Interleukin-1β/Interleukin10 Ratio Produced by Monocytes as a Biomarker of Neuroinflammation in Autism

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Abstract

Objective: Innate immune abnormalities have been frequently reported in children with autism spectrum disorders (ASD), but a role of innate immunity in ASD is not well understood. This study explored a possible role of innate immunity in ASD clinical features and co-morbidities.

Methods: Purified peripheral blood monocytes (PBMo) from ASD (N=125) and non-ASD (N=36) subjects were cultured overnight with or without stimulants of innate immunity, and production of pro-inflammatory and counter-regulator cytokines were assessed. Behavioral symptoms were assessed by aberrant behavioral checklist (ABC) at the time of PBMo sampling.

Results: ASD PBMo revealed highly variable IL-1β/IL-10 ratios, in contrast to a tight range of IL-1β/IL-10 ratios in non-ASD control cells. There was no association between cytokine levels or IL-1β/IL-10 ratios and ABC subscale scores when ASD data was analyzed, as a whole. However, when ASD data was separated into high, low, or normal (equivalent to controls) IL-1β/IL-10 ratio groups, IL-1β levels were positively associated with stereotype in the high ratio group. In contrast, IL-1β and IL-10 levels were negatively associated with irritability, lethargy, and hyperactivity in the normal ratio group. The low ratio group revealed a negative association between IL-1β levels and lethargy. When longitudinal changes in cytokine production from PBMo were studied in selected ASD subjects, fluctuating ratios were found in ASD subjects with deviated (high or low) IL-1β/IL-10 ratios, but ratios remained stable in ASD subjects with normal ratios. ASD subjects with deviated ratios were found to have higher frequencies of non-IgE mediated food allergy (NFA) (p<0.05) and seizure disorder (p<0.01) than those with normal ratios.

Conclusion: IL-1β and IL-10 produced by innate immune responses play crucial roles in the neuroimmune network. Thus the deviated (high or low) IL-1β/IL-10 ratio from ASD monocytes may be a promising candidate biomarker for assessing changes of neuroimmune regulations and risk of comorbidities (NFA and seizure disorders) in ASD.

Keywords: Autism spectrum disorder (ASD); Biomarker; Cytokine, Interleukin-1β (IL-1β); Interleukin-10 (IL-10); Monocytes; Neuroinflammation

Abbreviations: ABC: Aberrant Behavior Checklist; ADI-R: Autism Diagnostic Inventory revisited; ADOS: Autism Diagnostic Observational Scale; ASD: Autism Spectrum Disorder; BMDM cells: Bone Marrow Derived Microglial Cells; CNS: Central Nervous System; CHFQ: Children’s Sleep Habit Questionnaires; Fpies: Food Protein Induced Enterocolitis Syndrome; IL: Interleukin; MIA: Maternal Immune Activation; NCCPC: Non-Communicating Children’s Pain Checklist; NJMS: New Jersey Medical School; NFA: Non-IgE Mediated Food Allergy; PB: Peripheral Blood; PBMCs: Peripheral Blood Mononuclear Cells; PBMo: Peripheral Blood Monocytes; Poly I:C: Polyinosinic:Polycytidylic Acid; PRR: Pattern Recognition Receptor; PST: Prick Skin Testing; SPUH: Saint Peter’s University Hospital; TLR: Toll Like Receptor; TNF: Tumor Necrosis Factor; VABS: Vineland Adaptive Behavioral Scale.

Introduction

Mounting evidence has indicated that immune mediated inflammation likely has a role in the onset and development of autism spectrum disorders (ASD) [1]. This is partly due to the high prevalence of co-morbid conditions that are associated with immune mediated inflammation in ASD subjects, and convincing evidence indicating a role of maternal inflammation in ASD [1,2]. In animal models of ASD, mothers who were induced to have inflammation during mid-gestation, had offspring that developed ASD symptoms after birth. This animal model of ASD is known as the maternal immune activation (MIA) model, and has been widely used [3,4]. Additional evidence of immune mediated inflammation comes from the analysis of transcriptome and protein expression in the post-mortem brains of ASD subjects [5]. However, immune activation was only observed in some, but not all ASD brains examined, indicating the possible presence of an ASD subset, which may be categorized as immune or inflammatory autism [5].

Even with these intriguing studies, the etiology of immune abnormalities in ASD children has been difficult to elucidate [1]. This is partly attributed to the fact that current diagnostic measures of ASD mainly rely on behavioral observation, resulting in markedly heterogeneous ASD subjects. Only a small fraction of ASD patients have defined gene mutations that result in an ASD phenotype [6]. In most ASD subjects, complex gene-gene and gene-environmental interactions likely play a role, as typically seen in polygenic diseases [7].

