VDTP Innovation Passport Application

Full-Text References Pack
June 2025

This reference pack accompanies the Innovation Passport application submitted to the UK Medicines and Healthcare products Regulatory Agency (MHRA) for **Vitamin D Transport Protein (VDTP)**, a novel immune-modulating therapy developed by **Vita Vitalia Ltd** for Autism Spectrum Disorder.

In accordance with MHRA **ILAP submission guidance**, this document consolidates five full-text references cited throughout the application. These references provide supporting evidence for VDTP's mechanism of action, safety rationale, relevance to autism-related immune dysfunction, and the broader scientific basis for its proposed development as a biologic.

Each article is presented in full text, in the order referenced within the application, and reflects a balance of primary research, clinical observations, and mechanistic studies.

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Exploring Immune Modulation in Autism: The Role of Vitamin D Transport Proteins

Autism Spectrum Disorders (ASD) are complex conditions at the intersection of neuroscience, immunology, and human experience. While the exact causes of ASD remain under investigation, growing evidence suggests that imbalances in the immune system, such as persistent inflammation, overactive immune cells in the brain, and disrupted chemical signalling between cells, may play a role in its development and symptoms.

ASD is a spectrum disorder, meaning that individuals can experience a wide range of symptoms that vary in severity and presentation. These symptoms typically fall into four key areas:

- **Speech & Communication** – Many individuals with ASD face delayed speech development, difficulty forming sentences, or challenges in expressing their thoughts and emotions. Some may have limited verbal ability, while others may develop unusual speech patterns, such as echolalia (repeating words or phrases).
- **Social Skills** – Difficulties understanding social cues, making eye contact, forming relationships, or engaging in reciprocal conversations are common. Some individuals may prefer solitary activities, while others struggle with interpreting emotions and body language.
- **Sensory & Awareness** – Individuals with ASD often have heightened or reduced sensitivity to sensory input, such as loud noises, bright lights, certain textures, or strong smells. Some may also experience differences in cognitive processing, attention span, or problem-solving abilities.
- **Health & Behaviour** – Many individuals with ASD experience co-occurring conditions, such as anxiety, gastrointestinal issues, sleep disturbances, or repetitive behaviours (e.g., hand-flapping, rocking, or insistence on routines). Some individuals may struggle to regulate their emotions, resulting in meltdowns or shutdowns in response to sensory or social stressors.

Given the variability in ASD symptoms, research into immune system involvement has gained interest as a potential factor in neurodevelopmental regulation. This study explores an emerging area of research: Vitamin D Transport Proteins (VDTP) and their role in immune modulation. VDTP is a vital protein in all higher animals, essential for vitamin D transport and immune regulation. It plays a key role in maintaining immune balance and defending against disease, making it a promising target for potential therapeutic interventions in ASD.

The Prevalence of Autism Spectrum Disorders

Autism Spectrum Disorders (ASD) are complex neurodevelopmental conditions characterised by challenges in communication, social interaction, and repetitive behaviours. According to the CDC, ASD affects approximately 1 in 36 children in the U.S. [1]. In the United Kingdom, the prevalence is estimated at 1% of the population [2]. At the same time, rates in the European Union vary significantly, ranging from 0.7% in Southern Europe to as high as 1.5% in Scandinavian countries [3].

In recent decades, reported ASD cases have steadily increased, partly due to improved diagnostic criteria and greater awareness. However, rising prevalence rates also burden healthcare systems, educational institutions, and families, highlighting the urgent need for effective interventions.

The Economic Burden of ASD

Caring for individuals with ASD presents significant financial challenges. In the UK, the annual cost of supporting a child with ASD is estimated at £6,815–£29,767, depending on age, while support for adults with ASD can exceed £49,804 per year. Over a lifetime, the cost of care reaches £1.5 million for individuals with ASD and intellectual disabilities and £0.9 million for those without intellectual disabilities [4].

Special education services, parental productivity loss, and increased healthcare needs are major contributors to these costs. Prescription medication usage for ASD also varies across Europe, with Spain reporting the highest rates (100 prescriptions per 10,000 children), followed by the UK (63) and Germany (11).

The Emotional Burden on Families

Beyond financial stress, families of children with ASD often experience emotional strain, with parents facing higher divorce rates and increased mental health challenges. Studies indicate that many parents, particularly mothers who serve as primary caregivers, experience higher rates of depression, social isolation, and financial insecurity. These pressures are even more pronounced for single mothers, who often lack adequate support systems.

Given these economic and emotional burdens, there is an urgent need for effective interventions that improve quality of life, not only for individuals with ASD but also for their families.

The Role of Immune Modulation in ASD

Growing research suggests that immune system dysfunction plays a role in ASD, with studies identifying chronic inflammation, oxidative stress, and mitochondrial dysfunction as common biological markers in affected individuals [5,6]. This has increased interest in immune modulation as a potential therapeutic strategy.

One promising avenue involves Vitamin D Transport Proteins (VDTP), which may regulate immune responses more comprehensively than previously studied immune modulators, such as GcMAF [7], which primarily activates macrophages.

To better understand the role of immune proteins, it is essential to distinguish between the key proteins discussed in this study [8]:

- Vitamin D Binding Protein (VDBP): A naturally occurring protein produced in the body that binds and transports vitamin D while also playing a role in immune regulation.
- GcMAF (Gc protein-derived Macrophage Activating Factor): A laboratory-modified form of VDBP where sugar molecules are enzymatically removed to stimulate macrophage activity, enhancing immune response.
- Vitamin D Transport Protein (VDTP): This is a maternal-infant immune transfer protein found in colostrum and breast milk. It naturally provides immune modulation during early life while maintaining tolerance, potentially making it more suitable for neuroimmune conditions such as Autism Spectrum Disorder (ASD).

Study Design & Participants

This open-label study investigated the potential effects of Vitamin D Transport Proteins (VDTP) in Autism Spectrum Disorder (ASD) and related conditions. A total of 37 participants, diagnosed with ASD, received a Cytonic Food Supplement (CFS) containing VDTP.

The study was conducted under a Research-Use Only (RUO) framework, ensuring transparency and adherence to ethical standards. Participants and caregivers provided informed consent, with the option to withdraw at any time.

Data Collection & Outcome Measures

The Autism Treatment Evaluation Checklist (ATEC) [9], a widely used standardised tool, was used to track symptom progression across four core domains:

- Speech & Communication – Vocabulary use, sentence formation, verbal interaction.
- Sociability – Eye contact, social engagement, emotional responsiveness.
- Sensory & Cognitive Awareness – Sensory processing, cognitive focus, adaptability.
- Health & Behaviour – Sleep patterns, digestion, emotional regulation, anxiety.

ATEC assessments were conducted at baseline and regular intervals throughout the study. ATEC assessments and caregiver feedback were collected via a secure online platform (www.mafandmore.com), providing insights into daily behavioural improvements and functional changes.

ATEC Outcome Overview

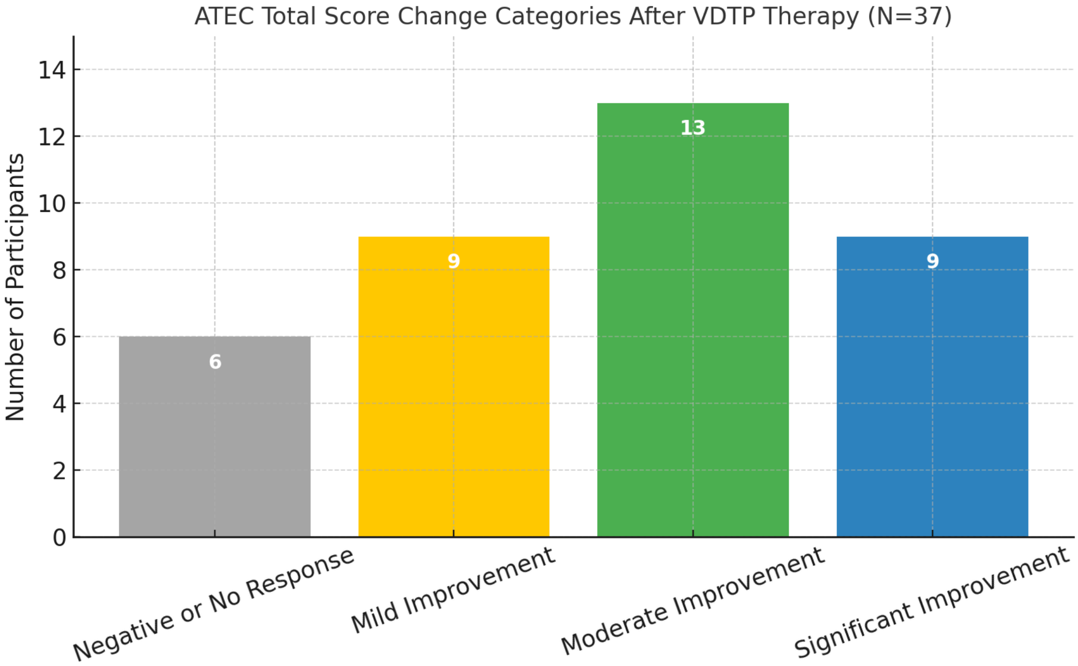

Figure 1. Individual participant data from n=37 children with ASD. Outcome categories are based on total ATEC score reduction, grouped as No Response, Mild, Moderate, or Significant Improvement.

Based on overall ATEC score reductions, participants fell into the following outcome categories:

- **6 participants (16%)** showed *negative or no response*
- **9 participants (24%)** had *mild improvement (1–29%)*
- **13 participants (35%)** showed *moderate improvement (30–49%)*
- **9 participants (24%)** demonstrated *significant improvement (≥50%)*

Domain-Specific Average Improvements (ATEC Scores)

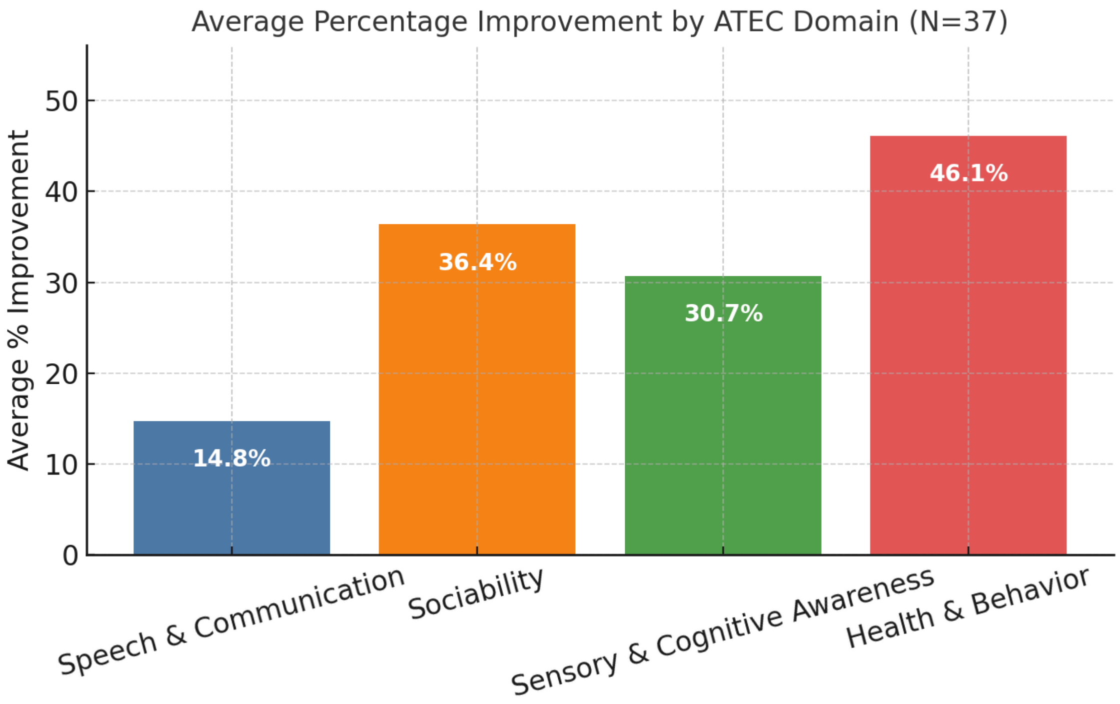

Figure 2 - Average percentage improvements across four ATEC domains *(Speech & Communication, Sociability, Sensory & Cognitive Awareness, and Health & Behaviour) following VDTP therapy.*

Average percentage improvements are shown below across the four core ATEC domains: Speech & Communication, Sociability, Sensory & Cognitive Awareness, and Health & Behaviour.

🗣️ **Speech & Communication:** 14.8%

🤝 **Sociability:** 36.4%

🧠 **Sensory & Cognitive Awareness:** 30.7%

🏥 **Health & Behaviour:** 46.1%

These findings suggest that VDTP may support a broad spectrum of symptom improvements, especially notable gains in health and sociability domains.

Table 1 - ATEC Results

Sex	Age	Diagnosis	ATEC Start	ATEC End	Change	% Change	Improvement
Male	13	ASD	3	0	3	100.00%	Significant
Male	10	ASD	61	7	54	88.50%	Significant
Male	6	ASD	81	13	68	84.00%	Significant
Male	6	ASD	12	2	10	83.30%	Significant
Male	5	ASD	31	6	25	80.60%	Significant
Male	13	ASD	15	4	11	73.30%	Significant
Male	5	ASD	52	18	34	65.40%	Significant
Male	12	ASD	33	14	19	57.60%	Significant
Male	23	ASD	46	20	26	56.50%	Significant
Male	10	ASD	21	11	10	47.60%	Moderate
Male	26	ASD	17	9	8	47.10%	Moderate
Male	5	ASD	96	52	44	45.80%	Moderate
Male	5	ASD	18	10	8	44.40%	Moderate
Male	5	ASD	67	39	28	41.80%	Moderate
Male	11	ASD	77	47	30	39.00%	Moderate
Male	7	ASD	36	22	14	38.90%	Moderate
Male	8	ASD	70	43	27	38.60%	Moderate
Male	6	ASD	35	22	13	37.10%	Moderate
Male	13	ASD	55	36	19	34.50%	Moderate
Male	14	ASD	27	18	9	33.30%	Moderate
Male	11	ASD	30	20	10	33.30%	Moderate
Male	5	ASD	26	18	8	30.80%	Moderate
Male	8	ASD	83	61	22	26.50%	Mild
Male	4	ASD	36	27	9	25.00%	Mild
Male	9	ASD	33	25	8	24.20%	Mild
Female	6	ASD	25	20	5	20.00%	Mild
Male	6	ASD	60	50	10	16.70%	Mild
Male	10	ASD	84	70	14	16.70%	Mild
Male	5	ASD	40	34	6	15.00%	Mild
Female	6	ASD	46	43	3	6.50%	Mild
Male	13	ASD	73	70	3	4.10%	Mild
Male	9	ASD	34	34	0	0.00%	No Response
Male	5	ASD	74	74	0	0.00%	No Response
Male	8	ASD	39	42	-3	-8.00%	No Response
Male	10	ASD	50	53	-3	-6.00%	No Response
Male	9	ASD	25	31	-6	-24.00%	No Response
Male	5	ASD	62	72	-10	-16.00%	No Response

Beyond the Numbers: Genuine Caregiver Experiences with VDTP

In addition to ATEC data, qualitative feedback provides vital insights into the daily-life improvements experienced by families, reflecting the real impact of VDTP.

"Within a few days of VDTP, our once nonverbal son began to talk. He now has at least a dozen words."

"My son’s ATEC score dropped from 93 to 15 after a month on VDTP—not even at full dose yet!"

"Our daughter, diagnosed with autism at age 2, has made great strides, especially since starting VDTP 18 months ago."

"ATEC dropped from 81 to 13—my son has made incredible progress with speech and learning."

"He made his first friend at school and is learning to read—this has never happened before!"

"Improvement in handwriting after two months on VDTP! Before-and-after photos show a clear difference."

"His speech and social skills therapists reported that my son is more attentive, more spontaneous, and better at conversation.”

"After starting VDTP, my son said 'Bless you' when I sneezed for the first time ever!"

"After using VDTP, my child is much happier, engaged, and showing a greater interest in socializing."

"One spray of VDTP daily, and my child is now able to play independently and with peers."

"His immune system is the strongest it has ever been. He used to get sick often, but not anymore."

"More interest in learning and participating in activities. His teacher says he’s much more engaged in class."

"He no longer runs off in social situations—before, we could never take him out safely!"

"VDTP significantly reduced aggressive behaviour and improved his cognitive ability to understand explanations."

"After years of struggling, my child is now potty trained since starting VDTP."

"His cognition and awareness have improved considerably—he understands instructions and responds appropriately."

"After three weeks of VDTP, my son has started using more words and even answering questions."
"We switched to 15000ng spray, and my daughter is showing big improvements in communication and writing."

"Since introducing VDTP, my child is calmer, less anxious, and showing significant reductions in repetitive behaviors."

"More social engagement and conversation initiation—he actually wants to play with his sister now!"
"We have seen a huge improvement in motor planning, independence, and general well-being."

"After five weeks, my child is now spontaneously forming simple sentences, something he never did before."

"His tantrums have reduced significantly, and he seems much happier and more at ease."

"For the first time ever, my son helped another child who fell—an amazing display of empathy!"

"We are so grateful—VDTP has given us hope that our child can recover and thrive!"

Clinical Implications

Immune dysfunction, chronic inflammation, and metabolic dysregulation have been identified as underlying factors in many ASD cases. The study aligns with growing evidence suggesting that targeted immune support can play a role in symptom management. VDTP's potential benefits may stem from its role in:

- Modulating immune responses without overstimulating inflammatory pathways.
- Enhancing gut-immune interactions, which are frequently disrupted in ASD.
- Supporting metabolic and mitochondrial function, which may alleviate symptoms linked to oxidative stress.

While this study provides encouraging preliminary data, controlled clinical trials are necessary to validate these effects and establish optimal dosing, long-term safety, and potential biomarker correlations.

Regulatory & Ethical Considerations

The study was conducted under a Research-Use Only (RUO) framework to comply with existing regulations. Despite promising results, regulatory agencies maintain a cautious stance on immune-modulating compounds. The challenges encountered during this research, ranging from legal scrutiny to regulatory ambiguity, highlight the need for greater transparency and collaboration between scientists, regulators, and patient advocacy groups.

Ethically, the study underscores the necessity of prioritising patient-centred research that respects scientific rigour while addressing real-world needs. Suppressing novel approaches to ASD treatment should not hinder scientific inquiry, especially when potential benefits outweigh known risks.

Future Directions

This research marks an essential step toward understanding how immune modulation may improve ASD symptoms. To build on these findings, future studies should:

1. Conduct Clinical Trials: Implement double-blind, placebo-controlled studies to establish the efficacy and safety of the treatment.
2. Investigate Biomarkers: Measure inflammatory cytokines, metabolic markers, and microbiome changes to refine mechanistic insights.
3. Explore Individualised Responses: Stratify participants based on maternal immune history, metabolic profiles, and immune status to identify the most responsive subgroups.
4. Expand Collaborative Research: Engage with academic institutions and independent research bodies to further validate and explore VDTP’s potential applications.

Conflict of Interest

The lead researcher, Trevor Banks, is the inventor of the extraction and purification process used to create the Cytonic Food Supplement, which contains VDTP. While this study was conducted under a non-commercial framework, the researcher's association with the product presents a potential conflict of interest. The findings and conclusions presented are based solely on the collected data and are intended to contribute to the scientific understanding of immune modulation in Autism Spectrum Disorder (ASD).

Disclaimer

This study is intended for research and educational purposes only. The findings do not constitute medical advice, diagnosis, or treatment recommendations. VDTP has not been approved as a medicinal product by regulatory authorities, and no efficacy or safety claims are made. Individuals considering interventions should consult with qualified healthcare professionals before making health-related decisions.

Acknowledgements

This research would not have been possible without the participation and trust of the families involved. Deep gratitude is extended to the caregivers who provided invaluable feedback and shared their experiences. Special thanks to the scientific collaborators, regulatory advisors, and independent supporters who believed in this work and contributed to its realisation.

I am also deeply thankful to my collaborators, particularly Vanja, whose encouragement, culinary mastery, and shared laughter sustained me through the most challenging moments. A special thanks to Sharon, Maja, Christiane, Kim, Vanja, Helena, Jelena, Beata, Courtenay, Keith, Leslie, John Smith, and all at Common Law Court, Dan, Klaus, and Robert. Your unwavering support and dedication have been instrumental in shaping this journey and making meaningful strides in understanding and supporting those affected by autism. I am deeply thankful for this.

