if there is no second hit in the offspring within the lifespan of microglia, then MIA will not exert any effect on the pathogenesis of ASD. Experimentally, it is possible to conduct *in vivo* studies on mice to predict an optimal time-window during which MIA offspring will be the most affected by a second hit.

Clinically, before human MIA offspring enters adolescence, a period with greater risks of a second hit, the microglia which possess the memory of MIA may have already renewed itself. On that account, another key question would be whether the innate immune memory can be inherited by daughter cells and for how long? In other words, if the IIM cannot be inherited, there is no need to worry about the effect of MIA, which would expire before adolescence. On the other hand, if IIM can be inherited, it is important to study for how many generations it can be passed onto, e.g. to compare the IIM *in vitro* between cells from microglial cell lines, and cells from the same cell line but treated with substances that stop cell proliferation. Subsequently, future studies can also consider developing drugs to accelerate the turnover of microglia so that the effect of MIA can be nullified before at-risk individuals reach the sensitive time window.

What might also be interesting is to investigate whether IIM can be passed on from MIA offspring to their offspring. Specifically, since epigenetic reprogramming of murine macrophages underlies the molecular foundation for innate immune memory (Netea et al., 2016; Netea et al., 2020), evidence is needed for whether this reprogramming can also influence the germline in rodent models. Overall, the integration of the two-hit rodent model of ASD and the innate immune memory of microglia is an ongoing topic and more experiments are required to answer the influence of IIM in microglial cells on the pathogenesis of ASD.

Conclusions

Taken together, the reproductive origin of ASD from an immunological point of view posits that the prenatal risk factors can induce immune activation in pregnant women, which leads to cytokine dysregulation in the fetal brain. Microglia retain the memory of the immune challenge, thereby enhancing the susceptibility to a second immune challenge in the offspring during childhood or adolescence. As a result, the risk of ASD is enhanced in the adult MIA offspring, which might be inherited by their offspring and persist in the family (see figure 7).

The current paper is the first to integrate the prenatal risk factors and to suggest that they all act on the pathogenesis of ASD via maternal immune activation. This framework offers a more straightforward and unified understanding of the reproductive origin of ASD from the regulatory role of the innate immune memory of microglia in cytokine signaling.

Subsequently, possible treatments targeted at the innate immune memory of microglia might be a breakthrough for prevention of ASD. Given a higher risk of immune activation in pregnant women during the 2019-nCoV outbreak, we advocate the initiation of clinical trials regarding IIM treatment in MIA offspring to prevent a potential surge of ASD incidences. Future studies should also focus on developing a biomarker as an indication of confirmational changes in microglial transcriptomic profile to offer selective treatment. It would be important to combine both maternal and postnatal immune activations of different nature, timing, and severity to study the exact reproductive origin of ASD and other neurodevelopmental disorders.

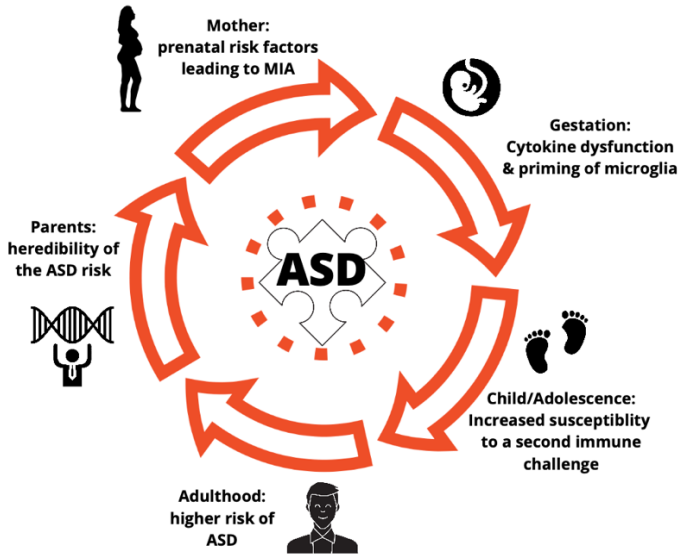


Figure 7. A complete illustration of the reproductive origin of ASD from an immunological perspective (modified from Estes & McAllister, 2016).

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