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The immune abnormalities reported in ASD involve almost every arm of the immune system, however, such immune abnormalities are found only in a portion of ASD subjects. This makes it difficult to understand which immune abnormalities have a crucial or primary role in ASD pathogenesis. However, among the immune abnormalities reported in the literature, dysregulated innate immune responses have been most consistently described [1]. Supporting evidence of the role that innate immunity plays in ASD is summarized as follows:

1) In the MIA model, ASD like behavioral symptoms are induced in offspring by provoking maternal inflammation during pregnancy by activating innate immunity. The most common method used in the MIA model is the injection of an agonist of toll like receptor 3 (TLR3), to pregnant rodents. TLRs are major receptors of innate immunity, and activation of TLRs by their agonists lead to sterile inflammation [3]. TLR3 is typically activated by various viral byproducts. Other types of stimulants of innate immunity have also been used for creating MIA models [3]. Findings in the MIA models are also consistent with the results of epidemiological study that maternal illness associated with immune mediated inflammation increases the risk of attention deficit hyperactivity disorder (ADHD) [8]. MIA model results may also be consistent with higher levels of inflammatory cytokines in ASD cord blood than in cord blood from typically developing children [9].

2) Macrophage/monocyte lineage cells, major innate immune cells, have been most consistently reported to show abnormalities in ASD subjects [1].

3) Inflammation triggered by innate immunity has been shown to play a role in other neuropsychiatric diseases, such as depression and schizophrenia, especially in the onset and early stages [10-13].

4) Our previous studies examined the association between clinical phenotypes of ASD and immune abnormalities. These studies found that clinical features indicating immune abnormalities are unlikely to be associated with specific pathogens, but are more likely to be associated with dysregulated innate immune responses [14-16]. Others also reported that under the LPS stimulated cultures, increases in production of certain cytokines by PBMCs, are associated with ASD severity in a total of 50 young ASD subjects [17].

Based on the above-described findings, we hypothesized that in ASD children who have a component of immune mediated inflammation, intrinsic innate immune abnormalities would be detected. We also hypothesized that such intrinsic abnormalities would be better characterized with the use of purified PBMo, major innate immune cells in the peripheral blood (PB). In this study, we have examined the cytokine production by PBMo in a total of 125 ASD subjects, in comparison to their co-morbid conditions and ASD behavioral symptoms. Our results indicate that the IL-1β/IL-10 ratios produced by PBMo, under various culture conditions, can be a promising biomarker for a deregulated neuroimmune network and a risk of certain co-morbid conditions.

### Methods and Materials

#### Study subjects:
The study followed the protocols approved by the Institutional Review Board at our institutions, Rutgers-New Jersey Medical School (NJMS) and Saint Peter's University Hospital (SPUH).

**ASD subjects:** ASD subjects were recruited in the Pediatric Allergy/Immunology clinic. Diagnosis of ASD in the study subjects was made at various autism diagnostic centers, including ours. The ASD diagnosis was based on the Autism Diagnostic Observation Scale (ADOS) and/or Autism Diagnostic Interview-Revisited (ADI-R), and other standard measures. For those who lack verification of ASD diagnosis, the ADOS and/or ADI-R were administered to confirm the diagnosis. Any subjects with deafness/blindness, any motor disability, such as cerebral palsy, or medical conditions with known gene mutations were excluded from the study. ASD subjects were also evaluated for their behavioral symptoms, sleep habits, and adaptive skills, by using previously validated questionnaires. These include the Aberrant Behavior Checklist (ABC) [18], Children’s Sleep Habits Questionnaires (CSHQ) [19], and Vineland Adaptive Behavior Scale (VABS) [20]. If the VABS was done as part of a school/medical evaluation within 1 year of enrollment, such data were used as a part of behavioral evaluation. In most ASD subjects, information of their cognitive activity was obtained through school records.

**Non-ASD controls:** Typically, developing, non-ASD control subjects were recruited in the pediatric Allergy/Immunology Clinic.

Demographic information of the study subjects is summarized in Table 1.

#### Diagnosis of food allergy (FA): IgE mediated FA was diagnosed with reactions to offending food, by affecting skin, GI, and/or respiratory tract immediately after intake of offending food (within 2 hours), supported by prick skin testing (PST) reactivity, and/or presence of food allergen-specific IgE in the serum. NFA was diagnosed with resolution of GI symptoms following implementation of a restricted diet (i.e., avoidance of offending food), and recurrence of symptoms upon reintroduction of offending food, following the Food Allergy Diagnostic Guidelines [21]. NFA patients are per definition, non-reactive to PST, and negative for food allergen-specific, serum IgE [21].