Despite the regulatory and legal hurdles faced during this journey, the core motivation behind this research remains unchanged: to provide new avenues of support for individuals with ASD and their families. Science must move forward not as a challenge to regulation but as an ethical imperative to explore safe and effective interventions.

The findings of this study should not be seen as an endpoint but rather as a foundation for future inquiry. We must now ask: *How do we transition from observational success to scientifically validated therapy?* Answering this will require persistence, collaboration, and an unwavering commitment to scientific integrity.

Affidavit of Principal Researcher

I, Trevor Banks, affirm that the contents of this study, including all findings, insights, and testimonials, have been documented truthfully and to the best of my knowledge. This work represents the culmination of dedicated research and collaboration, driven by a sincere desire to support the autism community. I stand by the integrity of this study and its purpose to advance understanding and accessibility for those affected by autism.

Trevor Banks

Date: 19th May 2025

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Autism Insights

Libertas Academica
FREEDOM TO RESEARCH

ORIGINAL RESEARCH

Initial Observations of Elevated Alpha-N-Acetylgalactosaminidase Activity Associated with Autism and Observed Reductions from GC Protein—Macrophage Activating Factor Injections

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Abstract

Background: Autism spectrum disorders (ASD) are developmental disorders affecting 1:88 children, and which appear to be associated with a variety of complex immune dysregulations including autoimmunity. The enzyme, alpha-N-acetylgalactosaminidase (Nagalase) deglycosylates serum Gc protein (vitamin D_3 – binding protein) rendering it incapable of activating macrophage defenses. Increased Nagalase activity has been associated with a variety of malignancies, immune disorders and viral infections. Macrophage activating factor (GcMAF) has been repeatedly published as an intervention to lower serum Nagalase activity for a variety of cancer and HIV patients. GcMAF is a naturally occurring protein with well-established safety and therapeutic benefit(s) supported by numerous human studies.

Methods: Initially, parents of 40 individuals with ASD sought testing for Nagalase serum activity as part of an evaluation of immune dysregulation. Nagalase enzyme activity measurement was performed by the European Laboratory of Nutrients (ELN), Bunnik, the Netherlands, using an end-point enzymatic assay of a chromogenic substrate. Some parents of patients with elevated Nagalase activity opted for weekly GcMAF injections provided by Immuno Biotech Ltd., Guernsey UK (www.gcmaf.eu). GcMAF is purified from human serum obtained from the American Red Cross using 25-hydroxyvitamin D3-Sepharose high affinity chromatography. The protein is then further diluted to obtain therapeutically appropriate levels for patients based on their clinical presentations.

Results: Individuals with ASD (32 males and 8 females, n = 40, ages: 1 year 4 months - 21 years 2 months) had initial and post treatment assessment of Nagalase activity. Dosing of GcMAF was recommended based on previously reported response curves adjusted by the treating clinician for age, weight, and Nagalase levels. The average pre-treatment Nagalase activity of the autism group was 1.93 nmol/min/mg of substrate. This was well above the laboratory reported normal range of <0.95 nmol/min/mg. For the ASD group the average level at the time of second testing was 1.03 nmol/min/mg, reflecting an average reduction of 0.90 nmol/min/mg ($P < 0.0001$). Apart from the likely immunological benefits of lowering the Nagalase activity of these individuals, uncontrolled observations of GcMAF therapy indicated substantial improvements in language, socialization and cognition. No significant side-effects were reported during the course of injections.

Conclusions: In this first report of Nagalase activity in patients with autism, it appears that most individuals have substantially higher levels than the expected healthy ranges. Although Nagalase is a nonspecific marker of immune dysregulation, its observed levels in autism may have both etiological and therapeutic significance. Importantly, this is also the first report of reduction of Nagalase activity in an autism population with GcMAF injections.

Keywords: autism, immune dysfunction, Nagalase, macrophage activation factor, biomarker

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Introduction

Autism is a complex neurodevelopmental disorder which appears in the first three years of life. Once a rare disorder, it is now approaching epidemic, if not pandemic, proportions. A recent report from the US National Center on Birth Defects and Developmental Disabilities revealed a variable range of state by state prevalence from 4.8 to 21.2 per 1,000 for children aged 8 years (data from 2008).[1] These data reflect a remarkable 23% increase over a mere two-year earlier evaluation. While no consensus exists, this trend in autism is at least suggestive of an infective pathogen. Within this context, various organisms have been postulated to be involved, including: gastrointestinal infections,[2] Polyomaviruses,[3] Chlamydophila,[4] Bornaviruses,[5] Paramyxoviruses,[6] and Borrelia burgdorferi.[7] While any of these may contribute to a small percentage of autism cases, it seems unlikely that any of them individually represents the origin of this epidemic.

Despite this uncertainty, growing evidence supports significant immune dysfunction, including autoimmunity, in autism.[8] Simultaneously, oxidative stress[9] and mitochondrial dysfunction[10] are common findings in this population. One possible explanation for the pattern of immune dysregulation and oxidative stress observed in autism spectrum disorders (ASD) could be persistence of active pathogens, perhaps from the perinatal or a subsequent period of child development.[11] Viruses are also known to subvert intracellular calcium regulation to their own functional requirements and in that process to disrupt mitochondrial activity.[12]

Measurement of biomarkers related to immune dysregulation and putative infectious agents is a routine part of the author's (JB) evaluation of ASD in a clinical setting. Recently, evaluation of the activity of alpha-N-acetylgalactosaminidase (Nagalase) has been made commercially available as a diagnostic laboratory measurement. Nagalase has been published as a biomarker associated with various types of cancer,[22–24,29,30] systemic lupus erythematosus (SLE),[13] influenza,[14] and human immunodeficiency virus infection (HIV).[31] It is enzymatically distinct from hepatic galactosaminidase and appears to be far more biologically active. It is therefore a nonspecific biomarker, which appears to be an important indicator of secondary immune dysregulation.

Nagalase is a component of viral hemagglutinin and is released by the action of trypsin on hemagglutinin.[14] Since hemagglutinin is a common glycan-binding lectin of many viruses (including influenza, paramyxoviruses and polyomaviruses), several viruses may individually or jointly contribute to hemagglutinin-derived Nagalase activity in the blood.[15]

In the absence of recent viral infection or malignancies, elevated Nagalase activity likely represents a marker of viral production of hemagglutinin protein being acted upon by inflammatory cell mediated trypsin activity; as such it may represent viral persistence, active transcription, and inflammation. Viral protein transcription is one potential mechanism of autoimmunity.[16,17] Beyond this, Nagalase is an enzyme that deglycosylates the Gc protein also known as vitamin D binding protein (VDBP), rendering it incapable of conversion to active GcMAF (Gc protein-derived Macrophage Activating Factor) and thereby preventing its regulation of macrophage activation.[18] It is noteworthy that vitamin D deficiency, either in pregnancy or during postnatal development, is an apparent risk factor for autism.[19] The impact of Nagalase on VDBP transportation of vitamin D is not known. However, vitamin D deficiency is a known risk factor for autoimmunity.[20]

In light of the possible involvement of immune abnormalities, autoimmunity, vitamin D deficiency, and potential viral persistence, Nagalase screening was added by JB and other physicians to the biomarker profile[21] of children presenting for biomedical evaluation of autism-related disorders and co-morbidities.

Methods

A retrospective chart review for analysis of routine Nagalase testing was accomplished on the initial cohort of patients tested by the clinician (JB). All records were reviewed by JB for confirmation of test results, confirmed diagnosis of autism, time intervals between testing, dosing of subsequent GcMAF used, and the observed clinical/parental/therapist/teacher responses. All patients met the criteria for autism (299.00 DSM-IV revised) and were diagnosed by either a child neurologist or developmental psychologist, in addition to receiving the evaluation of the clinician. No significant changes were made to the participants' treatments apart from the introduction of GcMAF during the timeframe reported in this study.

Additional pre-screening assessments

In addition to meeting DSM-IV revised criteria for autism and having independent determination of autistic severity performed by non-affiliated practitioners, the clinician in this study used an in-house severity scoring system. This consisted of 20 questions (Table 4) designed to evaluate relative severity in the standard domains required for the diagnosis of autism (ie, language, socialization, and stereotypies), as well as other meaningful determinants. Within each category the clinician and parents agreed on a score as follows: 1 = normal or near normal; 2 = mild; 3 = moderate; 4 = severe.

All of the participants assessed in this initial evaluation were in the 3–4 range for question 1 (General Impression of Autism Severity or Delayed Development). This was also true for the core domains of autism (questions 2, 4, and 10). Additionally, all participants scored a 4 (severe) for question 9, indicating a lack of imaginative or age-appropriate play. Significant variability was observed for most of the other domains in the questionnaire, especially in the areas related to motor (both fine and gross), where the greatest initial variability was observed. Specific assessments within each domain were beyond the limited scope of this initial retrospective analysis and would instead be appropriate for a future prospective investigation.

The parents provided written informed consent for phlebotomy and evaluation of potential medical comorbidities occurring in their children with autism. Specifically, Nagalase was discussed with the parents as a potential marker of immune dysregulation. Upon agreement by the parents, sufficient venous blood was withdrawn to fill a 9 ml EDTA tube, which was then immediately inverted at least 5 times. The tube was then centrifuged for 10 minutes at 3000 rpm to separate the plasma. After separation, approximately 3 ml of the clear plasma was transferred to the plasma collecting tube, which was then immediately frozen to −20 °C for at least 24 hours. The specimen was then shipped frozen overnight to an intermediary laboratory in New Jersey, United States. Collected specimens were kept frozen during further shipping to ELN.

The cohort in this initial study consisted of 40 patients whose records included both pre- and post-treatment Nagalase blood test results. Since this was a preliminary and retrospective evaluation of both Nagalase activity and the response to GcMAF, these initial data and observations reflect only the period between the first and second Nagalase testing for patients.

Of the 40 subjects, there were 32 males (ages at the point of first testing ranged from 1 year and 4 months to 21 years) and 8 females (ages ranged from 4 years 7 months to 18 years, median = 6.93 ± SD 5.08 years).

Nagalase assay

Following the procedure published by Yamamoto et al[22,23], Nagalase activity was determined by using an endpoint enzymatic assay using a chromogenic substrate. ELN established a reference range of 0.32–0.95 nM/min/mg of substrate based on serum collected from healthy volunteers, a range nearly superimposable to that previously reported which was between 0.35 and 0.65 nM/min/mg.[24]

GcMAF preparation

Patients obtained commercially available GcMAF from Immuno Biotech Ltd., Guernsey UK (http://www.gcmaf.eu). Immuno Biotech prepared the GcMAF according to the procedure described in their published materials.[24] Briefly, Gc protein was isolated from purified human serum obtained from the American Red Cross, using either 25-hydroxyvitamin D3-Sepharose high affinity chromatography or actin-agarose affinity chromatography. The bound material was eluted and then further processed by incubation with three immobilized enzymes. The resulting GcMAF was filter sterilized. The protein content and concentration was assayed using standard Bradford protein assay methods.[25] At the end of the production process, the GcMAF was checked for sterility in-house and externally by the UK Health Protection Agency and independent laboratories.[26]

GcMAF activity assay

Activity assay of GcMAF was based on a live macrophage cell proliferation method using water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, Calbiochem®). Cells were cultured in the presence of a range of GcMAF concentrations and incubated for 72 hours. The soluble MTT was added, and the cells incubated for a further period of time.

The resulting color change, due to the conversion of the MTT, was measured using a plate reader at 460 nm. The activity of the GcMAF could then be determined by comparison of the negative and positive controls using the RAW 264.7 murine macrophage cell line [Sigma-Aldrich®]. Positive macrophage activation was demonstrated by apoptosis of MCF7 breast cancer cells (Lawrence Berkeley National Laboratory). An internally known positive standard was also included in the assay as a control. Further testing of this GcMAF product's activity was determined measuring its biological effects in both human peripheral blood mononuclear cells[27] and human breast cancer cells.[28]

After appropriate written informed consent by the parents, GcMAF was injected subcutaneously on a weekly basis using a 31 gauge insulin syringe. In adults with HIV or several different types of cancer, the typical dose described for response given either intramuscular or intravenous administration has been a minimum of 100 ng/wk. Despite levels of Nagalase activity well into the range of many cancer or HIV patients, the clinician elected not to exceed 100 ng/wk so as to prevent putative over-stimulation of macrophages. All patients started on low doses that were increased gradually over the course of treatment. The doses ranged from 4 to 100 ng per wk and were adjusted based on clinical response, age, body weight, and the initial level of Nagalase activity.

To further assess the clinical responses, all parents were interviewed at intervals of no longer than a month using the improved Clinical Global Impression Scale (iCGI) defined by Kadouri et al and described below.[29]

Improved response format for the clinical global impression of improvement scale

The improved response format for the Clinical Global Impression of Improvement scale is as follows: 5 = Very considerable improvement; 4 = Considerable improvement; 3 = Moderate improvement; 2 = Slight improvement; 1 = Very slight improvement; 0 = State unchanged; −1 = Very slight deterioration; −2 = Slight deterioration; −3 = Moderate deterioration; −4 = Considerable deterioration; −5 = Very considerable deterioration; −6 = Maximum deterioration.

Statistical methods

Due to the relatively small population overall and only 8 females in this sample, the group was evaluated as a whole and not segregated based on gender or age. Statistical comparison between pre and post treatment levels of Nagalase was performed by two-tailed, paired difference *t*-test and by using standard formulas in Microsoft Excel® 2010. Since one subject had a Nagalase result significantly higher than the mean, consideration for skewing artifact was made. Adjusting for the skew effect changed the median of the group from 1.71 to 1.68, which was not statistically significant.

Results

The average pre-GcMAF treatment Nagalase activity was 1.93 nM/min per mg, with a median of 1.68 nM/min per mg (SD ± 1.21 nM/min per mg), and with a range of 0.90 nM/min per mg to 7.80 nM/min per mg (Table 1). At the point of time of subsequent testing (average interval 100 days, ±SD 32 days), the average Nagalase activity during GcMAF treatment was 1.03 nM/min per mg, with a median of 0.90 nM/min per mg (±0.67 nM/min per mg), and with a range of 0.44 to 4.40 nM/min per mg. This reflects an average reduction of 0.90 nM/min per mg ($P < 0.0001$). Of this original cohort only 2 of 40 (5%) were observed to be initially within the laboratory reference range (0.90 and 0.92 nM/min per mg).

Because of the standard laboratory turnaround time and the necessary time to discuss treatment options with the parents, the actual number of weekly injections was substantially less than the number of weeks in the interval of retesting. The average number of weekly injections was 14 (±4 weeks SD).

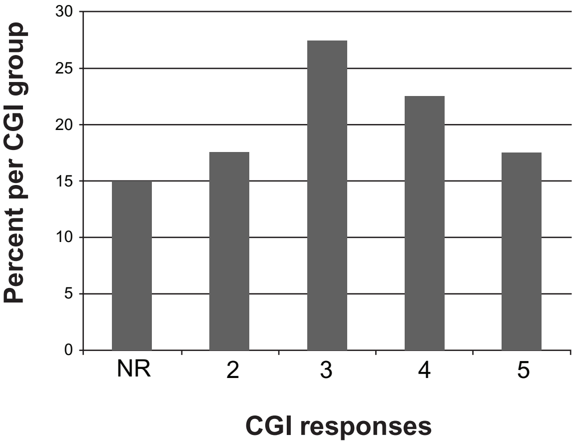
At the time of retesting, the Nagalase levels of 24 of the 40 patients (60%) had decreased to within the laboratory reference range of <0.95 nM/min per mg. In view of these results, a minimum of 16 patients (40%) would be considered not to have received adequate therapeutic effect and would therefore be candidates for continued intervention. Only 1 of 40 (2.5%) failed to respond with significant reduction of Nagalase activity (pre/post Nagalase difference of only 0.10 nM/min per mg). The families of the two patients whose initial Nagalase levels were within the upper part of the laboratory reference range both elected to initiate GcMAF therapy. Both patients experienced significant reductions in Nagalase activity, one with a considerable response (iCGI = 4), while the other was rated as a non-responder.

The initial levels of Nagalase activity in the group of patients that we studied ranged from the upper range of normal to beyond levels typically observed in metastatic cancer patients[30,31] and HIV-infected patients.[32] Despite concerns about autoimmunity in autism, none of the patients observed in this study experienced significant side-effects, and none were required to suspend

Table 1. Nagalase dataset for pre-post GcMAF therapy with iCGI response per subject.

Gender	**Nagalase**						**Pre/post Nagalase difference**	**iCGI**
Male/ female	**Pre draw date**	**Age in years**	**Pre result**	**Post 1 draw date**	**Post 1 result**	**Days between pre—post 1**		
m	6/21/2011	6.3	0.90	9/7/2011	0.47	76	–0.43	4
m	5/3/2011	21.2	1.00	7/22/2011	0.44	79	–0.56	5
m	4/28/2011	6.95	1.30	9/21/2011	0.72	143	–0.58	3
m	6/3/2011	9.75	2.20	12/23/2011	1.30	200	–0.90	2
m	5/3/2011	7.15	1.90	8/25/2011	0.76	112	–1.14	4
f	5/3/2011	11.1	1.90	8/25/2011	1.00	112	–0.90	5
f	5/3/2011	9.2	1.90	8/24/2011	1.20	111	–0.70	5
m	5/3/2011	4.3	1.70	8/25/2011	1.10	112	–0.60	4
m	4/29/2011	4.4	1.00	8/25/2011	0.76	116	–0.24	3
m	6/7/2011	6.05	1.20	8/16/2011	0.79	69	–0.41	4
f	6/15/2011	5.1	1.66	8/31/2011	0.40	76	–1.26	–1
m	8/25/2011	12.3	1.69	11/23/2011	0.47	88	–1.22	1
m	10/4/2011	11.5	7.80	11/30/2011	4.40	56	–3.40	3
m	6/24/2011	7	1.50	8/24/2011	0.90	60	–0.60	4
f	4/21/2011	12.05	1.98	8/5/2011	0.81	104	–1.17	3
m	6/16/2011	1.3	1.50	9/21/2011	1.00	95	–0.50	4
m	6/16/2011	5.6	2.60	9/21/2011	2.50	95	–0.10	2
m	5/11/2011	1	2.80	9/2/2011	1.80	111	–1.00	0
m	9/21/2011	18	1.30	11/30/2011	0.92	69	–0.38	3
m	5/6/2011	3.6	3.00	8/3/2011	1.00	87	–2.00	5
m	5/13/2011	16.5	1.20	9/21/2011	0.80	128	–0.40	4
m	5/13/2011	3.9	1.60	7/6/2011	1.10	53	–0.50	3
f	6/8/2011	4.6	0.92	9/21/2011	0.62	103	–0.30	0
m	6/29/2011	10.7	1.00	9/27/2011	0.90	88	–0.10	3
m	4/6/2011	4.85	1.40	7/21/2011	0.81	105	–0.59	3
f	4/13/2011	4.7	3.90	8/19/2011	1.60	126	–2.30	5
f	5/6/2011	3.65	1.10	8/5/2011	0.61	89	–0.49	2
m	4/19/2011	18.3	4.00	6/29/2011	1.40	70	–2.60	4
m	5/27/2011	3.05	2.60	8/18/2011	1.40	81	–1.20	3
m	5/19/2011	5.2	1.20	11/23/2011	0.96	184	–0.24	3
m	5/5/2011	9.1	1.79	8/10/2011	0.57	95	–1.22	2
f	6/22/2011	18.2	1.90	12/1/2011	1.20	159	–0.70	0
m	6/8/2011	16.4	1.82	8/25/2011	0.62	77	–1.20	2
m	5/19/2011	10.5	2.90	8/11/2011	0.93	82	–0.97	3
m	7/15/2011	6.8	1.73	8/23/2011	0.51	38	–1.22	4
m	5/6/2011	6.9	2.90	7/21/2011	1.20	75	–1.70	5
m	6/8/2011	10.15	1.20	9/21/2011	0.82	103	–0.38	1
m	6/8/2011	8.1	1.10	9/21/2011	0.68	103	–0.42	2
m	4/29/2011	4.1	1.00	8/26/2011	0.89	117	–0.11	2
m	4/6/2011	3.55	1.20	8/24/2011	0.91	138	–0.29	5
Average		8.33	1.93		1.03	99.63	–0.90	2.98
Median		6.93	1.68		0.90	95.00		3.00
S.D.		5.08	1.21		0.67	32.81	0.70	

Table 2. iCGI response to GcMAF after an average of 14 weekly subcutaneous injections.