#### Diagnosis of asthma and allergic rhinitis: Allergic rhinitis (AR) and allergic conjunctivitis (AC) were diagnosed with positive PST reactivity, and/or presence of allergen-specific IgE in the serum. AR was diagnosed with resolution of AR symptoms following implementation of a restricted diet (i.e., avoidance of offending food), and recurrence of symptoms upon reintroduction of offending food, following the Food Allergy Diagnostic Guidelines [21]. NFA was diagnosed with resolution of AR symptoms following implementation of a restricted diet (i.e., avoidance of offending food), and recurrence of symptoms upon reintroduction of offending food, following the Food Allergy Diagnostic Guidelines [21]. NFA was diagnosed with resolution of AR symptoms following implementation of a restricted diet (i.e., avoidance of offending food), and recurrence of symptoms upon reintroduction of offending food, following the Food Allergy Diagnostic Guidelines [21].

#### Antibody deficiency syndrome: Specifc polysaccharide antibody deficiency (SPAD) was diagnosed by the absence of detectable antibody (Ab) titers (more than 1.3 µg/mL) to more than 11 of 14 serotypes of Streptococcus pneumonia, following a booster dose of Pneumovax® [25], a standard diagnostic measure for SPAD.

#### Sample obtainment: Peripheral blood (PB) samples were obtained by venipuncture after obtainment of informed consent. Efforts were made

### Table 1: Demographic information of the study subjects

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Age (year) Median (range)</th>
<th>Gender (male: female)</th>
<th>Ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASD subjects (N=125)</td>
<td>12.3 (2.6-27.0)</td>
<td>97:28:00</td>
<td>9 AA, 16 Asians, 8 mixed, 92 W</td>
</tr>
<tr>
<td>Normal control (N=36)</td>
<td>11.9 (N=36)</td>
<td>97:28:00</td>
<td>3 Asians, 4 mixed, 29 W</td>
</tr>
</tbody>
</table>

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to obtain the PB samples at the time of routine blood work in order to minimize the numbers of venipuncture in all the study subjects. For the non-ASD control subjects, only 1 sample was obtained. For ASD subjects with fluctuating behavioral symptoms and varying GI symptoms, we attempted to obtain at least 2 samples, one when behavioral symptoms were at what was considered their baseline and another when parents reported exacerbation of behavioral symptoms. Venipuncture was conducted by the physician and if requested, the site of venipuncture was numbed by applying a topical lidocaine/prilocaine cream (Emla cream®).

Cell cultures: Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. PBMCs were purified by negatively selecting PBMCs depleting T, B, natural killer, and dendritic cells, using magnetic beads labeled with anti-CD3, CD7, CD16, CD19, CD56, CD123, and glycoporphin A (monocyte separation kit II – human, MILTENYI BIOTEC, Cambridge, MA).

Innate immune responses were assessed by incubating purified PBMo (2.5 × 10^5 cells/ml) overnight with a TLR4 agonist (LPS; 0.1 µg/ml), CD16, CD19, CD56, CD123, and glycoporphin A (monocyte separation kit II – human, MILTENYI BIOTEC, Cambridge, MA).

Levels of pro-inflammatory (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, IL-12p40, and IL-23) and anti-inflammatory cytokines (IL-10, IL-17, IL-6, IL-10, IL-12p40, and IL-17A, TNF-α) were obtained from BD Biosciences and R & D Systems (sTNFRII) cytokines in the culture supernatant were measured (ELISA). The ELISA, OptEIA™ Reagent Sets for IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-12p40, and TNF-α (BD Biosciences), and for sTNFRII, IL-17 (IL-17A), and TGF-β were obtained from BD Biosciences and R & D Systems (sTNFRII) cytokines in the culture supernatant were measured (ELISA). The ELISA, OptEIA™ Reagent Sets for IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-12p40, and TNF-α (BD Biosciences), and for sTNFRII, IL-17 (IL-17A), and TGF-β were obtained from BD Biosciences and R & D (Minneapolis, MN), respectively. IL-23 ELISA kit was purchased from eBiosciences, San Diego, CA. Intra- and inter-variations of cytokine levels were less than 5%.

Statistical analysis

For comparison of values win the 2 groups, Wilcoxon rank sum test or Mann-Whitney test were used. For differences in frequency between the two groups and correlation of two data sets, Fisher exact test, and Spearman’s test were used, respectively. A p value of <0.05 was considered nominally significant.