Notes: Y-axis represents the percentage of total in each group. NR = non-responder (–1 to 1), 2 = slight improvement (generally described as better eye contact, increased interaction with the environment and/or family), 3 = moderate improvement (Based on parental and teacher/therapists input, there was improved communication, increased acquisition of new skills, vocabulary and noticeable increase in sociability), 4 = considerable improvement (To obtain this level of response, patients were noted by parents, teachers and the clinician to have substantially better communication, generally going from single word utterances to several word sentences, as well as showing evidence of improved academic processing), 5 = Very considerable improvement (This response was demonstrated at school, during therapies, home and outside the home as substantial improvement to the point that many or most of the criteria of autism were no longer present).

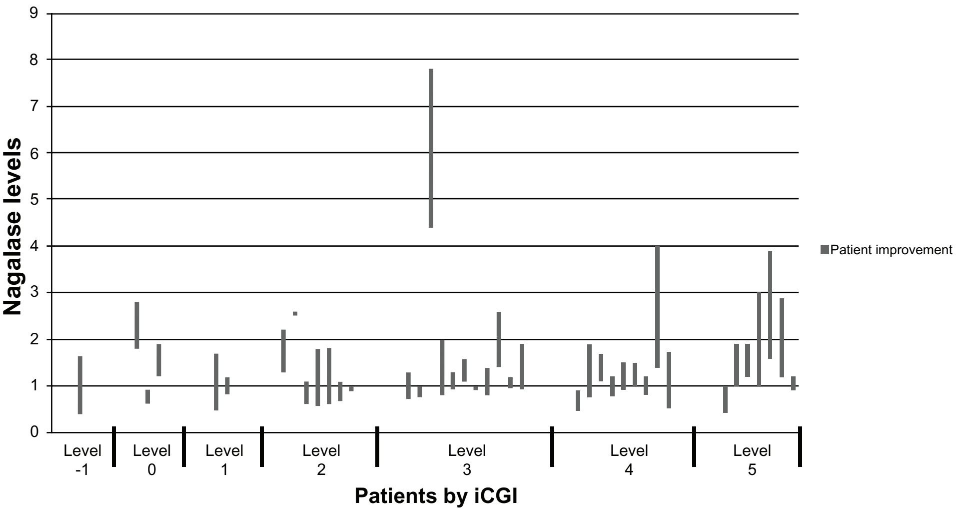
or drop out of treatment. During the first few weeks of treatment, 3 of 40 patients (7.5%) experienced low to moderate rise in body temperature, typically occurring 24 to 48 hours after the GcMAF injection and lasting less than 24 hours. Parents were instructed to use ibuprofen only if the temperature exceeded 102° F (approximately 39°C), and two were treated during the first few weeks. By the second month, no patients experienced significant febrile events. Interestingly, during the first 3 weeks, 6 of 40 patients (15%) were observed to have rashes compatible with viral exanthemas (generally on the trunk and in fine papules more commonly than maculae). Petechiae were not observed. These rashes could represent the manifestation of latent or persistent viral infections interacting with activated macrophages.

Discussion

Since this is an open-label, non-controlled, retrospective analysis, caution must be employed when ascribing cause and effect to any treatment outcome. However, the response to GcMAF was robust with regard to Nagalase reduction, as well as symptomatic improvements as shown by the iCGI. Despite the short observational time period, the result that 67.5% of the group responded in the 3 to 5 CGI-I range was unexpectedly substantial (Tables 2 and 3).

In this small population, it does not appear that an obvious association exists between the iCGI response and the change in the Nagalase activity (Table 3). Further statistical analysis was therefore not deemed appropriate.

Table 3. Pre-post Nagalase by iCGI response level.



Notes: The vertical line represents the change in Nagalase activity (all changed by going down over the time interval between pre-post testing). The smallest observed change in Nagalase activity was –0.1 nM/min per mg. The data are divided into groups based on the noted iCGI responses observed.

Table 4. Questionnaire designed to evaluate relative severity in the standard domains required for the diagnosis of autism.

1. General Impression of autism severity or delayed development?
2. Expressive language?
3. Difficulty following verbal commands?
4. Flapping or self-stimulation?
5. Sensory Issue (touch texture etc.)
6. Difficult transitions?
7. Tantrums?
8. Obsessive and/or compulsive behaviors?
9. Lack of imaginative or age appropriate play?
10. Lack of desire for social interaction?
11. Hyperactivity?
12. Inattention?
13. Lack of eye contact?
14. Problems Sleeping?
15. Sound sensitivity—ear covering?
16. Feeding problems?
17. Gross motor problems (abnormal walking or running gait)?
18. Fine motor problems (tripod grasp, buttons, zippers, snaps)?
19. Anxiety or panic around doctors or about bloodwork?
20. Problems with bowel movements?

Conclusion

The changes in Nagalase activity in response to GcMAF treatment in this ASD population reflected similar robust responses observed using GcMAF in the treatment of HIV infection and cancer. However, autism represents a developmental disorder with substantial delays in core domains of cognitive activity (language, socialization, and behavior) and is generally felt to be a life-long condition. Therefore, these initial observations give support to the notion that autism *per se* may be the consequence of treatable underlying pathophysiology. Given that ASD are now affecting more than 1% of US children, the observed response to GcMAF warrants urgent and further prospective evaluation.

Although Nagalase is a non-specific marker believed to be derived from viral hemagglutinin, it may be useful as a biomarker of therapeutic significance in ASD, and as such also warrants further investigation. Regardless of any immediate clinical improvement, the reduction of Nagalase to more desirable levels is of potential benefit to these patients, since Nagalase is known to impair immune defenses.

Author Contributions

Primary author, maintenance of the database, statistical analysis, and clinician: JB.Nagalase laboratory methodologies and assistance with statistical methods: EV. GcMAF preparation methods: LT.

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Competing Interests

JB prescribes testing and recommends therapies for children with autism. His son and stepson have autism. EV previously had a financial interest in ELN, the laboratory which tests measures Nagalase. LT is employed by Immuno Biotech, Ltd. (the laboratory isolating and purifying the GcMAF protein). However, in the case of ELN, EV had no knowledge of the therapies being used nor of the names of any patients whose data were being analyzed. Further, in the case of LT, neither she nor any employee of Immuno Biotech had any knowledge of the Nagalase results or the patient/parent names used in this study.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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ORIGINAL ARTICLE

Microglial Activation in Young Adults With Autism Spectrum Disorder

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Context: A growing body of evidence suggests that aberrant immunologic systems underlie the pathophysiologic characteristics of autism spectrum disorder (ASD). However, to our knowledge, no information is available on the patterns of distribution of microglial activation in the brain in ASD.

Objectives: To identify brain regions associated with excessively activated microglia in the whole brain, and to examine similarities in the pattern of distribution of activated microglia in subjects with ASD and control subjects.

Design: Case-control study using positron emission tomography and a radiotracer for microglia—$[^{11}C](R)$-(1-[2-chrorophynyl]-*N*-methyl-*N*-[1-methylpropyl]-3 isoquinoline carboxamide) ($[^{11}C](R)$-PK11195).

Setting: Subjects recruited from the community.

Participants: Twenty men with ASD (age range, 18-31 years; mean [SD] IQ, 95.9 [16.7]) and 20 age- and IQ-matched healthy men as controls. Diagnosis of ASD was made in accordance with the Autism Diagnostic Observation Schedule and the Autism Diagnostic Interview–Revised.

Main Outcome Measures: Regional brain $[^{11}C](R)$-PK11195 binding potential as a representative measure of microglial activation.

Results: The $[^{11}C](R)$-PK11195 binding potential values were significantly higher in multiple brain regions in young adults with ASD compared with those of controls ($P < .05$, corrected). Brain regions with increased binding potentials included the cerebellum, midbrain, pons, fusiform gyri, and the anterior cingulate and orbitofrontal cortices. The most prominent increase was observed in the cerebellum. The pattern of distribution of $[^{11}C](R)$-PK11195 binding potential values in these brain regions of ASD and control subjects was similar, whereas the magnitude of the $[^{11}C](R)$-PK11195 binding potential in the ASD group was greater than that of controls in all regions.

Conclusions: Our results indicate excessive microglial activation in multiple brain regions in young adult subjects with ASD. The similar distribution pattern of regional microglial activity in the ASD and control groups may indicate augmented but not altered microglial activation in the brain in the subjects with ASD.

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AUTISM SPECTRUM DISORDER (ASD) is a group of neurodevelopmental disorders characterized by pervasive abnormalities in social interaction and communication and by repetitive and restricted behavioral patterns and interests. Autism spectrum disorders include autistic disorder, Asperger disorder, and pervasive developmental disorder not otherwise specified.[1] Recent population-based surveys[2,3] showing that ASD is more common than previously believed have aroused serious public concern worldwide. Although the neurobiologic basis for ASD remains poorly understood, a growing body of research[4,5] suggests that immune abnormalities are a contributing factor to the development of ASD. Several genetic studies link ASD with genes that are associated with various immune functions, including the HLA antigen[6] and the major histocompatibility complex class III molecule, such as complement C4B.[7,8] Systemic abnormalities of the immune system have been one of the most common and long-standing reported findings in subjects with ASD.[9,10] Notably, increased production of cytokines (eg,

For editorial comment see page 9

Table 1. Demographic Characteristics of the Subjects[a]

	Mean (SD) [Range]	
Variable	**Control (n = 20)**	**ASD (n = 20)**
Age, y[b]	22.6 (5.3) [17.8-35.5]	23.3 (4.0) [18.6-31.9]
WAIS-III full IQ[c]	102.8 (12.5) [81.0-131.0]	95.9 (16.7) [81.0-140.0]
ADI-R		
Social	NA	20.6 (5.1) [10.0-29.0]
Communication	NA	15.2 (4.4) [8.0-24.0]
Stereotype	NA	4.3 (2.2) [3.0-10.0]
ADOS		
Social	NA	6.4 (3.0) [4.0-11.0]
Communication	NA	6.2 (2.7) [2.0-13.0]
Stereotype	NA	1.0 (0.9) [0-3.0]
Faux Pas Test	NA	21.2 (8.6) [3.0-34.0]
Y-BOCS	NA	11.0 (6.4) [0-28.0]
DCDQ-J total	NA	60.4 (12.0) [42.0-73.0]

Abbreviations: ADI-R, Autism Diagnostic Interview–Revised; ADOS, Autism Diagnostic Observation Schedule; ASD, autism spectrum disorder; DCDQ-J, Japanese version of the Developmental Coordination Disorder Questionnaire; NA, not applicable; WAIS-III, Wechsler Adult Intelligence Scale, third edition; Y-BOCS, Yale-Brown Obsessive Compulsive Scale.

[a] All subjects were men.

[b] $P = .63$.

[c] $P = .15$.

interleukin 6 [IL-6], tumor necrosis factor, and macrophage chemoattractant protein-1) has been observed in peripheral samples and the brains of ASD subjects.[11-16] In general, plasma cytokine levels in ASD subjects are widely distributed and show substantial overlap with control subjects, implying that there is a subset of ASD subjects with high levels of such cytokines. In addition, several studies[17-20] have identified specific antibodies against human brain epitopes in the serum of mothers of children with ASD, as well as in children with ASD, although autoantibodies are found in only 10% to 15% of the children with ASD. These findings argue in favor of the participation of the immune system in the pathogenesis of a subset of ASD subjects.

Microglia are resident brain cells that sense pathologic tissue alterations.[21,22] The first microglial precursors colonize the brain during the embryonic and fetal phases of development.[23,24] They develop into brain macrophages and perform immune functions. Upon exposure of the brain to any form of insult, such as infection, trauma, or ischemia, the microglia are rapidly activated. When activated, microglia produce neurotoxic substances, including proinflammatory cytokines (ie, tumor necrosis factor and IL-1β) and oxygen species (ie, hydrogen peroxide and superoxide). However, under certain conditions, activated microglia can produce anti-inflammatory cytokines such as IL-10 and transforming growth factor-β, which have neuroprotective effects in experimental animal models of traumatic injury and stroke.[25,26] Furthermore, experimental studies[27,28] have demonstrated that microglia play a role in the maintenance of synaptic integrity in the uninjured brain.

Recently, Vargas and colleagues[16] determined the magnitude of neuroglial and inflammatory reactions and their cytokine expression profiles in brain tissues from the cerebellum, midfrontal, and cingulate gyrus obtained at autopsy from children and adults with ASD. Immunocytochemical examination revealed marked activation of microglia and astroglia. Microglial responses were diffusely distributed in the cortex and subcortical areas, as well as the cerebellum, and were present as microglial nodules or as part of a prominent accumulation of perivascular macrophages. More recently, Morgan and colleagues[29] quantitatively assessed activated microglia in the dorsolateral prefrontal cortex of postmortem brains from children and adults with ASD. They found that the microglia were markedly or marginally activated in most cases examined. Transcriptomic analysis of the autistic brain by Voineagu and colleagues[30] has shown the presence of 2 modules in the ASD brain: a neuronal module enriched for known autism susceptibility genes, including neuronal-specific factors, such as ataxin 2-binding protein 1, and a module enriched for immune genes and glial markers. The latter immune-glial module has a less pronounced genetic component and thus is most likely either a secondary phenomenon or the result of environmental factors. Despite the striking features of microglial activation in the pathogenesis of ASD, to our knowledge, there is no information on the patterns and characteristics of the distribution of microglial activation in the whole brain in ASD subjects.

To address this issue, we conducted a positron emission tomography (PET) analysis using the radiocarbon ([^{11}C])-labeled (*R*)-(1-[2-chrorophynyl]-*N*-methyl-*N*-[1-methylpropyl]-3 isoquinoline carboxamide) ([^{11}C](*R*)-PK11195), a radiotracer that specifically binds to activated microglia.[31-33] This procedure permitted us to visualize the activated microglia in vivo in the whole brain. In this study, we initially determined the distribution of [^{11}C](*R*)-PK11195 binding potential (BP) in the whole brain of young adults with ASD and then identified several brain regions associated with the activation of microglia. Subsequently, we compared the levels of [^{11}C](*R*)-PK11195 BP in the identified brain regions. Because microglia may be prenatal in origin,[23,24] and because ASD is typically diagnosed by 3 years of age, we hypothesized that the regional variability of the [^{11}C](*R*)-PK11195 BP in the identified brain regions is similar between ASD and control subjects, whereas the magnitude of [^{11}C](*R*)-PK11195BP in ASD subjects is greater than that of controls in all the regions. To test this hypothesis, we carefully recruited subjects with no history of epilepsy or medication because epileptic seizures and psychotropic drugs can influence the condition of microglial activation.[34-37]

METHODS

SUBJECTS

The ethics committees of the Hamamatsu University School of Medicine approved this study. Written informed consent was obtained from all subjects and their guardians after they had been provided a detailed explanation of the study procedures. Twenty men with ASD and 20 age- and IQ-matched typically developing male subjects participated in this study (**Table 1**). All subjects were right-handed and had an IQ of greater than 80. None of the subjects were tobacco smokers, and none were taking any medication, including psychotropic drugs. All of them were physically healthy. At the time of scanning, all the subjects had no symptoms of inflammation and were not under stressful conditions. All the subjects with ASD were diagnosed by 2 trained child psychiatrists (K.N. and T.S.) according to the *DSM-IV-TR*.[1] The ASD diagnosis was confirmed for all cases using the Autism Diagnostic Interview–Revised (ADI-R)[38] and the Autism Diagnostic Observation Schedule (ADOS)[39] module-4 by trained clinicians (K.J.T. and K.M., respectively). As a result, 15 of 20 ASD subjects were diagnosed as having autistic disorder and the remaining 5 were considered to have pervasive developmental disorder not otherwise specified on the basis of the ADOS scores, although all 20 subjects met the ADI-R criteria for autistic disorder. None of the ASD subjects was classified as having regressive autism, the classification of which was based on clinical characteristics using both parental reporting and answers to questions on the ADI-R regarding language loss (question 11) and social skills (question 25). The ASD subjects did not have any other psychiatric comorbidity disorders, as confirmed by the Structured Clinical Interview for *DSM-IV* Axis I disorders.[40] In addition, they had no notable dysmorphism, neurocutaneous abnormalities, significant neurologic deficits, history of epileptic seizures, or disorders known to be associated with autism, such as fragile X syndrome, neurofibromatosis, or tuberous sclerosis. Fragile X syndrome was excluded by determining the CGG repeat number in the *FMR1* gene. We measured the markers of inflammation in the blood in the ASD subjects, including the serum C-reactive protein and white blood cell count. Both levels in all the ASD subjects were within normal range. None of the ASD subjects had any history of inflammatory or allergic diseases, except 2 subjects who had had atopic dermatitis in their childhood. One of the ASD subjects had a family history of major depression (his mother). In the remaining 19 subjects, there was no family history of any chronic inflammatory diseases or neuropsychiatric conditions. In the ASD subjects, the social cognitive disability and the degree of repetitive and/or obsessive behavior and interests were evaluated by the Faux Pas Test[41] and Yale-Brown Obsessive Compulsive Scale,[42,43] respectively. Current motor coordination problems were assessed by the Japanese version of the Developmental Coordination Disorder Questionnaire.[44] All control subjects were found to be mentally and physically healthy on the basis of comprehensive assessments of their medical histories and neuropsychiatric examinations.

MAGNETIC RESONANCE IMAGING AND PET PROCEDURES

As described previously,[33,45] we obtained 3-dimensional magnetic resonance images (MRIs) just before PET measurements using a 0.3-T MRI unit (MRP7000AD; Hitachi Medical) and a high-resolution brain PET scanner having an intrinsic resolution of 2.9 × 2.9 × 3.4 mm at full-width at half maximum and a 163-mm axial field of view, and yielding 47 PET images simultaneously (SHR 12000; Hamamatsu Photonics), respectively. All MRI and PET scans were set parallel to the anterior-posterior intercommissural line.[45] Before dynamic PET scanning, a 20-minute transmission scan was performed for attenuation correction using a ^{68}Ge/^{68}Ga source. Then, after a bolus intravenous injection of a 350-MBq dose of [^{11}C](*R*)-PK11195, we performed 32 serial PET scans (time frames: 4 × 30 second, 20 × 60 second, and 8 × 300 second) for 62 minutes. In quantitative PET brain imaging, the motion artifact is the important degrading factor. Therefore, we fixed the head of each subject by using a thermoplastic face mask, observed subjects carefully during each scan, and confirmed that all the subjects had remained immobilized.

IMAGE ANALYSIS AND KINETIC MODELING

The brain, particularly in cortical subregions, is known to be sensitive to a partial volume effect that sometimes occurs during the measurement of small brain structures and that leads to an underestimation of tracer activity. In this study, we used the following previously described procedure to minimize the contribution of the partial volume effect.[33,45] First, we adjusted the MRI voxel size to the PET voxel size 3-dimensionally using image-processing software (DrView; Asahi Kasei) on a Sun workstation (HyperSPARC ss-20; Sun Microsystems). Then, these reformatted MRIs with 3-dimensional scales and coordinates identical to those of the PET images were used as anatomic landmarks for the regions of interest (ROIs) setting. Subsequently, by referring to areas on the MRIs as anatomical landmarks, the ROIs were carefully drawn to avoid the involvement of either the sulci or ventricles. An investigator masked to the subject's condition placed 3 ROIs over the bilateral cerebellar cortices, midbrain, and bilateral thalami on the MRIs. These ROIs were then transferred onto the corresponding dynamic [^{11}C](*R*)-PK11195 images.

To assess activated microglial density in the brain, we analyzed the [^{11}C](*R*)-PK11195 time-activity curves (TACs) on the basis of a simplified reference tissue model[46,47] because the regional brain [^{11}C](*R*)-PK11195 BP (a ratio of binding and dissociation rate constants, k_3/k_4) estimated by the simplified reference tissue model is reported to correlate with the magnitude of microglial activity.[33,48] Because the decrease of TACs was sharpest in the cerebellar ROI among the 3 ROIs examined in the control group, we assumed that the specific binding would be the least in this region. A normalized input curve was first created by averaging the TACs from the ROIs placed over the bilateral cerebellar cortices in the control group. Then, the normalized mean input curve was used as the reference input function of the simplified reference tissue model in the ASD and control subjects because a desirable reference region free from specific binding was not evident in the ASD subjects.