Results

Cytokine production by peripheral blood monocytes (PBMo)

Highly variable IL-1β and IL-10 ratios in ASD subjects: An association between IL-1β and IL-10 in ASD cells was much more varied than in control cells: non-ASD control PBMo revealed a tight positive association between IL-1β and IL-10 under cultures without a stimulus (Figure 1A) or with stimuli (zymosan (Figure 1B), and β-lactam (Figure 1C). This finding is reflected in highly varied IL-1β/IL-10 ratios in ASD PBMo, in contrast to the narrow range of the IL-1β/IL-10 ratios in non-ASD control cells in all the culture conditions (medium only, or with LPS, zymosan, CL097, or β-lactam) (Figure 2).

In select ASD subjects, cytokine production by PBMo was longitudinally measured at multiple time points. The results show that IL-1β/IL-10 ratios fluctuated in some ASD subjects, while remained stable in others (Figure 3).

Subgrouping ASD cells based on IL-1β/IL-10 ratios: Given the findings described above, we assessed whether there was any association between cytokine profiles and ASD behavioral symptoms. ABC subscale scores, assessed at the time of each blood sampling, revealed no association with levels of IL-1β and IL-10 or IL-1β/IL-10 ratios when ASD samples were analyzed, as a whole (p>0.05). However, we observed changes in association between levels of IL-1β and/or IL-10 levels, and ABC subscale scores, when ASD PBMo samples were divided into 3 groups, based on IL-1β/IL-10 ratios, as follows:

- **Group 1**: Normal IL-1β/IL-10 ratios, defined as –1 SD<IL-1β/IL-10 ratios<1SD under all the culture conditions, or 1SD<IL-1β/IL-10 ratios<2SD under only one culture condition.
- **Group 2**: Low IL-1β/IL-10 ratios, defined as IL-1β/IL-10 ratios<1SD than control cells under at least 1 culture condition.
- **Group 3**: High IL-1β/IL-10 ratios, defined as IL-1β/IL-10 ratios>2SD than control cells at least under 1 culture condition and/or >1 SD under more than 2 culture conditions.

Association of behavioral symptoms and IL-1β and IL-10 levels changes, depending on IL-1β/IL-10 ratios: In Group 1 (normal IL-1β/IL-10 ratio group), negative associations were observed between IL-1β levels and ABC subscale scores (subscale I (irritability), subscale II (lethargy), subscale III (stereotypy), and subscale IV (hyperactivity)) under multiple culture conditions, as summarized in Table 2. This negative association was also observed between irritability and IL-10 levels in Group 1, in cultures with zymosan and β-lactam (Table 3). In contrast, in Group 3 (high ratio group), a positive, but not a negative association was observed between stereotypy and IL-10 levels in cultures with CL097, a TLR7/8 agonist (Table 2). In Group 2 (low ratio group), lethargy scores were negatively associated with IL-1β levels in cultures with LPS and zymosan (Table 2), and IL-10 levels in cultures with β-lactam (Table 3). We did not observe any significant association between the IL-1β/IL-10 ratios and ABC scores, except for a negative association with lethargy in Group 2 (Table 3). Positive and negative associations between IL-1β levels and stereotypy in representative cases are shown in Figure 4.

It should be noted that ABC subscale scores did not differ between Groups 1, 2, and 3, except for lower irritability and lethargy scores in Group 2 as compared to Groups 1 and 3 (Figure 5). This may be associated with the fact that low IL-1β/IL-10 ratios were associated with low lethargy and irritability scores in some ASD subjects who were longitudinally studied, as shown in a representative case (Figure 4, Case 1).

A higher frequency of comorbid conditions in the ASD subjects with high/low IL-1β/IL-10 ratios: In longitudinal studies, we found that the IL-1β/IL-10 ratios fluctuate over time in some ASD subjects, revealing high and low ratios, depending on time points (Figure 3). This makes it difficult to separate into ASD subjects into high, low, and normal IL-1β/IL-10 ratio groups, as defined in the previous section. Therefore, we divided ASD subjects into ASD subjects with high/low IL-1β/IL-10 ratios, and those with normal IL-1β/IL-10 ratios, when frequencies of comorbid conditions were evaluated. Results are summarized in Table 4. ASD subjects with high/low ratios revealed higher frequencies of non IgE mediated food allergy (NFA) and seizure disorders than those with normal ratios (Table 4). Specific antibody deficiency tended to be higher in the ASD subjects with the high/low IL-1β/IL-10 ratios (p=0.082). There was no difference in cognitive activity between these 2 ASD groups.
Figure 1: A positive association between IL-1β and IL-10 produced by ASD PBMo (N=178) and normal control cells (N=36) in the absence of stimulus (Panel A), or in the presence of zymosan (Panel B) or β-lactam (Panel C). We also observed a similar positive association with LPS in control cells (r=0.548, p<0.001), and less clearly in ASD cells (r=0.206, p<0.05).