Using biomedical imaging software (PMOD, version 3.0; PMOD Technologies), we constructed whole-brain parametric maps of the [^{11}C](*R*)-PK11195 BP for the subsequent voxel-based analysis using Statistical Parametric Mapping software (SPM5; http://www.fil.ion.ucl.ac.uk/spm). The [^{11}C](*R*)-PK11195 BP maps were normalized to the Montreal Neurological Institute space, as defined by the MRI T1 template implemented in SPM5. The extracerebral structures were then masked by demarcating cerebral regions on spatially normalized MRIs. Finally, the normalized and masked BP maps were smoothed with an 8-mm full-width at half maximum gaussian filter.

In addition to the voxel-based analysis, which is suitable for an exploratory examination of altered tracer distribution in the brain, we performed a volume of interest (VOI)–based analysis because it enabled us to generate quantitative differences in [^{11}C](*R*)-PK11195 BP in specific regions. For this purpose, we placed additional spherical VOIs of 5-mm radius, which centered on the peak voxel derived from the results of the voxel-based analysis, on [^{11}C](*R*)-PK11195 BP maps for each of the subjects. The VOIs selected were the bilateral cerebellum, brainstem, splenium of the corpus callosum, bilateral fusiform gyri, bilateral superior temporal gyri, and the bilateral anterior cingulate, bilateral orbitofrontal, left midfrontal, and right parietal cortices. Averaged [^{11}C](*R*)-PK11195 BP values for each VOI were obtained in the ASD and control groups.

VOXEL-BASED MORPHOMETRY

To investigate possible differences in brain structure between the ASD and control groups, we conducted voxel-based morphometry. For this purpose, we used a 3-T MRI scanner (Signa Excite;

General Electric Medical Systems) to obtain T1-weighted volumetric images scanned by the inversion recovery-prepared fast spoiled gradient recalled acquisition protocol as follows: repetition time = 11.0 milliseconds, echo time = 5.0 milliseconds, preparation time = 450 milliseconds, flip angle 20°, number of excitations = 1, field of view = 24.0 cm, matrix = 256 × 256, auto-zero-fill interpolation = 512, location per slab = 160, slice thickness = 1.2 mm, and voxel size = 0.94 × 0.94 × 1.2 mm. The T1-weighted volumetric images were analyzed using the VBM5.1 toolbox (http://www.fil.ion.ucl.ac.uk/spm/ext/) implemented in SPM5 with the default parameters. Estimates of the absolute gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF) volumes were obtained after the automatic brain segmentation procedure had been carried out by VBM5.1. The total intracranial volume was calculated as the sum of the volumes of the GM, WM, and CSF.

STATISTICAL ANALYSIS

The demographic and clinical variables of the ASD and control groups were compared by the unpaired *t* test using statistical software (PASW Statistics version 18; SPSS Japan Inc). The level of statistical significance was set at $P < .05$.

The voxel-based analyses of the $[^{11}C]$(*R*)-PK11195 BP maps were conducted using SPM5. For the SPM5 analysis of the $[^{11}C]$(*R*)-PK11195 BP maps, between-group comparisons were performed to explore regional differences in the $[^{11}C]$(*R*)-PK11195 BP using the *t* test for each voxel without a proportional scaling of the $[^{11}C]$(*R*)-PK11195 BP maps. We also performed exploratory correlation analyses between the regional changes in $[^{11}C]$(*R*)-PK11195 BP values and the severity of clinical features in ASD subjects using SPM5. The scores on the ADOS, ADI-R, Faux Pas Test, Yale-Brown Obsessive Compulsive Scale, and the Japanese version of the Developmental Coordination Disorder Questionnaire were variables of interest. To test hypotheses about the region-specific effects of these variables, the estimates were compared using 2 linear contrasts (positive or negative correlation). In the SPM5 analyses, values of $P < .05$ were statistically significant after adjustment for the false discovery rate in the whole-brain multiple comparisons.

In the VOI-based analyses, we tested the main effect of the diagnosis of ASD on $[^{11}C]$(*R*)-PK11195 BP values derived from 13 brain regions using 2-way analysis of variance, in which statistical significance was set at $P < .05$. For comparisons of clinical variables between subgroups of ASD subjects, a Mann-Whitney test was performed.

To assess the differences in segmented brain volumes between groups in the voxel-based morphometry analysis, we conducted a multivariate analysis of covariance using PASW software with group (ASD and control) as a between-subject factor, segmented brain regional absolute volume (GM, WM, and CSF) as a within-subject factor, and intracranial volume as a covariate. The statistical significance level was set at $P < .05$. Second, for the GM analysis, the normalized, modulated, and smoothed GM image segments in each group were entered into a voxel-wise 2-sample *t* test analysis in SPM5. An absolute threshold mask of 0.30 was used to avoid possible edge effects around the border between GM and WM. The statistical threshold was set at $P < .05$ after the false discovery rate correction. Data were presented as mean (SD).

RESULTS

Characteristics of all the subjects are summarized in Table 1. There was no significant difference in age or IQ between the 2 groups.

COMPARISON OF $[^{11}C]$(R)-PK11195 BP BETWEEN ASD SUBJECTS AND CONTROLS

The tissue TACs of $[^{11}C]$(*R*)-PK11195 are shown in **Figure 1**A. After the administration of $[^{11}C]$(*R*)-PK11195, the radioactivity in 3 ROIs over the cerebellum, midbrain, and thalamus of a representative control subject decreased with time. The TACs in an ASD subject decreased less sharply than those in the control subject, indicating a time-course accumulation of $[^{11}C]$(*R*)-PK11195 in the respective brain structures. Figure 1B shows MRI-PET fusion parametric images of $[^{11}C]$(*R*)-PK11195 BP in the representative control and ASD subjects. A marked increase in $[^{11}C]$(*R*)-PK11195 binding was observed across widespread areas of the brain of the representative ASD subject.

In the voxel-based analysis, we found greater $[^{11}C]$(*R*)-PK11195 BP in multiple brain regions in the ASD group than in the control group; the brain regions with increased $[^{11}C]$(*R*)-PK11195 BP included the cerebellum, brainstem (midbrain and pons), subcortical region (corpus callosum), limbic region (anterior cingulate cortex), and the frontal, temporal, and parietal regions (**Table 2** and **Figure 2**). Among the brain regions, the left cerebellum showed the most prominent *z* score. There were no voxels in which controls had a significantly higher $[^{11}C]$(*R*)-PK11195 BP compared with that of the ASD group. In the ASD group, there was no significant difference in $[^{11}C]$(*R*)-PK11195 BP between the 2 diagnoses—that is, autistic disorder (n = 15) or pervasive developmental disorder not otherwise specified (n = 5).

On the basis of the results of the voxel-based analysis, we then conducted VOI-based analysis. We placed 14 spherical VOIs of 5-mm radius, which centered on the peak voxels listed in Table 2. In accordance with the findings derived from the voxel-based analysis, the $[^{11}C]$(*R*)-PK11195 BP was significantly higher in ASD subjects than in control subjects throughout all VOIs (**Figure 3**; $F_{13,532} = 17.62, P < .001$). As shown in Figure 3, the mean $[^{11}C]$(*R*)-PK11195 BP was highest in the brainstem, followed by the left cerebellum, right orbitofrontal cortex, right anterior cingulate cortex, and other regions in the control group. The corresponding rank order was essentially the same in the ASD group. Thus, the pattern of distribution of $[^{11}C]$(*R*)-PK11195 BP values throughout the VOI was quite similar between the 2 groups. **Figure 4** shows a scatterplot of $[^{11}C]$(*R*)-PK11195 BP from the 4 VOIs (the left cerebellum, midbrain, right orbitofrontal cortex, and right anterior cingulate cortex) in the ASD and control groups. Although the overall average level of $[^{11}C]$(*R*)-PK11195 BP was higher in the ASD group than in the control group, the BPs of some ASD subjects overlapped those of the controls in the 4 VOIs.

CORRELATION BETWEEN $[^{11}C]$(R)-PK11195 BP AND SYMPTOMS IN ASD

Relationships between the regional changes in $[^{11}C]$(*R*)-PK11195 BP values and the clinical features of ASD subjects were evaluated by voxel-based exploratory correlation analyses using SPM5. There was no voxel for which significant correlations were observed between $[^{11}C]$(*R*)-

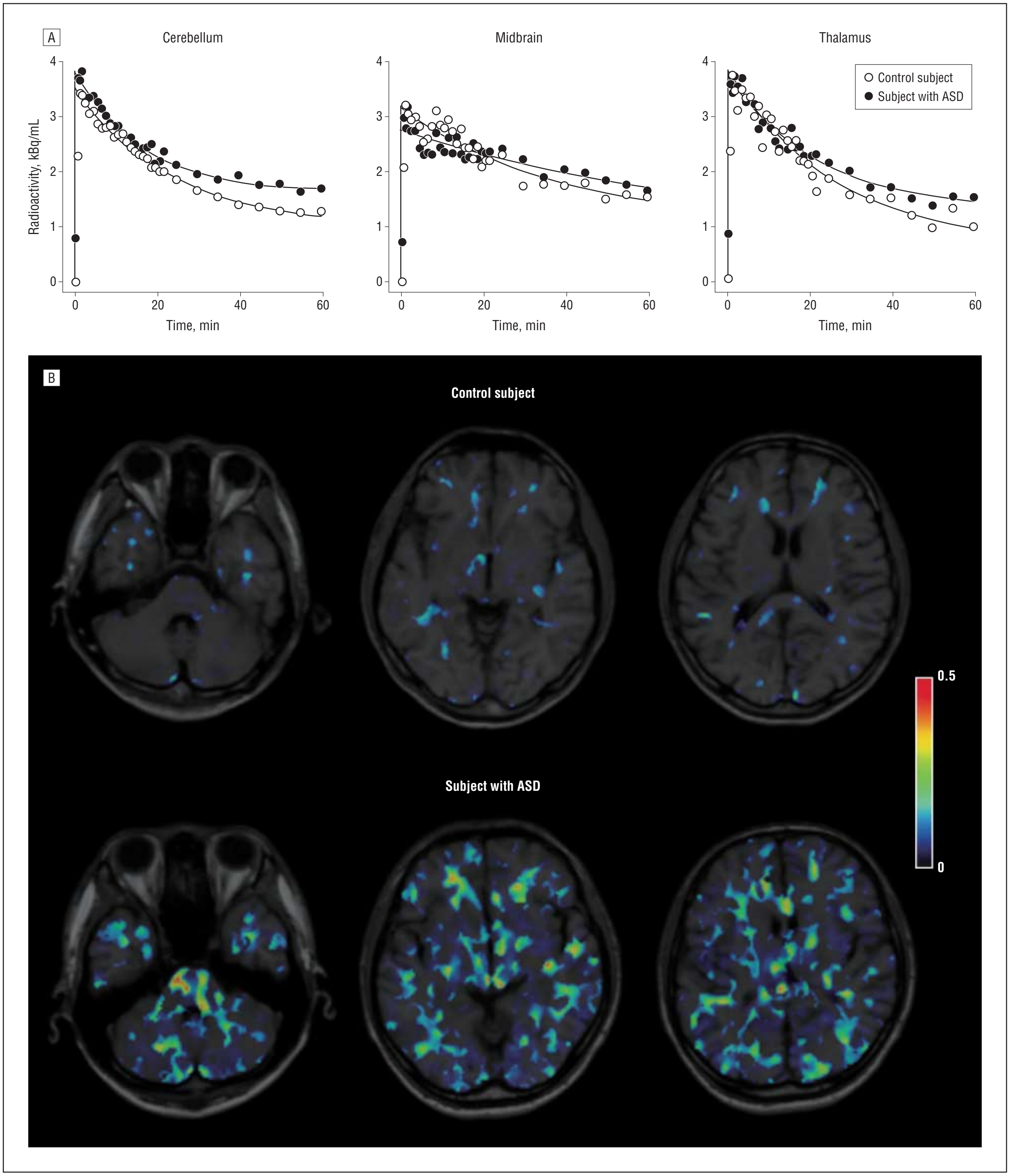

Figure 1. Results of positron emission tomography image analyses in a healthy control subject and a subject with autism. A, Scattergrams showing the time-activity curves of radiocarbon (^{11}C)-labeled (*R*)-(1-[2-chrorophynyl]-*N*-methyl-*N*-[1-methylpropyl]-3 isoquinoline carboxamide) ([^{11}C](*R*)-PK11195) for regions of interest in the cerebellum, midbrain, and thalamus in a subject with autism spectrum disorder (ASD) and a control subject. B, Magnetic resonance imaging–positron emission tomography fusion parametric images of [^{11}C](*R*)-PK11195 binding potential in a subject with ASD and a control subject. The left brain is shown on the right. The color bar indicates a level of binding potential.

PK11195 BP and the scores on the Faux Pas Test, Yale-Brown Obsessive Compulsive Scale, ADI-R, ADOS, or the Japanese version of the Developmental Coordination Disorder Questionnaire after the correction of whole-brain multiple comparisons (data not shown).

In the VOI-based analysis, we also conducted correlation analyses between [^{11}C](*R*)-PK11195 BP in each VOI and clinical valuables, and we found no significant correlations. We divided the ASD group into 2 subgroups, a High-BP and Not-High-BP group, on the basis of the

Table 2. Results of the Whole-Brain Voxel-Based Statistical Parametric Mapping Analyses of [^{11}C](*R*)-PK11195 Binding Potential: Increase in Binding in the Subjects With ASD[a]

	Coordinates			Voxel Level	
Brain Regions	**x**	**y**	**z**	**Corrected *P* Value**	***z* Score**
Cerebellum					
Left lobuli 7, 8, and 9	−10	−58	−38	.03	4.82
Right lobuli 7 and 8	32	−76	−48	.04	3.77
Brainstem (midbrain and pons)	10	−38	−42	.03	4.56
Frontal region					
Left middle frontal gyrus, BA10, BA46	−44	50	12	.03	3.89
Left orbitofrontal cortex, BA11	−8	48	−4	.03	3.93
Right orbitofrontal cortex, BA47	14	30	−16	.03	4.32
Temporal region					
Left superior temporal gyrus, BA22	−52	−28	4	.03	3.67
Right superior temporal gyrus, BA22	50	−20	−6	.03	4.22
Left fusiform gyrus, BA37	−48	−60	−14	.03	4.16
Right fusiform gyrus, BA37	38	−58	−16	.03	4.30
Parietal region					
Right parietal cortex, BA40	28	−48	54	.03	3.70
Limbic region					
Left anterior cingulate cortex, BA32	−6	38	18	.03	4.12
Right anterior cingulate cortex, BA32	18	10	46	.04	3.47
Subcortical region					
Corpus callosum	−2	−26	16	.03	4.11

Abbreviations: ASD, autism spectrum disorder; BA, Brodmann area; [^{11}C](*R*)-PK11195, radioactive carbon-labeled (*R*)-(1-[2-chrorophynyl]-*N*-methyl-*N*-[1-methylpropyl]-3 isoquinoline carboxamide).

[a] The significance thresholds at the voxel cluster levels were $P < .05$ after false discovery rate correction for multiple comparisons across the whole brain. Coordinates are given in millimeters based on the Montreal Neurological Institute brain template. Each location is a peak within a cluster (defined as the voxel with highest *z* score).

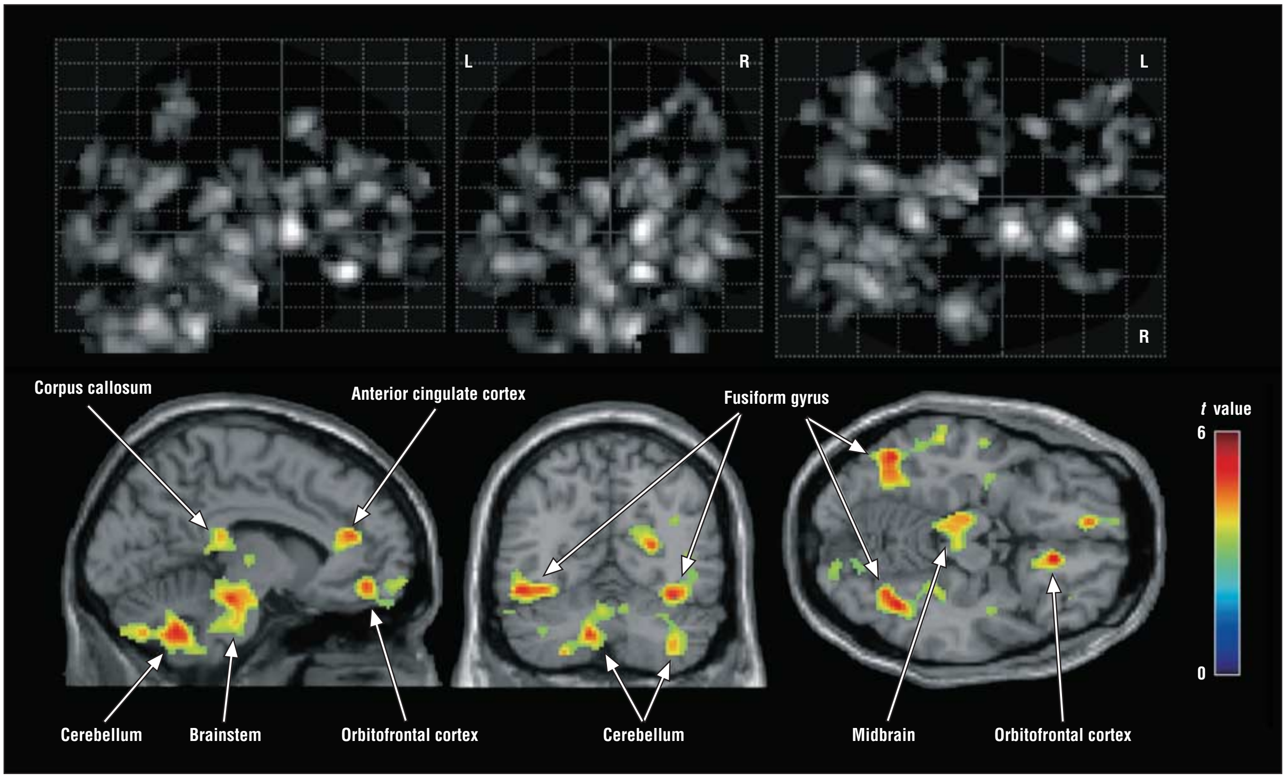

Figure 2. Results of the whole-brain voxel-based statistical parametric mapping analysis of the [^{11}C](*R*)-PK11195 binding potentials. Locations of clusters with significant increases in the group with autism spectrum disorder compared with the control group ($P < .05$, false discovery rate corrected) are shown on glass brain images and superimposed onto normal-template magnetic resonance images. L indicates left; and R, right.

[^{11}C](*R*)-PK11195 BPs in 4 VOIs respectively located in the left cerebellum, midbrain, right orbitofrontal cortex, and right anterior cingulate cortex. In the VOI at the left cerebellum, 12 ASD subjects had BPs that were more

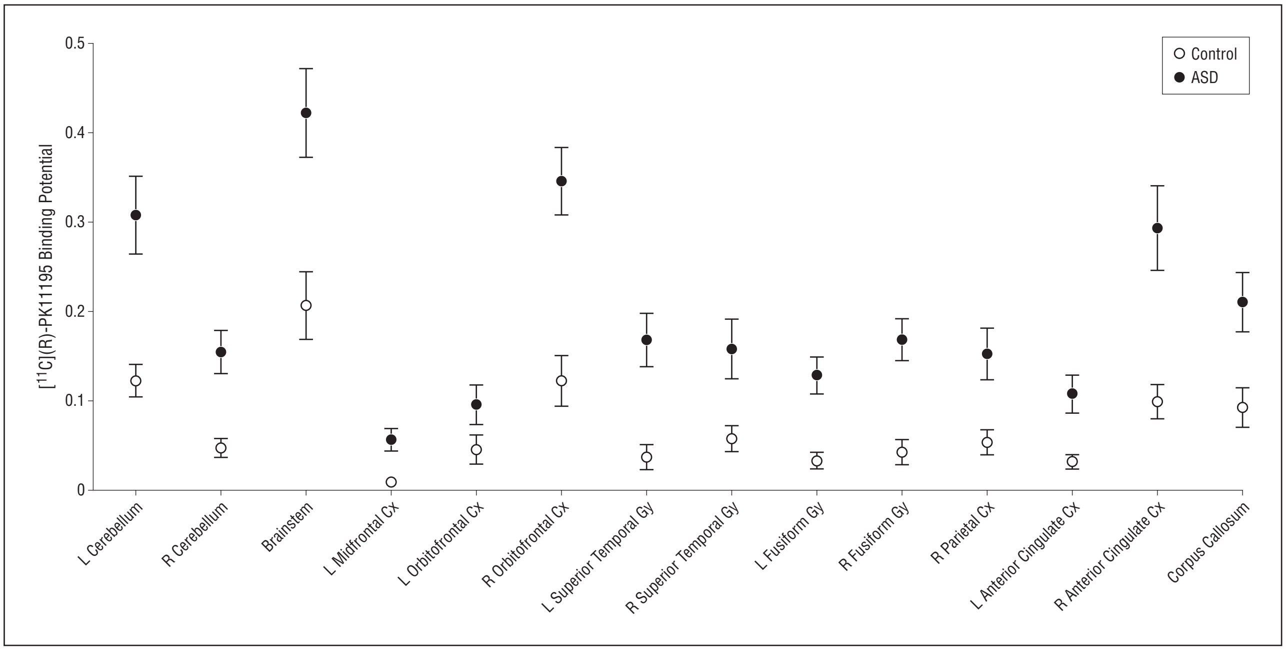

Figure 3. Regional brain [^{11}C](*R*)-PK11195 binding potential in the autism spectrum disorder (ASD) and control group. Subjects with ASD had significantly higher [^{11}C](*R*)-PK11195 binding potentials than those of controls ($F_{12,456} = 24.59$, $P < .001$). Error bars represent the SEM. Cx indicates cortex; Gy, gyrus; L, left; and R, right.

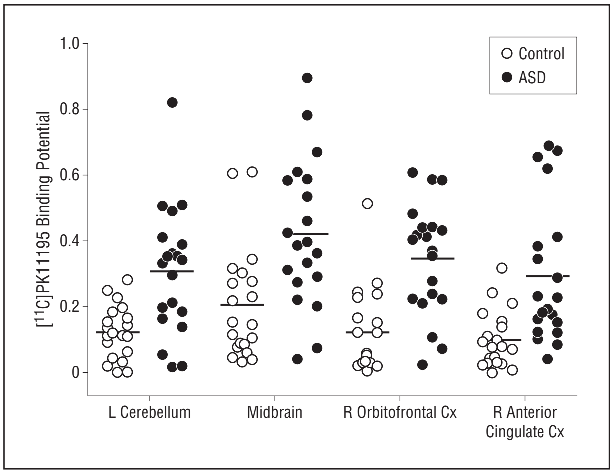

Figure 4. Scatterplot of regional [^{11}C](*R*)-PK11195 binding potential in the autism spectrum disorder (ASD) and control groups in 4 spherical volumes of interest placed over the left cerebellum, midbrain, right orbitofrontal cortex, and right anterior cingulate cortex.

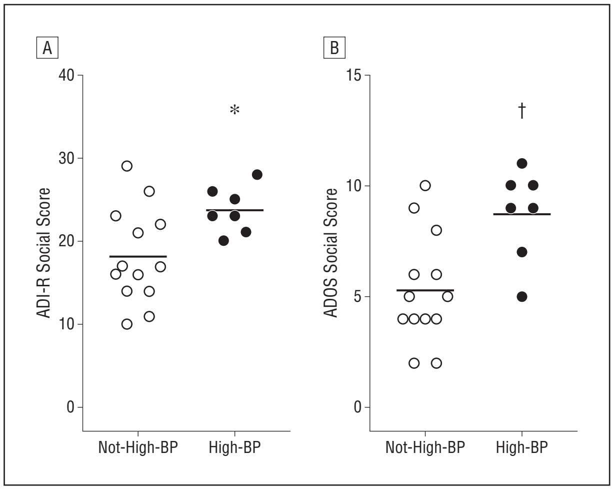

Figure 5. Comparison of social domain scores from Autism Diagnostic Interview–Revised (ADI-R) (A) and Autism Diagnostic Observation Schedule (ADOS) (B) between the High-Binding Potential (BP) and Not-High-BP subgroups in subjects with autism spectrum disorder. *$P = .03$ and †$P = .006$.

than 2 SDs higher than the mean BP of controls. The number of ASD subjects who had BPs that were more than 2 SDs higher than the mean value of the controls was 6 for the VOI in the midbrain, 10 for the VOI in the right orbitofrontal cortex, and 8 for the VOI in the right anterior cingulate cortex. Subjects with ASD who exhibited high BPs in at least 3 of the 4 VOIs were classified into a High-BP group (n = 7), and the remaining subjects were classified into a Not-High-BP group (n = 13). When clinical variables were compared between the High-BP and Not-High-BP groups, statistically significant differences were observed for the social scores of the ADI-R ($U = 19.0$, $P = .04$) and the ADOS ($U = 13.0$, $P = .01$) (**Figure 5**), suggesting that social disabilities might be more severe in the High-BP group.

COMPARISON OF REGIONAL VOLUME BETWEEN ASD SUBJECTS AND CONTROLS

The absolute volumes of the segmented brain regions were estimated in the control and ASD groups (GM: 676.3 [50.3] vs 705.8 [78.2] [control vs ASD]; WM: 421.7 [42.3] vs 439.7 [48.4]; CSF: 405.1 [47.1] vs 426.0 [50.2]; and intracranial volume: 1503.1 [123.7] vs 1571.5 [161.7]). The multivariate analysis of covariance revealed no significant differences in volume between the 2 groups (GM: $F_{1,37} = 0.006$, $P = .94$; WM: $F_{1,37} = 0.209$, $P = .65$; CSF: $F_{1,37} = 0.036$, $P = .85$). A voxel-wise 2-sample *t* test analysis of normalized and smoothed

GM images revealed no significant differences in GM volume between the 2 groups (data not shown).

COMMENT

Our PET measurements revealed that young adults with ASD had significantly increased [^{11}C](*R*)-PK11195 BP, a representative measure of the activation of microglia, in a wide range of brain areas, including the cerebellum, brainstem, anterior cingulate cortex, frontal cortex (orbitofrontal and midfrontal), temporal cortex (superior temporal and fusiform), parietal cortex, and corpus callosum. The microglial activation was greater in the ASD group than in the control group across all regions tested, although the most prominent increase was evident in the cerebellum. To our knowledge, this is the first in vivo evidence of the presence of excessive microglial activation in ASD subjects, and these findings support the contention that microglial activation may play a role in the pathogenesis of ASD.[16,29]

When we performed a VOI-based analysis on the [^{11}C](*R*)-PK11195 BPs for different brain regions associated with microglial activation, the pattern of distribution of [^{11}C](*R*)-PK11195 BP values throughout the VOIs was quite similar between the ASD and control subjects. The similar distribution of regionally activated microglia in the ASD and control groups may indicate the augmented but not altered microglial activation in the brain in the ASD subjects. Resident microglia, which are embryonic and fetal in origin, can be replenished intrinsically and do not require significant turnover from circulating blood progenitors (monocytes)[49] (see also the review by Chan et al[50]). Under pathologic conditions, however, microglia in neonates and adults are considered to derive from circulating blood monocytes originating primarily within the bone marrow.[50] In brain tissues from children and adults with ASD, macrophage chemoattractant protein-1, which can facilitate the infiltration and accumulation of blood monocytes in the brain,[51,52] is greatly increased.[16] It is also possible that microglia might respond to prolonged aberrant neuronal functioning in the ASD adults, providing trophic support to damaged cells or engaging in synaptic stripping to protect against excitotoxicity.[25-28] Taken together, the excessive activation of microglia in ASD subjects could begin in the prenatal period and last until adulthood. However, we propose that the critical period for the occurrence of excessive activation of microglia as a possible pathogenic factor for ASD may be during prenatal and early postnatal development of the brain because symptoms of ASD are manifested very early in life, typically by 3 years of age. To better understand the detailed mechanism underlying the long-running microglial activation, further studies, including experiments in animal models, may be helpful.

In the present PET assessment, young adults with ASD showed a prominent activation of microglia in the cerebellum. The cerebellum has been one of the foci of postmortem studies of autistic children and adults. Of the 30 postmortem cases of autism in which the cerebellum has been studied, 22 (73%) showed a reduced number of Purkinje cells, particularly in the hemispheres.[53-56] Pathologic abnormalities have been observed in both childhood and adult cases, with and without a history of seizures or medication usage. It is not known whether cerebellar lesions might have been present in the high-functioning young adults with ASD recruited for this study. Nonetheless, cerebellar activation of the microglia may reflect an association with cerebellar pathologic abnormalities, because when *N*-acetylaspartate, a putative marker of neuronal loss, was assessed by proton magnetic resonance spectroscopy, levels were significantly decreased in high-functioning adults with ASD.[57] An in vitro study has demonstrated that microglial activation can promote the death of developing Purkinje cells via reactive oxygen species[58]; however, it remains unclear whether this microglia-mediated mechanism would apply in cases of ASD.

The voxel-based correlation analysis failed to find a cluster in which [^{11}C](R)-PK11195 BP correlated significantly with any of the clinical features evaluated by the Faux Pas Test, Yale-Brown Obsessive Compulsive Scale, ADI-R, and ADOS. However, when ASD subjects were divided into High-BP and Not-High-BP subgroups before being entered into the VOI-based analysis, social disabilities as assessed by ADI-R and ADOS in the High-BP subgroup were significantly more severe than in the Not-High-BP subgroup. The results suggest that ASD subjects carrying more microglial activation may be more impaired in their cognitive skills. In a previous study, immune abnormalities in peripheral blood from severely affected children with ASD, especially the regressive type of autism, appeared to correlate with the disturbance of cognitive skills.[13,59] Considering the positive observation of the VOI-based analysis and the previous data in the ASD children with regression, the failure of the voxel-based correlation analysis was probably due to the selection of the ASD subjects, all of whom were high-functioning ASD subjects with no regression. Namely, the subject selection may have been inappropriate for comparison with studies of severely affected cases. The small subject population may be another reason for the lack of voxel-based correlation analysis. In this study, there was no correlation in the cerebellum between the [^{11}C](R)-PK11195 BP and motor coordination as assessed by the Developmental Coordination Disorder Questionnaire. Again, the selection of the high-functioning subjects and the small sample size may have contributed to the absence of correlation. Although there was no correlation of microglial activation with any of the clinical features, this could not exclude the recently emerging evidence that microglia play a crucial role in monitoring and maintaining synapses in the uninjured brain.[27,28] During development, microglia actively engulf synaptic material and play a major role in synaptic pruning.[60,61] Microglial activation might have led to impairment of synaptic function in the corresponding brain regions being associated with clinical features in ASD.[62-67]

Several limitations of our study bear mention. Our study was performed on a population basis and the subject group consisted entirely of high-functioning ASD subjects. That is, this study did not include ASD subtypes in which immunologic abnormality may be more prominent, although greater microglial activations are more likely to occur in more severe subtypes. Another potential weakness was the nature of the tracer used in this study, which has a significant nonspecific binding. Future studies on a wider range of autistic phenotypes using a new ligand with more specificity would be warranted.

In conclusion, the present PET measurements revealed marked activation of microglia in multiple brain regions of young adults with ASD. The results strongly support the contention that immune abnormalities contribute to the etiology of ASD. The similar patterns of distribution of regionally activated microglia in these ASD and control groups may indicate the augmented but not altered microglial activation in the brain in the ASD subjects.

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Reduced subcortical glutamate/glutamine in adults with autism spectrum disorders: a [^{1}H]MRS study

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Dysfunctional glutamatergic neurotransmission has been implicated in autism spectrum disorder (ASD). However, relatively few studies have directly measured brain glutamate in ASD adults, or related variation in glutamate to clinical phenotype. We therefore set out to investigate brain glutamate levels in adults with an ASD, comparing these to healthy controls and also comparing results between individuals at different points on the spectrum of symptom severity. We recruited 28 adults with ASD and 14 matched healthy controls. Of those with ASD, 15 fulfilled the 'narrowly' defined criteria for typical autism, whereas 13 met the 'broader phenotype'. We measured the concentration of the combined glutamate and glutamine signal (Glx), and other important metabolites, using proton magnetic resonance spectroscopy in two brain regions implicated in ASD—the basal ganglia (including the head of caudate and the anterior putamen) and the dorsolateral prefrontal cortex—as well as in a parietal cortex 'control' region. Individuals with ASD had a significant decrease ($P<0.001$) in concentration of Glx in the basal ganglia, and this was true in both the 'narrow' and 'broader' phenotype. Also, within the ASD sample, reduced basal ganglia Glx was significantly correlated with increased impairment in social communication ($P=0.013$). In addition, there was a significant reduction in the concentration of other metabolites such as choline, creatine (Cr) and *N*-acetylaspartate (NAA) in the basal ganglia. In the dorsolateral prefrontal cortex, Cr and NAA were reduced ($P<0.05$), although Glx was not. There were no detectable differences in Glx, or any other metabolite, in the parietal lobe control region. There were no significant between-group differences in age, gender, IQ, voxel composition or data quality. In conclusion, individuals across the spectrum of ASD have regionally specific abnormalities in subcortical glutamatergic neurotransmission that are associated with variation in social development.

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Introduction

Autism spectrum disorder (ASD) is characterized by deficits in social reciprocity, communication impairments, and restricted, repetitive interests and behaviours.[1] Recent research suggests an approximate prevalence of 0.6–1.5% in the general population.[2]

At present, however, therapeutic options for ASD are limited because the pathophysiology of ASD is unclear, leading to a paucity of treatment targets for the core symptoms. Numerous studies have reported abnormalities in brain anatomy and function of ASD individuals (e.g. see Hallahan *et al.*[3] and Barttfeld *et al.*[4]), but the underlying molecular basis of these differences is unknown.

There is, however, emerging evidence suggesting that ASD may be associated with abnormalities in excitatory glutamate and inhibitory γ-amino-butyric acid (GABA) neurotransmission.[5] The balanced interaction between glutamate and GABA transmission is essential for regulating cognition, learning, memory and emotional behaviours. An imbalance between glutamate excitation and GABA inhibition, leading to hyper-excitation, has been linked to ASD.[6–8]

There is also evidence for an association between ASD and genetic variation in the glutamatergic and GABAergic systems. For example, there are reports of associations between ASD and variants in genes coding for glutamate receptors[9,10] and glutamate transporter proteins,[11] although not in all studies.[12] Also, recent work on fragile X syndrome, the most common monogenetic syndrome associated with ASD, points to the potential importance of metabotropic glutamate receptors (mGluR1) as possible treatment targets in ASD.[13]

Unfortunately, it is not possible to quantify glutamate and GABA concentrations in post-mortem studies, because they degrade rapidly after death. Progress can be made, however, as *in vivo* proton magnetic resonance spectroscopy ([^{1}H]MRS) can be used to quantify a range of neural metabolites, including glutamate and its metabolic product glutamine (Glu + Gln—henceforth abbreviated Glx).

There are six published [^{1}H]MRS studies reported on Glx in ASD. Of these, four investigated children, one reported a widespread decrease in cortical Glx[14] and another reported a nonsignificant reduction in Glx in the left thalamic region.[15] However, two other studies found no differences in any region studied: one investigated the frontal, temporal and parietal cortex and basal ganglia,[16] and the other the frontal cortex and basal ganglia.[17]

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Only two published [^{1}H]MRS studies have measured Glx in adults with ASD. Page *et al.*[18] reported that adults with ASD had a significantly *higher* concentration of Glx than controls in the right amygdala-hippocampal complex. In contrast, Bernardi *et al.*[19] found a significantly *lower* Glx in the right anterior cingulate cortex.

These prior investigations suggest that ASD individuals may have differences in brain Glx, but the results are inconsistent. Possible explanations for this mixed picture are that some of these studies investigated relatively small samples, and they examined different age groups and/or brain regions. Also, no study of adults has yet addressed whether any of these putative differences are present across the behavioural spectrum (i.e. in both the 'core' disorder, and those with the 'broader phenotype').

This is potentially of importance because while, in the past, autism was generally treated as a 'categorical' diagnosis, it is now understood to likely cover a spectrum of severity. For example, the biological relatives of people with ASD often show an attenuated 'broader phenotype' of mild social, cognitive and neurobiological abnormalities.[20]

This clinical (and likely aetiological) heterogeneity has led some to suggest that we refer to 'the autisms' rather than to 'autism' and search for final common pathways through which various causative agents may lead to disorder.[21] However, previous [^{1}H]MRS studies of adults with ASD have treated all participants with ASD as a single group (although one study in children did not).[22] Thus, it is unclear whether putative abnormalities in Glx are present across the spectrum, that is, whether they are a potential common pathway, and/or relate to particular core symptoms.

Hence, in this study, we used [^{1}H]MRS to investigate differences in brain glutamate and other metabolites in adults with ASD. We compared controls with ASD people diagnosed with the narrow 'core' disorder, who scored above cutoff on research diagnostic criteria, and those with a broader phenotype, who only met some of the criteria.

We focused on regions previously implicated in ASD pathology and symptomatology: the basal ganglia and the dorsolateral prefrontal cortex (DLPFC). For example, (1) anatomical and metabolic abnormalities have been reported in both of these interconnected areas in ASD;[23,24] (2) the basal ganglia have been linked with social and emotional differences[25] and compulsive and repetitive behaviours[26] and (3) the DLPFC has been linked to deficits in executive function[27] and theory of mind.[28] Hence, we also correlated [^{1}H]MRS measures that differed significantly between groups with scores on the Autism Diagnostic Interview—Revised (ADI-R) interview.

We also included a 'control' region, in the medial parietal lobe, which has not been linked to ASD and where no differences were seen in a previous [^{1}H]MRS study.[18]

Materials and methods

Participants. We recruited 42 adult participants: 28 individuals with ASD and 14 healthy controls matched for age, gender and IQ (see Table 1). All participants had an IQ above 65. We recruited only participants who reported being right-handed, to avoid possible lateralization effects given our use of unilateral [^{1}H]MRS voxels.

Table 1 Participant demographic and clinical characteristics

	Narrow ASD	*Broader ASD*	*Control*	*F*	P-*value*
Number	15	13	14	N/A	
Number female	1	1	3		
Age (years)	29 (6.0)	27 (6.4)	34 (8.8)	2.86	0.07
FSIQ	95 (13)	103 (16)	107 (21)	1.63	0.21
VIQ	95 (16)	101 (20)	106 (19.0)	1.21	0.31
PIQ	96 (19)	106 (12)	107 (21)	1.47	0.24
ADI-R A domain	16.5 (4.2)	8.7 (2.4)	N/A		
ADI-R B domain	10.7 (2.4)	6.3 (2.6)			
ADI-R C domain	3.9 (0.8)	1.8 (1.5)			

Abbreviations: ADI-R, Autism Diagnostic Interview—Revised; Domain A, social interaction; FSIQ, full-scale IQ; Domain B, communication; Domain C, restricted and repetitive patterns of behaviour; HFA, high functioning autism; N/A, not applicable; PIQ, performance IQ; VIQ, verbal IQ.
Values are expressed as mean (s.d.), unless otherwise indicated.
Note: The healthy control group did not receive an ADI-R assessment. Therefore, there are no ADI-R scores for this group.

The 28 participants in the ASD group were further divided into two subtypes on the basis of their symptom profile. Fifteen were diagnosed with the 'narrowly defined phenotype' of autism based on the fact that they met the ADI-R cutoff criteria in all three symptom domains and fulfilled the diagnostic criteria for childhood autism or Asperger's syndrome according to the ICD-10 Research Classification of Mental and Behavioural Disorders[1] (criteria F84.0 and F84.5, respectively). The other 13 individuals were classified as having the 'broader phenotype', that is, they did not meet the ADI-R cutoff in one domain (see Table 1), but fulfilled the ICD-10 diagnostic criteria for atypical autism (F84.1).

All individuals with ASD were recruited through London's Maudsley Hospital Behavioural Genetics Clinic, a specialist diagnostic service.

Potential participants were excluded if they had a comorbid psychiatric or medical disorder affecting brain development (e.g. epilepsy or psychosis), a history of head injury, a genetic disorder associated with ASD, for example, tuberous sclerosis or fragile X syndrome, or an IQ below 65. Participants with ASD suffering from anxiety or depressive disorders were not excluded, given the high frequency of these comorbidities in ASD. According to participant self-report, all participants were medication naive at the time of scanning.

All participants provided written informed consent. Ethical approval for this study was provided by South London and Maudsley/Institute of Psychiatry NHS Research Ethics Committee, study reference 1997/087.

[^{1}H]MRS data acquisition. [^{1}H]MRS data were acquired on a 1.5 T GE HDx magnetic resonance imaging (MRI) scanner (GE Medical Systems, Milwaukee, WI, USA) equipped with TwinSpeed gradients.