Figure 2: IL-1β/IL-10 ratios in ASD and control cells in the absence of stimullus (medium only) or in the presence of stimuli (LPS, zymosan, CL097, and β-lactam).
Figure 3: Longitudinal changes in the IL-1β/IL-10 ratios in 6 representative ASD cases. Cases 1-4 revealed fluctuating IL-1β/IL-10 ratios, while Cases 5-6 revealed stable ratios.

<table>
<thead>
<tr>
<th>Group</th>
<th>I Irritability (I)</th>
<th>II Lethargy</th>
<th>III Stereotypy</th>
<th>IV Hyperactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.0421</td>
<td>0.0228</td>
<td>0.0191</td>
<td>0.1252</td>
</tr>
<tr>
<td>LPS</td>
<td>-0.1873</td>
<td>-0.4776 (p&lt;0.05)</td>
<td>-0.048</td>
<td>-0.1853</td>
</tr>
<tr>
<td>Zymosan</td>
<td>-0.3731 (p=0.0795)</td>
<td>-0.4637 (p&lt;0.05)</td>
<td>0.1442</td>
<td>-0.1958</td>
</tr>
<tr>
<td>CL097</td>
<td>-0.0144</td>
<td>-0.231</td>
<td>-0.1109</td>
<td>-0.0547</td>
</tr>
<tr>
<td>β-lactam</td>
<td>-0.2106</td>
<td>-0.3759 (p=0.0771)</td>
<td>-0.1164</td>
<td>-0.2869</td>
</tr>
</tbody>
</table>

Table 2: Association between IL-1β levels by PBMo and ABC subscale scores in Groups 1-3. The data points in the groups 1-3 are as follows: Group 1 (N=50), Group 2 (N=23), and Group 3 (N=39). We did not include data points where ABC scores were not available for analysis. R (correlation coefficient) by Spearman’s test.
Figure 4: Scores of ABC subscale I, II, III, and IV in groups 1, 2, and 3. Grouping of SBC subscale data were based on IL-1β/IL-10 ratios as detainted in the results section.

<table>
<thead>
<tr>
<th>Group 11</th>
<th>I Irritability</th>
<th>II Lethargy</th>
<th>I Irritability</th>
<th>II Lethargy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.3776</td>
<td>0.0241</td>
<td>0.3387</td>
<td>0.0456</td>
</tr>
<tr>
<td>LPS</td>
<td>0.115</td>
<td>0.2391</td>
<td>0.0437</td>
<td>0.1156</td>
</tr>
<tr>
<td>Zymosan</td>
<td>0.0222</td>
<td>0.0437</td>
<td>0.0187</td>
<td>0.1156</td>
</tr>
<tr>
<td>β-lactam</td>
<td>-0.0051</td>
<td>-0.0873</td>
<td>-0.1255</td>
<td>-0.04739</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2</th>
<th>I Irritability</th>
<th>II Lethargy</th>
<th>I Irritability</th>
<th>II Lethargy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>-0.2247</td>
<td>-0.2032</td>
<td>-0.0777</td>
<td>-0.24756</td>
</tr>
<tr>
<td>LPS</td>
<td>-0.2546</td>
<td>-0.2084</td>
<td>-0.23724</td>
<td>-0.24887</td>
</tr>
<tr>
<td>Zymosan</td>
<td>-0.3431 (p&lt;0.05)</td>
<td>-0.2582</td>
<td>0.19692</td>
<td>-0.12748</td>
</tr>
<tr>
<td>CL097</td>
<td>-0.2142</td>
<td>-0.0518</td>
<td>-0.05632</td>
<td>-0.19132</td>
</tr>
<tr>
<td>β-lactam</td>
<td>-0.3562 (p&lt;0.05)</td>
<td>-0.2059</td>
<td>0.09165</td>
<td>0.10877</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3</th>
<th>I Irritability</th>
<th>II Lethargy</th>
<th>I Irritability</th>
<th>II Lethargy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0.0636</td>
<td>0.1533</td>
<td>0.03203</td>
<td>0.17868</td>
</tr>
<tr>
<td>LPS</td>
<td>0.0241</td>
<td>0.2391</td>
<td>0.03087</td>
<td>0.13725</td>
</tr>
<tr>
<td>Zymosan</td>
<td>0.0022</td>
<td>0.0437</td>
<td>0.11562</td>
<td>0.11858</td>
</tr>
<tr>
<td>CL097</td>
<td>0.0869</td>
<td>0.054</td>
<td>0.0093</td>
<td>0.21392</td>
</tr>
<tr>
<td>β-lactam</td>
<td>-0.0051</td>
<td>-0.0873</td>
<td>-0.1255</td>
<td>-0.04739</td>
</tr>
</tbody>
</table>