The scanning protocol included a structural MRI scan, namely a three-dimensional fast inversion-recovery-prepared gradient echo acquisition (number of slices = 146, slice thickness = 1.2 mm, inversion time (TI) = 300 ms, repetition time (TR) = 11 ms, echo time (TE) = 5 ms, field of

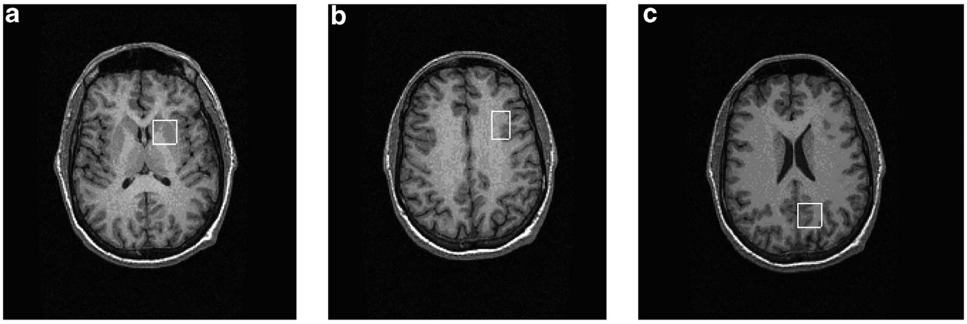

Figure 1 Examples of the location of proton magnetic resonance spectroscopy ([^{1}H]MRS) voxels. Three voxels were positioned in (**a**) left basal ganglia ($20 \times 20 \times 15\,\text{mm}^3$) to include the head of the caudate, putamen and internal capsule, (**b**) left dorsolateral prefrontal cortex ($16 \times 24 \times 20\,\text{mm}^3$) and (**c**) left medial parietal lobe ($20 \times 20 \times 20\,\text{mm}^3$).

view = 310 mm, flip angle = 18°, matrix = 256×160 over a 310×194 mm field of view, giving $1.2 \times 1.2 \times 1.2\,\text{mm}^3$ voxels). This structural MRI was used for the localization of the spectroscopy voxels in each participant.

Single voxel [^{1}H]MRS spectra were then acquired, using a point-resolved spectroscopy sequence. Point-resolved spectroscopy parameters were: TR = 3000 ms and TE = 30 ms. A voxel of interest was positioned in the left basal ganglia ($20 \times 20 \times 15\,\text{mm}^3$). This voxel included parts of the head of the caudate, the anterior putamen and the internal capsule. Voxel of interests were also positioned in the left DLPFC ($16 \times 24 \times 20\,\text{mm}^3$) and in the left medial parietal lobe ($20 \times 20 \times 20\,\text{mm}^3$), using previously described methods.[29] See Figure 1 for an illustration of the location of the voxels.

Data processing. [^{1}H]MRS spectra were processed using the LCModel software version 6-1-0 (Stephen Provencher Incorporated, Oakville, Canada). LCModel uses a linear combination of model spectra of metabolite solutions *in vitro* to analyse the major resonances of *in vivo* spectra. In this case, a basis set of alanine, aspartate, creatine (Cr), γ-aminobutyric acid (GABA), glutamine, glutamate, glycerophosphocholine, mI, lactate, *N*-acetylaspartate (NAA), *N*-acetyl-aspartylglutamate, scyllo-inositol and taurine, together with a baseline function, were used for the analysis. Each spectrum was reviewed to ensure adequate signal-to-noise ratio, as well as the absence of artefacts. Note that the NAA resonance at 2 p.p.m. contains both NAA and *N*-acetyl-aspartylglutamate; we report here results reflecting the combination of NAA + *N*-acetyl-aspartylglutamate, and use the term NAA for brevity.

Calculation of absolute metabolite concentrations. Metabolite concentrations for NAA, Cr, Glx and choline (Cho) were calculated, in institutional units, as follows. The raw metabolite estimates (LCModel output) were first corrected by reference to calibration data from a phantom, containing an aqueous solution of known NAA concentration. One phantom [^{1}H]MRS spectrum was acquired at the end of each scanning session. The amplitude of the phantom NAA peak was used to derive a correction factor, by which all metabolite values for the scan were multiplied.

Furthermore, partial volume effects (group differences in proportions of gray matter, white matter and cerebrospinal fluid, CSF, in the [^{1}H]MRS voxels) are a potential confound in spectroscopy. This could be especially relevant to the present investigation, given previously reported volumetric differences between ASD individuals and controls, for example, in the basal ganglia.[23,26]

Therefore, to guard against such confounds, we determined the percentage of gray matter, white matter and CSF within each [^{1}H]MRS voxel for each participant. We first segmented the T1-weighted structural MRI using an automated procedure, *spm_segment*, part of the Statistical Parametric Mapping software package (SPM2; http://www.fil.ion.ucl.ac.uk/spm/software/spm2/; Wellcome Trust Centre for Neuroimaging, London, UK).

The position of each individual [^{1}H]MRS voxel relative to the corresponding structural was determined, using positional coordinates embedded in the raw spectra data files. The % grey, white and CSF composition of each voxel was then calculated automatically using in-house software. Finally, all metabolite concentrations were corrected for the amount of CSF in the voxel—under the assumption that CSF only contains negligible quantities of the metabolites of interest—by multiplying values by an individual correction factor = $1/(1 - \text{Proportion}_{\text{CSF}})$, where $\text{Proportion}_{\text{CSF}}$ could range from 0 to 1, calculated separately for each voxel from each participant. This was applied after correcting for phantom NAA values (see above).

In summary: $\text{Metabolite}_{\text{corrected}} = \text{Metabolite}_{\text{raw}} \times (\text{PhantomNAA}_{\text{known}}/\text{PhantomNAA}_{\text{observed}}) \times (1/(1 - \text{Proportion}_{\text{CSF}}))$.

Statistical analysis. Age and IQ were compared using one-way analysis of variance (ANOVA) across the three groups (healthy control, broad ASD and narrow ASD).

Differences in mean metabolite concentrations were calculated using a series of one-way ANOVAs, with group as a between-subjects factor. One such ANOVA was performed for each of the four metabolites, in each of the three voxels, a total of 12 ANOVAs. As this procedure involves multiple (12) comparisons, we applied a Bonferroni correction to guard against Type I errors. We report results both before and after this correction.

Planned *post hoc* independent sample *t*-tests were then applied, in metabolites where a significant between-group difference was found on the ANOVA, to evaluate differences between (a) narrow ASD and healthy controls, (b) broader ASD and healthy controls and (c) between the two ASD groups (narrow vs broad).

Table 2 Voxel tissue composition of grey matter, white matter and CSF

								ANOVA	
Region	*Measure*	*Narrow ASD*	*s.d.*		*s.d.*	*Control*	*s.d.*	*F*	P*-value*
Basal ganglia	Grey matter	62.20%	12.94%	64.99%	6.01%	54.31%	16.49%	2.65	0.084
	White matter	35.68%	13.07%	33.04%	5.12%	43.84%	17.13%	2.73	0.078
	CSF	2.09%	1.45%	1.92%	1.57%	1.82%	1.66%	0.16	0.849
DLPFC	Grey matter	45.35%	14.48%	45.32%	14.01%	2.17%	14.37%	0.38	0.685
	White matter	48.79%	17.57%	49.37%	16.70%	54.06%	17.34%	0.68	0.685
	CSF	5.67%	4.03%	5.07%	3.39%	4.03%	3.58%	0.76	0.476
Parietal lobe	Grey matter	44.47%	7.94%	47.61%	7.62%	44.89%	6.33%	1.03	0.366
	White matter	35.16%	14.10%	34.47%	11.98%	38.39%	11.43%	0.35	0.706
	CSF	19.34%	14.28%	15.75%	6.86%	16.09%	7.56%	0.48	0.625

Abbreviations: ANOVA, analysis of variance; ASD, autism spectrum disorder; CSF, cerebrospinal fluid; DLPFC, dorsolateral prefrontal cortex.

We examined possible correlations between concentrations of metabolites that differed significantly from controls in these *t*-tests against ADI-R domain scores, across the whole combined ASD group, using Pearson's product–moment correlation coefficients.

All analyses were performed using SPSS 15.0 software (SPSS, Chicago, IL, USA).

Results

Demographics. Groups did (Table 1) not differ significantly in age, full-scale IQ, verbal IQ or performance IQ.

Tissue composition and data quality. Groups did not differ significantly in mean voxel % grey matter, white matter or CSF in any of the three voxels (Table 2). This is unsurprising as, although volumetric differences have been observed in ASD in the basal ganglia[26] and cortex,[30] these were of small magnitude, and would not be expected to materially affect composition of hand-placed voxels.

To verify that the quality of the [^{1}H]MRS data did not vary between groups, we compared the LCModel 6-1-0 Cramer-Rao Lower Bound estimate standard deviations for each metabolite in each voxel, using a one-way ANOVA across the three groups. This revealed no significant differences (all $F(2,42) < 2.8$, all $P > 0.07$.) See Figure 2 for an example of a [^{1}H]MRS spectrum after model fitting.

Finally, because of the potential risk of 'drift' in extended [^{1}H]MRS investigations (in which metabolite estimates on the same scanner change over long periods of time), we compared the timings of scans across the three groups, in terms of days after the first scan of the series. This revealed no significant difference (one-way ANOVA $F(2,44) = 0.739$, $P = 0.484$). Scan date was also not correlated with the value of any metabolite in any voxel (all Pearson's $r < 0.23$, all $P > 0.13$).

Metabolite differences

Basal ganglia. There was a significant group effect in every metabolite concentration we measured in this voxel (ANOVA) (Table 3). Two of these effects—Cr and Glx—survived conservative Bonferroni correction for multiple comparisons over all metabolites and voxels. *Post hoc* independent sample *t*-tests showed that Glx concentrations were significantly lower in both the 'restricted' and 'broader' ASD phenotypes compared with controls (see Figure 3). Both Cr and NAA showed a similar pattern. Cho was also lower in both ASD groups as compared with controls, but this only reached statistical significance in the broader ASD phenotype.

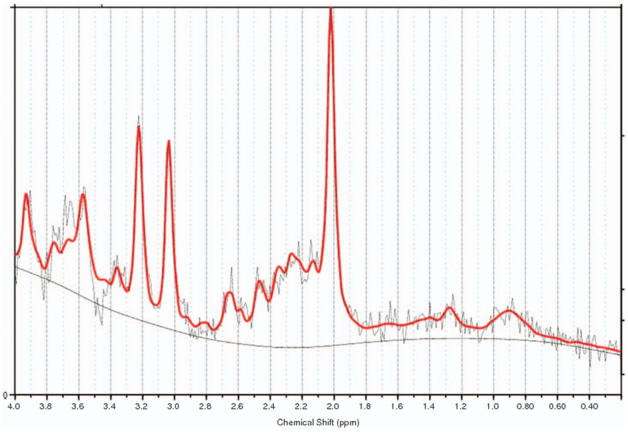

Figure 2 Example of a proton magnetic resonance spectroscopy ([^{1}H]MRS) spectrum showing LCModel 6-1-0 fit.

There were no significant differences between the two ASD subgroups in any metabolite concentration.

To establish whether the finding of lower Glx remained significant after controlling for tissue composition and the other metabolite concentrations, we performed a univariate GLM covarying for basal ganglia grey/white matter, Cr, NAA, Cho, age and IQ. The findings remained significant ($F = 3.530$, $P < 0.05$).

Dorsolateral prefrontal cortex. There was no significant effect of group for Glx or Cho. However, there were significant effects of Cr and NAA, although neither of these differences survived conservative Bonferroni correction. *Post hoc* tests revealed that Cr was significantly lower in both ASD groups relative to healthy controls, whereas NAA was significantly lower only in the 'narrow' ASD phenotype. However, there were no significant differences between the two ASD subgroups.

Table 3 Metabolite concentrations in BG, DLPFC and parietal cortex [^{1}H]MRS voxels and group differences

					ANOVA		Post hoc t-*tests*—P-*values*[b]		
Region	*Metabolite*[a]	*Narrow ASD*	*Broader ASD*	*Control*	*F*	P-*values*	*Narrow ASD* vs *controls*	*Broad ASD* vs *controls*	*Broad ASD* vs *narrow ASD*
Basal ganglia	Glx	10.12 (1.14)	10.43 (1.26)	12.34 (1.45)	12.63	<**0.0001**[c]	<0.001	0.001	0.25
	Cho	1.205 (0.19)	1.098 (0.14)	1.324 (0.19)	5.83	0.006[d]	0.212	0.004	0.44
	Cr	5.097 (0.65)	5.323 (0.53)	6.435 (1.25)	10.02	**0.0003**[c]	<0.001	0.004	0.47
	NAA	5.454 (0.81)	5.486 (0.42)	6.458 (1.52)	4.54	0.017[d]	0.030	0.045	0.32
DLPFC	Glx	7.492 (1.11)	7.156 (1.51)	8.007 (1.42)	1.36	0.27	—	—	0.32
	Cho	1.129 (0.21)	1.110 (0.16)	1.271 (0.27)	2.35	0.11	—	—	0.93
	Cr	4.074 (0.48)	4.021 (0.49)	4.602 (0.60)	5.32	0.009[d]	0.027	0.017	0.64
	NAA	5.954 (0.79)	5.955 (0.47)	6.660 (0.95)	4.07	0.025[d]	0.047	0.057	0.22
Parietal	Glx	10.607 (2.79)	9.790 (1.65)	11.028 (1.62)	1.31	0.28	—	—	0.41
	Cho	1.112 (0.37)	1.048 (0.13)	1.149 (0.25)	0.53	0.59	—	—	0.524
	Cr	5.461 (1.42)	5.140 (0.81)	5.382 (0.70)	0.39	0.68	—	—	0.19
	NAA	7.998 (2.32)	7.472 (0.76)	7.887 (1.06)	0.48	0.62	—	—	0.31

Abbreviations: ANOVA, analysis of variance; ASD, autism spectrum disorder; BG, basal ganglia; Cho, choline; Cr, creatine; DLPFC, dorsolateral prefrontal cortex; Glx, glutamate and glutamine; [^{1}H]MRS, proton magnetic resonance spectroscopy; NAA, *N*-acetylaspartate.

[a] Data are expressed as mean (s.d.). [b] Post hoc *t*-tests comparing ASD cases with controls were only performed when ANOVA was significant at uncorrected $P<0.05$. [c] Bold figures show values significant at $P=0.05$ level, with Bonferroni correction over 12 comparisons, that is, uncorrected $P<0.0047$. [d] Significant at $P=0.05$ level before Bonferroni correction.

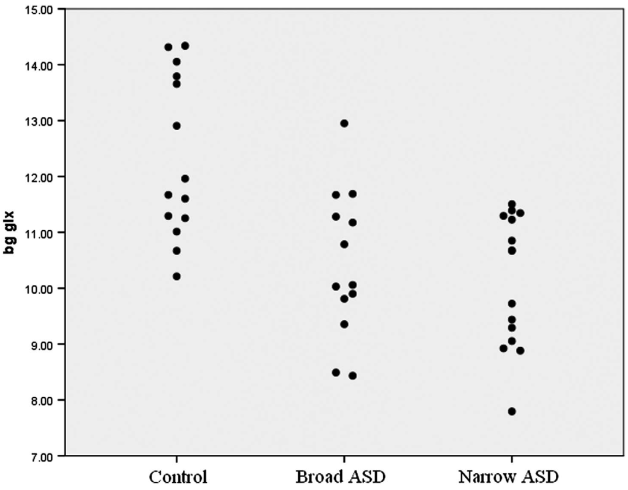

Figure 3 Individual participant data showing basal ganglia glutamate and glutamine (Glx) by group. ASD, autism spectrum disorder.

Parietal region. As predicted, no significant differences were found in any metabolite concentration between any of the groups, even before Bonferroni correction.

Relationship to behavioural variables. Across the combined ASD group (both broader and narrow phenotype), lower basal ganglia concentration of Glx was significantly correlated with worse scores on the ADI-R Communication Scale (i.e. more abnormal Glx concentrations were associated with greater communication impairment *(*$r=-0.465$, $P=0.013$, $n=28$; see Figure 4). This correlation was specific to this metabolite and this symptom domain: no correlations were seen in other metabolites or domains.

We further examined this association within each group separately. There was no significant correlation in the narrowly defined autism group ($r=-0.224$, $P=0.422$), but there was in those with the broader phenotype ($r=-0.805$, $P=0.001$).

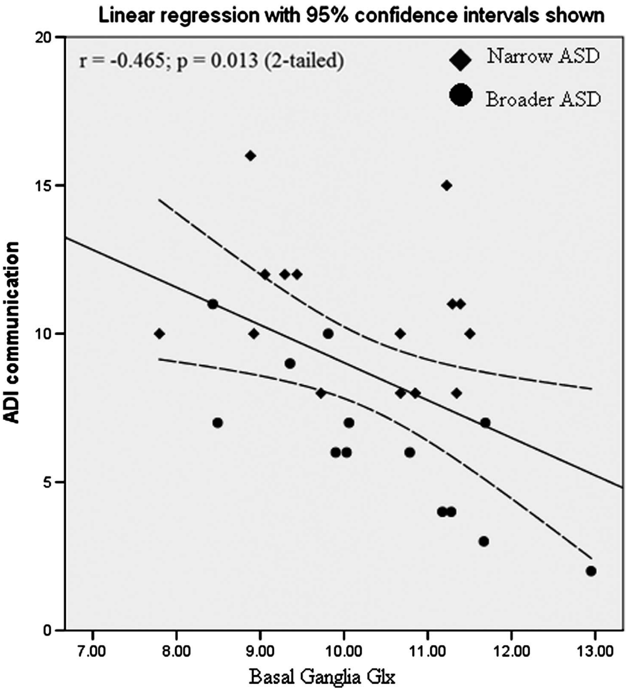

Figure 4 Association between basal ganglia glutamate and glutamine (Glx) and Autism Diagnostic Interview—Revised (ADI-R) Communication Subscale Score in individuals with autism spectrum disorder (ASD).

Discussion

We found that adults with ASD have a significantly reduced Glx concentration in the basal ganglia as compared with controls. Our preliminary evidence further suggests that that this reduction was (1) regionally specific, that is, there were no significant differences in the other cortical regions we examined; (2) a potential final common pathway in ASD, as it was present in both ‘narrow’ and ‘broadly’ defined ASD; and (3) was associated with some aspects of clinical variation (social communication).

We suggest that it is unlikely that our findings can be fully explained by potential confounds, such as differences in voxel tissue composition, age, IQ or medication. There were no significant between-group differences in voxel grey matter, white matter and CSF, and all metabolite values were corrected for CSF %. Demographic variables such as age, gender and IQ were not different between the groups, all participants were right handed and all of the individuals we studied were psychotropic medication naive according to self-report.

This is the first [^{1}H]MRS study to report on Glx in the basal ganglia of adults with ASD. Our finding of reduced Glx in the basal ganglia (predominantly the lentiform nuclei) agrees with studies finding reductions of Glx in the cingulate cortex and the thalamus,[19] but contrasts with our previous finding of *increased* Glx in the amygdala-hippocampal cortex in adults with ASD.[18] Also, similar to prior studies, we found no differences in the parietal cortex.[18]

Taken together, these results demonstrate that, rather than being a ‘global’ neurobiological abnormality, Glx changes seen in ASD are highly regionally specific, suggesting that the underlying neurobiological cause(s) are also localized. Reductions in Glx could result simply from a local reduced density of glutamatergic synapses and neurons, such as reduced storage capacity and turnover, but could also be the product of alterations in glutamate and glutamine metabolism.

In neurons, glutamate is synthesized from glutamine via glutaminase, but after release in the synapse, glutamate is converted back into glutamine in glial cells, by glutamine synthetase. Glutamate is also converted to GABA by the neuronal enzyme glutamate decarboxylase (GAD).[5]

Alterations in GAD expression could be a potential explanation for the fact that we observed reduced Glx in the basal ganglia. This hypothesis would also help to reconcile these results with the suggestion that individuals with ASD have an *inhibitory* signalling deficit and an *increased* ratio of excitatory glutamate to inhibitory GABA transmission.[8,31] It is possible that, while the Glx signal was reduced, the *ratio* between glutamate and GABA was still increased. Reduced glutamate would be expected to lead to a corresponding reduction in GABA synthesis, as glutamate is the precursor of GABA. If GAD activity were reduced, one would expect a lower GABA:Glx ratio.

Unfortunately, there have not yet been any studies examining GAD expression in the basal ganglia in ASD, but studies of other brain regions have shown regionally specific differences associated with the disorder. For example, GAD has been reported to be *decreased* in cerebellar Purkinje cells,[32] but increased in cerebellar interneurons,[33] in ASD.

A further possibility is that the observed differences in Glx are secondary to alterations in other neurotransmitter systems. For example, the basal ganglia are densely innervated by serotonergic projections, which exert complex modulatory effects on glutamate and GABA release.[34] We and others reported reduced density of cortical 5HT2A (serotonin 2A) receptors and serotonin transporter in the cortex and midbrain,[35–37] (although see Girgis *et al.*[38]) the same pattern has been found in the parents of children with ASD.[39] Further, some have reported that children with ASD have significant differences in serotonin synthesis.[40] Hence, it could be that serotonergic abnormalities underlie the differences in Glx we observed—either indirectly via influences on neurodevelopment or through direct action on glutamate metabolism.

Also, in the context of previous findings, our results suggest that within ASD age may be an important moderator of both cortical and subcortical differences in brain Glx. Specifically, prior [^{1}H]MRS studies in *children* with ASD reported widespread decreases in cortical Glx[14]—but no differences in the basal ganglia.[16,17] This is the opposite of the pattern we observed in adults, namely no differences in the cortical regions (DLPFC and parietal lobe), but a reduction in the basal ganglia. This is consistent with the idea of autism as a disorder of brain maturation.[41] We were unable to address age effects directly, as this study did not include children—but this is a focus of our ongoing studies.

The correlation between basal ganglia (Glx) and the severity of social communication impairments in ASD is consistent with the known involvement of this area in various aspects of language and communication. For example, functional imaging studies demonstrated that the caudate and putamen are involved in ‘higher level’ aspects of language, such as inferring with the implied as opposed to literal meaning of sentences,[42] and resolving ambiguous sentences—functions that are characteristically impaired in ASD. This correlation was specific to the basal ganglia, however, with no significant correlation seen in the DLPFC, an area also known to be involved in communication and Theory of Mind.[28] One explanation for this could be that alterations in neurotransmitters other than glutamate are responsible for DLPFC dysfunction in autism; serotonin is one possibility, as a previous study found that lower cortical 5HT2A receptor binding is associated with communication impairments in ASD.[37]

We are only able to report a correlation between ASD in adults (and in particular, Social Communication symptoms as measured by the ADI-R) and reduced basal ganglia Glx levels. Hence, we cannot be certain whether the differences in Glx are the *cause* of the ASD symptoms. It is possible that they represent a downstream *effect* of the symptoms if, for example, the lack of social interaction or high-level language use engaged in by people with ASD led, over time, to neuroplastic changes in corticobasal circuits. However, we do not believe that this can explain all our results, because the ADI is a measure childhood (developmental) symptoms in the first years of life.

In addition to the findings related to glutamate, discussed above, we also observed reduced levels of Cho, Cr and NAA in the basal ganglia, and of Cr and NAA in the DLPFC, in individuals with ASD, although only the basal ganglia Cr difference survived conservative Bonferroni correction. The finding of reduced Cr and NAA in both the basal ganglia and in the DLPFC is consistent with previous [^{1}H]MRS studies in this area in ASD.[14,15,22] However, we have previously[29] reported *increased* NAA, Cr and Cho in the *medial* prefrontal cortex of adults with Asperger’s syndrome, underlining that the differences seen are regionally specific. Also in line with prior work,[18,29] we did not observe any significant metabolite differences in the parietal cortex control region. This confirms that the effects observed in the basal ganglia and the DLPFC are regionally specific.

As both Cr and NAA are involved in neuronal energy metabolism, our finding of local reductions in these metabolites in the basal ganglia and the DLPFC may indicate either metabolic dysfunction in these areas or a reduced density of metabolically active neurons. If the latter, this may also explain the observed reduction in Cho in the basal ganglia (as this is a component of cell membranes).

Another important implication of our finding of reduced Cr in ASD in the basal ganglia and DLPFC is that it may be invalid to express the concentration of metabolites such as NAA and Glx as ratios to Cr in the same voxel. This approach is commonly used in [^{1}H]MRS studies,[22,43] as it is widely assumed that Cr is constant; our findings suggest the this is not true in adults with ASD.

However, our study does have a number of limitations. We obtained [^{1}H]MRS data on a 1.5 T MRI scanner. At 1.5 T, it is not possible to distinguish between the compounds that contribute to the 'Glx' signal, that is, glutamate and glutamine. Future studies at 3 T or higher are needed to distinguish these compounds, but previous studies have cautiously attributed reductions in Glx to glutamate, as glutamate constitutes the most abundant central neurotransmitter.[14,15,18]

Another limitation is that we only recruited people with a normal or above-normal IQ, and excluded those with a history of epilepsy or seizures. This served to increase the homogeneity of the sample and ensure that any differences observed were associated with symptoms of ASD *per se*, but it means that the results may not be representative of the entire ASD population, as many people with ASD also suffer from a below-normal IQ and/or epilepsy.[1] Future studies should examine this population.

In summary, we found preliminary evidence that adults with ASD (both narrowly and broadly defined) have significant differences in brain glutamate and/or glutamine metabolism. This may be a final 'common pathway' in the disorder, and underpin some clinical symptoms. Further work is required to determine the cause(s) of this putative abnormality.

Conflict of interest

The authors declare no conflict of interest.

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Disclaimer. The corresponding author (JH) had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Immune Dysfunction and Autoimmunity as Pathological Mechanisms in Autism Spectrum Disorders

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REVIEW
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Immune Dysfunction and Autoimmunity as Pathological Mechanisms in Autism Spectrum Disorders

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Autism spectrum disorders (ASD) are a group of heterogeneous neurological disorders that are highly variable and are clinically characterized by deficits in social interactions, communication, and stereotypical behaviors. Prevalence has risen from 1 in 10,000 in 1972 to 1 in 59 children in the United States in 2014. This rise in prevalence could be due in part to better diagnoses and awareness, however, these together cannot solely account for such a significant rise. While causative connections have not been proven in the majority of cases, many current studies focus on the combined effects of genetics and environment. Strikingly, a distinct picture of immune dysfunction has emerged and been supported by many independent studies over the past decade. Many players in the immune-ASD puzzle may be mechanistically contributing to pathogenesis of these disorders, including skewed cytokine responses, differences in total numbers and frequencies of immune cells and their subsets, neuroinflammation, and adaptive and innate immune dysfunction, as well as altered levels of immunoglobulin and the presence of autoantibodies which have been found in a substantial number of individuals with ASD. This review summarizes the latest research linking ASD, autoimmunity and immune dysfunction, and discusses evidence of a potential autoimmune component of ASD.

Keywords: autism, immune, dysregulation, autoimmunity, neurodevelopment, behavior

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INTRODUCTION

First defined as a distinct syndrome in 1943 by child psychiatrist Kanner (1943), autism spectrum disorders (ASD) are a group of heterogeneous neurological disorders that are clinically characterized by deficits in social interactions, communication, and stereotypical behaviors (Baio et al., 2018). Recent prevalence of ASD has risen dramatically to 1-in-59 US children, with preponderance toward males (Baio et al., 2018). Although increased awareness of the disorder and changing diagnostic criteria have undoubtedly contributed to the increase in prevalence (King and Bearman, 2009), researchers agree that these cannot solely account for such a significant rise in occurrence (Hertz-Picciotto and Delwiche, 2009). While the cause of the majority of ASD remains elusive, it likely involves a combination of genetic, epigenetic, and environmental factors. Twin studies have found a high concordance rate among monozygotic twins, with a much lower rate among dizygotic twins, implicating genetics as a factor; however, when combined, genetic interactions/mechanisms account for only 10–20% of ASD cases (Abrahams and Geschwind, 2008), this may suggest other heritable factors or shared genetic and environmental influences may be involved. These genetic markers may also be present in healthy individuals, again suggesting other risk factors in the pathogenesis of most cases of ASD. The rate for dizygotic twins is higher than non-twin siblings, and may suggest that shared prenatal environmental factors such as maternal infection, diet, and household chemical exposures may play a significant role in the etiology of ASD (Hallmayer et al., 2011). Environmental factors during pregnancy including maternal inflammation, air pollution and pesticide exposure have been associated with an increased risk of developing ASD and could be responsible for epigenetic changes identified in ASD (Loke et al., 2015); however, no single etiological factor has risen to the forefront. Research in ASD is confounded by the fact that these are highly heterogeneous disorders that likely have multiple etiologies and converging pathophysiological pathways. There is a growing need to determine which factors might be involved in the development of these disorders, as individuals with ASD and their families experience increased stress and decreased quality of life (Estes et al., 2013; Kuhlthau et al., 2014), and the increased prevalence of ASD has created a significant burden on health care (Lavelle et al., 2014) with an economic impact projected to exceed $400 billion by 2025 (Leigh and Du, 2015).

Although the specific etiologies of ASD remain unknown, many hypotheses regarding causation of ASD abound, including the potential involvement of the immune system. Just over a decade ago, we hypothesized that ASD may in fact be an autoimmune disorder (Ashwood and Van de Water, 2004; Ashwood et al., 2006). At that time, immune studies were limited and results had been somewhat inconsistent between various laboratories. Since then, a significant amount of research linking ASD and aberrant immune function has taken place, and although inconsistencies still exist, a clearer picture of the importance of immune involvement in ASD has emerged. This comprehensive review summarizes the latest research linking ASD and immune dysfunction, and discusses evidence of potential autoimmune mechanisms of ASD.

FAMILIAL AUTOIMMUNITY

Diverse autoimmune diseases within a nuclear family suggest familial autoimmunity (Anaya et al., 2007). Historically, studies have shown an increased prevalence of familial autoimmune disorders in ASD. The first study to identify a connection was in 1971, where researchers found a child with ASD who had several family members with multiple autoimmune disorders (Money et al., 1971). A later study found that 46% of ASD families had two or more members with autoimmune disorders, and as the number of autoimmune disorders within the family rose from one to three, the odds ratio for a risk of a child with ASD increased from 1.9 to 5.5. This study found that autoimmune prevalence was significantly increased in mothers and first-degree family members of ASD subjects, with type I diabetes (T1DM), rheumatoid arthritis (RA), hypothyroid and systemic lupus erythematosis (SLE) being the most common disorders found (Comi et al., 1999). A 2003 study also found a specific link to hypothyroid/Hashimoto's thyroiditis and RA in parents with ASD offspring (Sweeten et al., 2003a); however, others only found such an increase was paternally linked (Micali et al., 2004).

Since then, both self-reported and registry-based studies have shown additional links to autoimmunity in the family of children with ASD. A study in 2006 found autoimmune thyroid disease to be a significant risk factor, especially if the family member was the mother (Molloy et al., 2006a). This and other self-reported studies have some limitations as they may include biased or inaccurate recall. Utilization of health registries have allowed researchers to obtain larger datasets from patients based on medical records in population based case-control studies. In 2007, Mouridsen et al. found maternal ulcerative colitis and paternal T1DM were associated with infantile autism, (Mouridsen et al., 2007). In addition, mothers with autoimmunity were more likely to have a child with intellectual disability (ID), defined as an IQ of less than 50 (Mouridsen et al., 2007). In a large nationwide study that included all children born in Denmark from 1993 to 2004 (689,196 children, 3325 with ASD), researchers found that maternal RA and celiac disease increased risk of ASD in offspring (RA incidence rate ratio (IRR): 1.70 [95% CI: 1.07–2.54]; celiac disease IRR: 2.97 [95% CI: 1.27–5.75]), and an increased risk of infantile autism was found to be associated with family history of T1DM in either parent [IRR:1.78; 95% CI:1.16–2.61] (Atladottir et al., 2009). A further study utilizing the Danish registries looked specifically at both maternal and paternal RA and risk for ASD through 2007. Their data show that the risk of ASD increased by approximately 30% in cases of parental RA (maternal: HR 1.31 and 95% CI 1.06–1.63; paternal: HR 1.33, 95% CI 0.97–1.82). They conclude that a genetic component, specifically the Human Leukocyte Antigen − antigen D Related (HLA−DR) B1*04 alleles found commonly in both RA and ASD, may be playing a significant role in the development of ASD along with environmental factors (Rom et al., 2018). Registry-based studies have also shown an almost 50% higher odds-ratio of a child having an ASD diagnosis by age 10 if either parent had any autoimmune disease (maternal OR =1.6 [95% CI = 1.1–2.2]; paternal OR = 1.4 [95% CI = 1.0–2.0]), with maternal T1DM, idiopathic thrombocytopenic purpura (ITP), myasthenia gravis and rheumatic fever carrying the highest risks (Keil et al., 2010).

Confirming the link to autoimmune thyroid disorders previously found in case reports, a nested case control study in Finland identified a significantly increased risk of developing ASD in children who were born to mothers positive for anti-thyroid peroxidase antibodies (TPO-Ab+) during pregnancy. This study looked at a population of children born in Finland between 1987 and 2005, which included 1,132 confirmed cases of childhood autism. When compared to 967 matched controls, they found odds for developing ASD without ID were increased nearly 80% among children born to mothers who were TPO-Ab+ during pregnancy (OR = 1.78, 95% CI = 1.16–2.75). Interestingly, the odds were not increased for development of ASD with comorbid ID (Brown et al., 2015).

These large population studies rely on medical reporting and abstraction of medical data, as well as only reporting past and present autoimmunity, therefore they may not cover younger mothers who develop autoimmunity after the study period has ended. Nonetheless, a consistent pattern has emerged, as multiple meta-analyses have confirmed the familial autoimmunity and ASD link. A 2016 meta-analysis of mainly cased-control studies found significant positive associations of ASD with maternal autoimmunity during pregnancy (pooled OR: 1.34, 95% CI 1.23–1.56) and maternal autoimmune thyroid conditions (pooled OR: 1.29, 95% CI 1.14–1.45) (Chen et al., 2016). Familial T1DM and autoimmune thyroid disease were also associated with higher rates of regressive autism, versus those with developmental delays evident during infancy (Scott et al., 2017). Overall, combined family history of autoimmune disorders increased the risk of ASD by 28%, with most significant increased risks associated with psoriasis 59%, RA 51%, T1DM 49%, and hypothyroid 64% (Wu et al., 2015)].

In summary, the results of these familial studies do not single out one autoimmune disease and risk for ASD; however, a clear familial autoimmune component has emerged. The significant overlap of autoimmunity within the family members of ASD subjects may suggest an involvement of inherited immune factors. Maternal autoimmunity could also be playing a role in the gestational immune environment that has been found to significantly influence neurodevelopment. Summary of these studies can be found in **Table 1**.

GESTATIONAL IMMUNE INFLUENCES

Maternal Autoantibodies

The gestational environment is protected by the placenta, a selective barrier that allows for nutrient uptake and waste elimination, and provides protection from pathogens while allowing protective immune factors such as immunoglobulin-G (IgG) to cross into the amniotic fluid compartment (Garty et al., 1994). This passage could be facilitating the transfer of maternal IgG that target fetal brain antigens and could play an etiological role in ASD by blocking or activating proteins in the fetal brain, or initiating a cascade of neuroinflammation. In addition to an increased prevalence of familial and maternal autoimmunity in ASD, a subset of mothers of children with ASD (10–12%) have been found to harbor autoantibodies with reactivity to fetal brain components (summarized in **Table 2**), and these antibodies induce ASD-like pathology in animal models (Martin et al., 2008; Braunschweig et al., 2012b; Bauman et al., 2013).

The first evidence of such antibodies was found in 2003 in the serum of a mother with two children on the autism spectrum. These antibodies were found to be reactive to Purkinje cells and other neuronal proteins in rodent brain tissue (Dalton et al., 2003). Since then, several studies have identified maternal antibodies with reactivity to various brain proteins of different molecular weights in mothers of ASD subjects. For example, Zimmerman et al. found multiple patterns of reactivity to rat brain proteins of low kilodalton (kDa) weight, and one at 250 kDa (Zimmerman et al., 2007). When measured by Western blot for reactivity to human and rodent fetal brain tissue, sera from ASD mothers had significant reactivity to a 36 kDa protein present in both human and rodent fetal brain and dense banding at 61 kDa (Singer et al., 2008). Braunschweig et al. observed immunoreactivity to proteins at approximately 37 and 73 kDa exclusively in the mothers of children with ASD, and found these to be associated with increased language deficits in the child. Additional reactivity to a pair of bands in the region of 39 and 73 kDa was associated with increased irritability and self-injurious behavior (Braunschweig et al., 2008, 2012a). Multiple studies have since confirmed the presence of maternal autoantibodies (MAbs) with paired reactivity to 37 and 73 kDA proteins exclusive to mothers of children with ASD (Croen et al., 2008; Nordahl et al., 2013; Rossi et al., 2013). Consistent with these studies, Piras et al. found that single (or combinations of) maternal anti-brain antibodies correlated with severity of language and other behavioral impairments, and that the presence of a specific autoantibody at 62 kDa in the child correlated with the presence of autoantibodies in the mother (Piras et al., 2014). Brimberg et al. confirmed that anti-brain antibodies are significantly more prevalent in mothers of children with ASD than typically developing children, and a majority of these women who harbor anti-brain antibodies also harbor anti-nuclear antibodies common to autoimmune disorders (Brimberg et al., 2013). Although the presence of these maternal autoantibodies were associated with risk of ASD in offspring, these studies are limited in that no clear mechanism was identified, which limits our understanding of how they might contribute to the etiology of ASD.

Animal models have allowed us to identify some pathogenicity of these autoantibodies (**Table 3**). Passive transfer of anti-brain antibodies from mothers of children with ASD to animals during gestation led to ASD-like pathology in both rodent (Singer et al., 2009; Braunschweig et al., 2012b) and primate offspring (Martin et al., 2008; Bauman et al., 2013), Target antigens to these maternal autoantibodies have since been identified as lactate dehydrogenase A and B (LDH—37 kDa band), cypin (previously undetected 44 kDa band), stress-induced phosphoprotein 1 (STIP1-upper 73 kDa band), collapsin response mediator proteins 1 and 2 (CRMP1/2-lower 70 kDa band) and Y-box-binding protein (YBX1–39 kDa band), with individual and combinations of maternal autoantibodies specific to these antigens increased in mothers of children with ASD. Increased stereotypical behavior and overall impairment were observed in children of mothers who possessed combinations of these autoantibodies (Braunschweig et al., 2013). Using structural magnetic resonance imaging (MRI), Nordahl et al. showed enlarged brain volume in male children born to mothers harboring anti-brain antibodies (Nordahl et al., 2013). In rodents, maternal autoantibodies administered during gestation were found to be able to migrate into the cortical parenchyma and alter coronal development by binding to radial glial cells in the ventricular zone and increased the number of neuronal precursor cells in the subventricular zone, increasing brain size and weight. Administration of autoantibodies also led to decreased numbers of mature dendritic spines in the adult cortex of mice, with STIP1 blockade being the likely culprit due to its importance in neuritogenesis, the sprouting of neurites that later develop

TABLE 1 | Studies identifying association of familial autoimmunity and ASD.