Table 3: An association between IL-10 levels or IL-1β/IL-10 ratios by PB Mo and ABC subscales I and II scores. 

1The data points in the groups 1-3 are the same as shown in Table 2, continued. 
2R (correlation coefficient) by Spearman’s test
Discussion

Our study revealed deviations (higher or lower) in IL-1β/IL-10 ratios, in some, but not all ASD subjects, as compared to non-ASD, typically developing controls, when purified PBMo were used as a cellular source. These ratios could fluctuate over time in ASD subjects with deviated ratios, but remain stable in ASD subjects with normal ratios. Moreover, high and low IL-1β/IL-10 ratios are correlated with changes in positive or negative association between ABC behavioral scores and levels of IL-1β and IL-10 produced by PBMo.

Inflammatory mediators generated by innate immune responses, such as IL-1β, IL-6, and TNF-α, have been known to play key physiological roles in acute stress responses, allowing the body to prepare for environmental stressors [10]. However, chronic and dysregulated stress responses can cause detrimental effects, and have even been implicated in the onset and progress of many neuropsychiatric conditions. For example, chronic stress responses caused by environmental factors have been implicated in the development of depression [10]. Apart from stress responses, inflammation in the other organ systems, can also affect the brain functions. This could occur via soluble inflammatory mediators, activated immune cells migrating into the brain, and peripheral nervous system feedback [10,27-29]. These factors are also implicated in neuropsychiatric symptoms manifested in patients with systemic autoimmune and/or inflammatory conditions [27,30]. However, how the immune system affects brain function in these conditions remains poorly understood.

Innate immunity is thought to be closely involved in generating stress responses and brain inflammation. Microglial cells are major innate immune cells in the brain, and belong to macrophage-monocyte lineage cells. They play a role in the physiological homeostasis of the central nervous system (CNS), and are associated with its development and plasticity. In addition, microglial cells are the initial sensor of stressors, sensing stimuli of innate immunity through pattern recognition receptors (PRRs), such as TLRs, and also being activated by inflammatory cytokines [1,12,29]. Activated microglial cells, in response to stressors, closely resemble classical activated or type 1 (M1) macrophages and produce large amount of pro-inflammatory cytokines and other mediators [31].

Recently, microglial cells were categorized into 2 groups, resident microglial cells and bone marrow derived microglial (BMDM) cells. Resident microglial cells are usually in a dormant state and are engaged in homeostasis of the CNS. In contrast, BMDM cells are thought to be derived from classically activated (M1) monocytes in the periphery, which are then recruited to the brain [32]. In chronic neurodegenerative conditions, BMDM cells have been shown to play a major role in CNS pathology [32]. Activation of microglial cells, as well as up-regulated expression of chemokines that augment recruitment of monocytes, have been found in the post-mortem brains of ASD subjects [33,34]. Both resident and BMDM cells can also be directly activated by stressors (i.e., stimuli of innate immunity) in the brain.

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Table 4: Frequencies of comorbid medical conditions in ASD subjects with high/low IL-1β/IL-10 ratios or those with normal ratios. *FPIES: Food Protein Induced Enterocolitis Syndrome; SAD: Specific Antibody Deficiency; AR: Allergic Rhinitis; †Results of Cognitive activity evaluation were obtained from recent school evaluation or by Woodcock-Johnson test.

<table>
<thead>
<tr>
<th>Condition</th>
<th>High/low IL-1β/IL-10 ratios (N=62)</th>
<th>High/low IL-1β/IL-10 ratios (N=62)</th>
<th>p-value (Fisher’s exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQ&lt;1%</td>
<td>46 (74.2%)</td>
<td>39 (61.9%)</td>
<td>0.5746</td>
</tr>
<tr>
<td>Seizure disorder</td>
<td>10 (16.1%)</td>
<td>1 (1.6%)</td>
<td>0.0052</td>
</tr>
<tr>
<td>FPIES*</td>
<td>47 (75.8%)</td>
<td>24 (38.1%)</td>
<td>0.026</td>
</tr>
<tr>
<td>SAD</td>
<td>14 (22.6%)</td>
<td>5 (7.6%)</td>
<td>0.0623</td>
</tr>
<tr>
<td>AR</td>
<td>7 (11.3%)</td>
<td>5 (7.9%)</td>
<td>0.764</td>
</tr>
<tr>
<td>Asthma</td>
<td>8 (12.9%)</td>
<td>4 (6.3%)</td>
<td>0.367</td>
</tr>
</tbody>
</table>

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Figure 5: A comparison between changes in ABC subscale III (stereotypy) scores and IL-1β levels produced by PBMo in representative 3 ASD cases. Case 1 belongs to the high IL-1β/IL-10 ratio group (Group 3), while Cases 2 and 3 belong to the normal IL-1β/IL-10 group (Group 1). Changes in IL-1β levels were more drastic in the Case 1 and lower IL-1β level were observed when the subject show lower subscale III (Stereotypy) scores. While in cases 2 and 3, higher subscale III (Stereotypy) scores revealed lower IL-1β levels, although changes are not as drastic as observed in the Case 1.
Balanced IL-1β/IL-10 ratio | High IL-1β/IL-10 ratio | Low IL-1β/IL-10 ratio
---|---|---
Immune mediated inflammation may have a less role in ASD behavioral symptoms | No suppression | IL-10 mediated control
Aggravated behavioral symptoms by dysregulated inflammatory cytokine production | Suppression of aberrant behaviors | Increased risk of microbial infection due to excessive IL-10 production
Vulnerable to immune mediated co-morbid conditions | Fluctuating behavioral symptoms | A higher frequency of immune mediated comorbidities

**Figure 6**: Graphical summary of potential implications of our findings.

Based on this information, we hypothesized that PBMo can be utilized as surrogates of BMDM cells, for assessing innate immune abnormalities and dysregulation in the neuroimmune network in ASD subjects. Consistent with our previous findings [15], we observed variable cytokine production profiles in ASD subjects. The most notable differences observed between ASD and control PBMo were the production of IL-1β, an inflammatory cytokine, and IL-10, a counter-regulatory cytokine. In non-ASD control cells, we observed less variability in the values of these cytokines along with a tight positive association between IL-1β and IL-10 (Figure 1). This may indicate the presence of counter-regulatory measures in response to increases in IL-1β production, as we previously reported [16]. We also found increased variability in the levels of IL-1β and IL-10 produced by ASD PBMo, rendering the positive association between these two cytokines less evident (Figure 1). When IL-1β and IL-10 levels produced were expressed as IL-1β/IL-10 ratios, normal control cells revealed a tight range for the ratio, while in numbers of ASD subjects’ cells, L-1β/IL-10 ratios deviated from controls cells, having either higher or lower ratios, in all the culture conditions (Figure 2). In selected ASD cases, for whom cytokine production by PBMo was measured at multiple time points, we found fluctuating IL-1β/IL-10 ratios in ASD subjects with deviated IL-1β/IL-10 ratios, while in those with normal ratios, the ratios remained stable (Figure 3).

IL-1β is known to be a key mediator of acute stress responses. However, IL-1β also plays a crucial role in brain inflammation and subsequent brain pathology in multiple neurological conditions. In febrile seizures, IL-1β has been shown to play a major role [35]. Certain auto-inflammatory syndromes are caused by gene mutations that result in dysregulated, excessive IL-1β production [36]. These subjects are known to manifest various neuropsychiatric symptoms and seizures [36,37]. In rodent models of seizures, treatment-resistant seizures were shown to be associated with neuro-inflammation triggered by IL-1β [38]. In experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis, transmigration of monocyte-macrophage lineage cells, was also shown to trigger IL-1β-driven neuro-inflammation [39].

Counter regulatory cytokines, such as IL-10 also play an important role in maintaining immune homeostasis [40]. Dysregulated IL-10 production/expression has been reported in multiple autoimmune conditions, including psoriasis and inflammatory bowel disease [41]. Studies have shown that IL-10 knock-out mice spontaneously develop enterocolitis, resembling inflammatory bowel diseases [42]. Given these findings, it is expected that the balance between IL-1β and IL-10 production is tightly regulated at transcriptional and post-transcriptional levels. Therefore, our findings indicate a possibility that ASD children with higher IL-1β/IL-10 ratios may have impaired regulatory mechanisms for maintaining balanced IL-1β and IL-10 production by innate immune cells. If so, it raises another question as to whether IL-1β/IL-10 ratios are associated with any changes in ASD behavioral symptoms.

When we examined an association between ASD behavioral symptoms and the IL-1β/IL-10 ratios as a whole, no direct association was found. However, when we subdivided ASD samples groups with higher, lower, or equivalent levels of IL-1β/IL-10 ratios, as compared to non-ASD controls, we found significant differences in association between IL-1β/IL-10 levels and ABC subscale scores. We found a negative association between ABC subscales I (irritability), II (lethargy), III (stereotype), and IV (hyperactivity) scores and IL-1β levels produced, when ASD PBMo had the IL-1β/IL-10 ratios equivalent to normal controls. IL-10 levels were also negatively associated with ABC subscales I (irritability) and II (lethargy). In contrast, when ASD PBMo produced higher IL-1β/IL-10 ratios, the reverse was seen. That is, IL-1β levels were positively associated with ABC subscale III (Stereotypy). When ASD PBMo produced lower IL-1β/IL-10 ratios, negative association was seen between IL-1β levels and lethargy alone. Our results may indicate that when production of IL-1β and IL-10 is in balance, these cytokines produced by innate immune cells, are helpful in controlling behavioral symptoms, possibly suppressing aberrant behaviors. However, when balance between IL-1β and IL-10 is in disarray, IL-1β may exert deleterious effects on ASD behavioral symptoms, in part, due to the loss of the counter-regulatory action of IL-10 (Figure 6). When counter-regulatory actions of IL-10 are excessive with lower IL-1β/IL-10 ratios, ASD behaviors may be better controlled, as shown by less irritability and less lethargy (Figure 5). However, excessive IL-10 production may predispose such ASD subjects to frequent infection, given IL-10’s potent immune-suppressive actions. Then such immune insults may reverse the immune regulatory status to higher IL-1β/IL-10 ratios again, resulting in fluctuating IL-1β/IL-10 ratios as observed in some ASD subjects (Figure 3).

We also assessed if there was any association in changes in the IL-
1β/IL-10 ratios with co-morbid medical conditions. Since ASD subjects with deviated IL-1β/IL-1 ratios revealed high or low ratios, depending on time points, we compared the clinical features between ASD subjects with deviated IL-1β/IL-10 ratios vs. those with ratios equivalent to normal controls. ASD subjects with deviated ratios were found to have higher frequencies of non-IgE mediated food allergy (NFA) and seizure disorders than in those with normal ratios as summarized in Table 4. These results are intriguing, given the well-described role of IL-1β in febrile seizures and rodent models of seizures [35,38]. Our results also indicate that ASD subjects with the deviated IL-1β/IL-10 ratios may represent a subset of ASD subjects in whom dysregulated innate immune responses play a role in alterations in the neuro-immune network (Figure 6). The IL-1β/IL-10 ratios by PBMo may help identify such ASD subjects early, which could lead to control neuroinflammation in early stages.

Although behavioral symptoms were assessed with the use of well validated questionnaires, the numbers of ASD subjects longitudinally examined by both cytokine production profile and ABC scores were limited in this study. Assessment of ASD behavioral symptoms and cytokine profiles by PBMo prospectively in larger numbers of ASD subjects will be required to validate our initial results. It should also be cautioned that associations between the IL-1β/IL-10 ratios and clinical features found in ASD subjects may change with age. It will be critical, for future studies, to examine IL-1β/IL-10 ratios in randomly selected ASD subjects of variable ages, along with age-appropriate non-ASD controls, and track changes in their clinical features, prospectively, over several years. This strategy will allow for the assessment of which parameters of innate immunity may best serve as biomarkers for addressing the role of innate immunity plays in ASD.

In summary, our study revealed that the IL-1β/IL-10 ratios from PBMo are promising candidate biomarkers for addressing dysregulated neuro-immune network in ASD. This parameter can also be helpful in identifying ASD subjects at risk for co-morbid conditions associated with innate immune abnormalities at an early stage, especially, if tested at multiple time points. Identifications of such ASD subjects may lead to much needed treatment options that focus on controlling immune mediated inflammation.

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Author’s Contributions

HJ was responsible for the study design, recruitment of the study subjects, collection of clinical information and blood samples, analysis of the overall data, and preparation of most of this manuscript. LG conducted cytokine production assays, and assisted cytokine data analysis. SB was responsible for statistical analysis of clinical data and cytokine production data. All the authors read and approved the final forms of manuscript.

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