Subject details	Methods	Summarized findings	References
61 ASD 46 TD	Self-reported questionnaire: known AI within family	46% ASD families had 2+ members with AI ↑ ASD odds ratio (1.9 to 5.5) as number of family members with AI ↑ from 1-3 ↑ T1DM, RA, hypothyroid and SLE in ASD mothers and 1st degree relatives	Comi et al., 1999
101 ASD 101 AI 101 HC	Self-reported questionnaire: which 1st and 2nd degree relatives have AI	↑ frequency of AI in ASD families compared to AI and TD families ↑ AI including hypothyroid/Hashimoto's thyroiditis and RA in ASD parents	Sweeten et al., 2003a
79 ASD 61 DD	Self-reported questionnaire: ASD with familial AI and psychiatric history	No significant relationship of AI in ASD vs. DD 31.5% of ASD fathers vs 18.2% control had AI No difference found in mothers.	Micali et al., 2004
153 ASD 155 regressive ASD	Telephone interview: AI in 1st and 2nd degree relatives.	ASD with regression ↑ in families with 1st or 2nd degree AI relatives ↑ risk with familial AI thyroid diagnoses, especially maternal family members	Molloy et al., 2006a
111 ASD 330 TD	Registry based: Danish national hospital registry	↑ risk of ASD with maternal UC and paternal T1DM Mothers with AI more likely to have child with ID (IQ < 50)	Mouridsen et al., 2007
3325 ASD (1089 "infantile autism")	Registry based: all children born in Denmark 1993–2004	↑ risk of ASD with maternal RA and celiac disease ↑ risk of infantile autism associated with family history of T1DM, both parents.	Atladottir et al., 2009
1227 ASD 30,675 TD	Registry based: Three Swedish registries	↑ risk of ASD with AI in both parents ↑ risk of ASD with maternal T1DM, ITP, myasthenia gravis and rheumatic fever	Keil et al., 2010
967 ASD 967 TD	Nested case–control design: prospectively drawn maternal sera samples with registry-based ASD diagnoses from FiPS-A	↑ risk of ASD with maternal TPO-Ab presence during pregnancy	Brown et al., 2015
11 studies	Systematic review and meta-analysis	↑ risk of ASD with family history of all AI ↑ risk of ASD with familial hypothyroidism, RA, and psoriasis	Wu et al., 2015
10 studies	Systematic review and meta-analysis	↑ risk of ASD with maternal AI developed during pregnancy ↑ risk of ASD with maternal thyroid disease	Chen et al., 2016
206 ASD 33 regressive ASD	Medical chart review of ASD diagnosis and familial AI association	↑ risk of regressive ASD with familial AI Regressive ASD associated with familial T1DM and autoimmune thyroiditis	Scott et al., 2017

ASD, autism spectrum disorders; TD, typically developing; AI, autoimmunity; T1DM, Type 1 diabetes mellitus; RA, rheumatoid arthritis; C: SLE, systemic lupus erythematosus; healthy control; BAP, broad autism phenotype; IBD, inflammatory bowel disease; UC, ulcerative colitis; ID, intellectual disability; ITP, idiopathic thrombocytopenic purpura; FiPS-A, Finnish Prenatal Study of Autism; TPO-Ab, thyroid peroxidase antibody.

into dendrites (Martinez-Cerdeno et al., 2016; Ariza et al., 2017). Mice exposed to maternal autoantibodies during the embryonic stage displayed ASD-like behaviors including increased repetitive behaviors and altered social interactions (Camacho et al., 2014). Generation of endogenous autoantibodies prior to gestation to the fetal brain epitopes identified in ASD mothers also led to social deficits and increased repetitive grooming in adult offspring mice (Jones et al., 2018).

Although the origin(s) of these autoantibodies are unknown, they are more frequent in mothers carrying a functional variant of the Met Receptor Tyrosine Kinase (*MET*) promoter which leads to reduced production of MET receptor tyrosine kinase, a receptor involved in immune regulation (Heuer et al., 2011). Mothers carrying the variant *MET* allele had reduced interleukin (IL)-10, an important regulatory cytokine, suggesting that dysfunction in immune regulation may be driving the production of autoantibodies (Heuer et al., 2011). Understanding the development of these MAbs and the role of the proteins they target in neurodevelopment is important due to the substantial number of ASD cases found to involve these antibodies. Research in this area may lead to diagnostic tools that assess maternal risk, as well as possible treatments and early interventions for children with maternal autoantibody-related ASD.

TABLE 2 | Studies identifying presence of anti-brain autoantibodies in mothers of children with ASD.

Subject details	Methods	Summarized findings	References
Mother with 2 ASD children	IHC of sera binding to rodent brain.	Pilot study-maternal sera had reactivity to rodent Purkinje cells in cerebellum and large brain stem neurons.	Dalton et al., 2003
11 ASD mothers 10 TD mothers	Serum reactivity to prenatal, postnatal, and adult rat brain proteins by immunoblotting	↑ reactivity to prenatal rat brain in multiple patterns of low kDa weight, and one significantly higher at 250 kDa No reactivity to postnatal or adult rat brain	Zimmerman et al., 2007
100 ASD mothers 100 TD mothers	Serum reactivity to human and rodent fetal and adult brain tissues, GFAP, and MBP by immunoblotting	↑ reactivity at 36 kDa in both human fetal and rodent embryonic brain tissue. ↑ reactivity at 61 kD in human fetal brain tissue. ↑ reactivity at 36 and 39 kDa against human fetal brain in mothers whose children had regressive ASD.	Singer et al., 2008
61 ASD mothers 62 TD mothers 40 DD mothers	Plasma reactivity to human fetal and adult brain proteins by immunoblotting	↑reactivity to 73kDa and 37kDa to human fetal brain correlated with regressive ASD Reactivity to 37 kDa was higher in ASD mothers compared with TD and DD mothers. No reactivity with TD plasma to either tissue type.	Braunschweig et al., 2008
84 ASD mothers 49 DD mothers 160 TD mothers	Mid-pregnancy plasma reactivity to fetal brain protein by immunoblotting	↑reactivity at 39 kDa in ASD compared to DD and TD Reactivity at both 39 kDa and 73 kDa seen only in early-onset ASD	Croen et al., 2008
202 ASD mothers 163 TD mothers	PCR for MET rs1858830 allele genotyping. Measured MET protein and cytokines by Luminex from stimulated maternal PBMCs. Previous study results used for the associations to presence of auto-Abs	Presence of C allele associated with reactivity at 37 and 73-kDa to fetal brain proteins. Presence of C allele associated with ↓ MET protein expression and ↓ IL-10	Heuer et al., 2011
277 ASD (70 BAP) 189 age-matched TD (2-5 years) and their mothers	Maternal plasma reactivity to Rhesus macaque fetal brain protein medleys by immunoblotting, child plasma reactivity to Rhesus macaque cerebellum protein medley.	↑ reactivity to many including proteins with MW of 42, 49, 60, 80, and 100 kDa in plasma from ASD mothers No correlation with reactivity found in children Child results listed in **Table 4**.	Goines P. et al., 2011
204 ASD mothers 71 BAP mothers 102 DD mothers 183 TD mothers	Maternal plasma reactivity to Rhesus macaque brain at 3 gestational ages by immunoblotting	↑ paired reactivity at 37 and 73 kDa combined in ASD, not seen in TD ↑ paired reactivity at 39 and 73 kDa in ASD and BAP compared to TD and DD Paired reactivity at 37 and 73 kDa associated with language deficits. Paired reactivity at 39 and 73 kDa associated with increased irritability Reactivity to 39 kDa (alone or paired with 73 kDa) associated with BAP	Braunschweig et al., 2012a
37 ASD and TD mothers and their children ages 3-13 years	IHC for plasma reactivity to rhesus macaque brain tissue. Immunoblot reactivity to fetal and adult rhesus macaque brain proteins.	Reactivity at 37 and 73 kDa or 39 and 73 kDa found only in ASD mothers No significant differences in reactivity seen in ASD children vs. TD.	Rossi et al., 2013
Preschool aged males: 131 ASD (10 with 37/73 kDa IgG+ mothers) 50 TD, all negative for auto-Abs	MRI scan (during sleep) to evaluate total brain volume and compare maternal auto-Ab positive group to maternal auto-Ab negative groups	↑ abnormal brain enlargement in ASD, both groups ASD children with 37/73 kDa IgG+ mothers had more extreme abnormal brain enlargement compared to Ab negative ASD and TD groups, specifically in the frontal lobe.	Nordahl et al., 2013

(Continued)

TABLE 2 | Continued

Subject details	Methods	Summarized findings	References
246 ASD mothers 149 TD mothers	Plasma reactivity to fetal macaque brain verified by immunoblotting. Protein enrichment via PlasmPrep cell protein fractionation, 2-D electrophoresis and mass spectrometry.	6 brain proteins that has plasma reactivity were identified: lactate dehydrogenase A and B (LDH), cypin, stress-induced phosphoprotein 1 (STIP1), collapsin response mediator proteins 1 and 2 (CRMP1, CRMP2) and Y-box-binding protein (YBX1) Reactivity to any alone or in combination significantly was associated with ASD outcome ↑ stereotypical associated with reactivity to LDH, and combined reactivity to LDH/STIP1 or LDH/STIP1/CRMP1 ↑ overall impairment associated with reactivity to LDH and CRMP1	Braunschweig et al., 2013
2431 ASD mothers, 653 controls of child-bearing age	Plasma IHC reactivity to mouse brain	↑ presence of brain-reactive auto-Abs in ASD mothers compared to control women Presence of brain-reactive auto-Abs associated with anti-nuclear autoantibodies and increased prevalence of autoimmune diseases, especially RA and SLE.	Brimberg et al., 2013
333 ASD mothers 355 ASD 142 SIB	Child and mother plasma reactivity to Rhesus macaque brain tissue and human adult cerebellum by immune-blotting	Reactivity at 37, 39 and/or 73 kDa anti-brain auto-Abs associated with impaired language development, neurodevelopmental delay and sleep/wake cycle disturbances. Presence of the 62 kDa autoAb in the child associated with maternal reactivity at 39 and/or 73 kDa. Child results listed in **Table 4**.	Piras et al., 2014

ASD, autism spectrum disorders; IHC, immunohistochemistry; TD, typically developing; kDa, kilodalton; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; DD, non-ASD developmentally delayed; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells; BAP, broader diagnosis of autism spectrum disorder; IgG, immunoglobulin G; MRI, magnetic resonance imaging; 2-D, two dimensional; auto-Abs, auto-antibodies; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SIB, typically developing sibling.

Maternal Immune Activation (MIA)

Maternal infection during pregnancy has been implicated as a potential environmental risk factor for ASD (reviewed in Patterson, 2011). In case series reports, infections during pregnancy, such as rubella, measles or toxoplasmosis, can negatively impact early neurodevelopment of the fetus. In population based studies, viral, and bacterial infections occurring in the first or third trimester, respectively, or maternal fever during gestation pose an increased risk for later development of ASD in offspring (Atladottir et al., 2010; Zerbo et al., 2013). Both rodent and non-human primate models of maternal infection during gestation have supported epidemiological studies, showing alterations in ASD-associated behaviors and immune dysregulation that persisted into adulthood in offspring born to mothers exposed to viral or bacterial antigens during gestation (Schwartzer et al., 2013; Bauman et al., 2014; Meyer, 2014; Onore et al., 2014; Choi et al., 2016; Rose et al., 2017). Maternal asthma during pregnancy has also previously been linked with ASD (Croen et al., 2005; Lyall et al., 2014). Furthermore, in a cohort of 220 children with ASD, those whose mothers had a history of allergies or asthma during pregnancy displayed more severe social impairments (Patel et al., 2017). Animal models of maternal gestational asthma have validated both behavioral and immune abnormalities in offspring, including epigenetic alterations in methylation of immune pathway genes in microglia–the resident immune cells of the brain (Schwartzer et al., 2015, 2017; Vogel Ciernia et al., 2018).

The driving mediators of MIA-associated ASD pathology are most likely elevations in maternal cytokines and chemokines. In addition to their roles as immune-mediators, these signaling proteins play important roles in central nervous system (CNS) development and are involved in migration of neuronal precursors, neuronal maintenance, synaptic pruning and plasticity, thus they need to be tightly regulated (Deverman and Patterson, 2009). Cytokines that cross the placenta, such as IL-6 and IL-4, have the potential to alter epigenetic regulation of gene transcription (Nardone and Elliott, 2016). Elevations of cytokines and chemokines in both maternal serum during gestation and amniotic fluid are associated with increased risk of ASD in human subjects (Goines P. E. et al., 2011; Abdallah et al., 2012; Jones et al., 2016). Mechanistically, maternal cytokines such as IL-6 and IL-17 may be mediating inflammation either at the placenta or directly in the developing fetal brain (Smith et al., 2007; Hsiao et al., 2012; Choi et al., 2016). Moreover, it is possible that maternal inflammation may be contributing to the development of maternal autoantibodies (**Figure 1**). As well as cytokine driven responses, other downstream events occur that can effect immune and neuronal development. For instance, in the LPS model of MIA trace metal levels are altered included the sequestration of zinc in both the dams and offspring (Coyle et al., 2009; Kirsten et al., 2015; Kirsten and Bernardi, 2017).

Recent evidence in a mouse model of MIA suggest that maternal microbiota composition may be driving maternal inflammation to skew toward IL-17 production, inducing behavioral changes. Dams lacking certain microbiota did not produce IL-17 and their offspring did not exhibit aberrant behavioral phenotypes. IL-17 blockade was also effective in preventing behavioral abnormalities (Choi et al., 2016; Kim et al., 2017). This is a notable finding considering our understanding of IL-17 as a central driver of autoimmune disorders (Zhu and Qian, 2012). A better understanding of the role of IL-17 signaling

TABLE 3 | Preclinical studies of maternal autoantibodies and ASD-like pathology.

Pre-clinical model details	Summarized findings	References
Mouse model Sera from 1 ASD mother (auto-Ab+) and 4 TD mothers injected into pregnant MF1 mice at varied time points. Offspring behaviors and cerebellar chemistry measured with standard behavioral tests and MRS	ASD-sera exposed offspring exhibited: ↓ reflexes ↓ exploration ↓ spatial orientation ↓ creatine and choline concentrations in cerebellum No impairments in memory	Dalton et al., 2003
Non-human primate model Purified IgG from separately pooled from 21 ASD and 7 TD maternal sera, measured for presence of auto-Abs. 4 Rhesus macaques were injected IV with auto-Ab+ IgG from ASD maternal sera and 4 were injected with auto-Ab- IgG from TD maternal sera at GD 27, 41, and 55. Behaviors assessed at preweaning and postweaning time points.	ASD-IgG exposed offspring exhibited: ↑ whole-body stereotypies ↑hyperactivity No significant differences seen in social behaviors	Martin et al., 2008
Mouse model Purified IgG from separately pooled from 63 ASD and 63 TD maternal sera, injected IP into 26 pregnant C57Bl/6J mice total, 13 dams per group, at E13-E18. Offspring from untreated and from saline injected mice included as negative controls. Several behavioral and neurodevelopmental outcomes measured.	ASD-IgG exposed offspring exhibited: ↑ anxiety, startle, and hyperactivity ↑ Iba1 staining indicating microglia activation in E18 embryos ↑ BDNF at adolescence Social deficits seen at adulthood IL-12 detectable in E16 embryos	Singer et al., 2009
Mouse model Purified IgG from pooled from 3 ASD maternal sera positive for 37 kDa and 73 kDa fetal brain protein reactivity. Purified IgG also pooled from 3 TD maternal sera absent of reactivity. Single IV injection of purified IgG per group given to pregnant C57Bl/6J mice at GD 12, saline given as negative control. Offspring assessed for behavioral abnormalities and alterations in neurodevelopment.	ASD-IgG exposed offspring exhibited: ↓ weight and body length ↓ sensory and motor development prior to weaning ↑ anxiety ↑ vocalizations during separation-induced stress in males at PND8 Social deficits trended in males but did not reach significance No differences seen in stereotypical behaviors No differenced seen in numbers of CA1 hippocampal neurons	Braunschweig et al., 2012b
Non-human primate model Purified IgG from ASD maternal sera positive for 37 kDa and 73 kDa autoAbs and purified IgG from TD maternal sera absent of reactivity injected IV into two groups of pregnant Rhesus macaques at GD 30, 44, 58, 72, 86, and 100.	ASD-IgG exposed offspring exhibited: ↑ maternal protectiveness during pre-weaning stage ↑ inappropriate and frequency of social approach in juveniles ↑ brain volume in males compared with controls, mainly white matter and most profoundly in the frontal lobes.	Bauman et al., 2013
Mouse model Purified IgG from ASD maternal sera positive for autoAbs or from TD maternal sera absent of reactivity was injected directly into cerebral ventricles of E14 embryonic Swiss Webster mice. Behaviors measured with battery of behavioral assays	ASD-IgG injected adult offspring exhibited: ↑ grooming ↑ marble burying No differences in social approach, however ASD-IgG mice had↑ time spent with novel object compared to mice injected with TD-IgG	Camacho et al., 2014
Mouse model Biotinylated IgG from ASD maternal sera positive for 37 kDa and 73 kDa fetal brain protein reactivity or from TD maternal sera absent of reactivity was injected directly into cerebral ventricles of E14 or E16 embryonic Swiss Webster mice. Brain reactivity and quantification assessed with IHC and stereology.	Brain from ASD-IgG injected embryos exhibited: Reactivity to RG cells (neural stem cells) in VZ ↑proliferative Pax6+ RG cells in the SVZ ↑mitotic precursor cells in the SVZ of the ganglionic eminence (a neurodevelopmental structure that guides cell and axon migration) RG cells translocated much earlier than control mice ↑ brain weight and rostro-caudal length ↑somal volume in neurons	Martinez-Cerdeno et al., 2016
Mouse model Purified IgG from ASD maternal sera positive for 37 kDa and 73 kDa fetal brain protein reactivity or from TD maternal sera absent of reactivity was injected directly into cerebral ventricles of E14 embryonic Swiss Webster mice. Changes in dendritic arbor and spine population assessed with Golgi method and Neurolucida.	Adult brain from ASD-IgG injected embryos showed: ↓ length and volume of dendritic spines on neurons of the frontal cortex ↓ total number of spines on neurons in frontal cortex ↓ total number of spines on neurons in occipital cortex ↓ spine density of apical dendrites, and ↓ number of mature spines on basal and apical dendrites in occipital cortex	Ariza et al., 2017

(Continued)

TABLE 3 | Continued

Pre-clinical model details	Summarized findings	References
Mouse model Mixtures of 21 synthesized epitopes of LDH-A, LDH-B, STIP1, and CRMP1 (fetal brain target peptides) plus adjuvant were injected SC 5 times into dams prior to mating (MAR-ASD). Control females injected SC with saline plus adjuvant. Maternal sera tested for verification of endogenous autoAb production. Offspring behaviors measured with behavioral assays	Epitope-specific antibodies were successfully produced and persisted in dams through end of lactation MAR-ASD offspring exhibited: ↑ weight and head width ↑ repetitive behaviors ↓ social behaviors, including male-female social interactions	Jones et al., 2018

ASD, autism spectrum disorder; autoAb, autoantibody; MRS, magnetic resonance spectroscopy; IgG, immunoglobulin G; TD, typically developing; IV, intravenous; GD, gestational day; IP, intraperitoneal; Iba1, Ionized calcium binding adaptor molecule 1; E13, embryonic day 13; BDNF, brain derived neurotrophic factor; IL-12, interleukin-12; IHC, immunohistochemistry; RG cells, radial glial cells; VZ, ventricular zone; SVZ, subventricular zone; LDH, lactate dehydrogenase; STIP1, stress-induced phosphoprotein 1; CRMP1, collapsin response mediator protein 1; SC, subcutaneous.

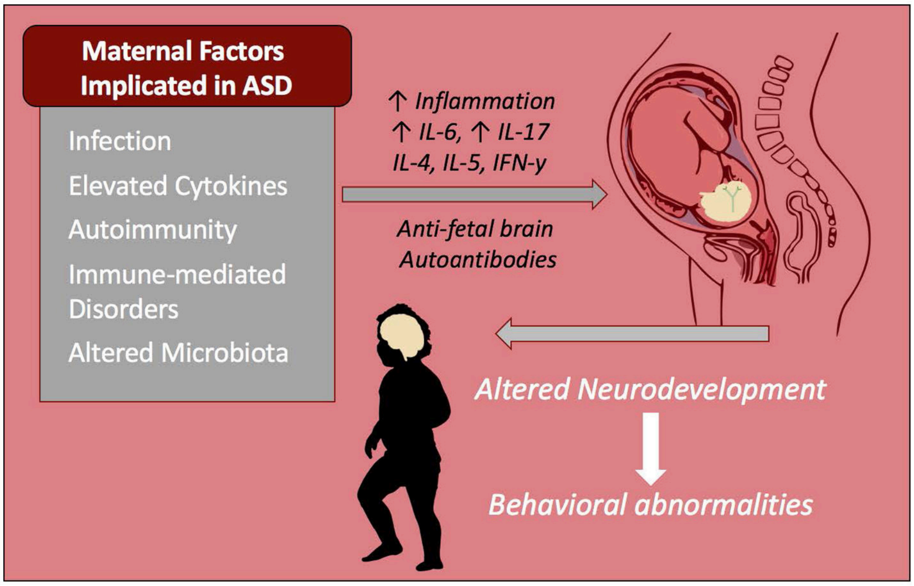

FIGURE 1 | Maternal immune influences during gestation increase risk of ASD. Infection and immune-mediated/autoimmune disorders in the mother are known risk factors that increase the chances of a child developing ASD. These inflammatory factors as well as altered maternal microbiota may be contributing to increased inflammatory cytokines and/or autoantibodies that react to fetal brain tissue. These factors alter the immune profile and neurodevelopment of the child and are linked to behavioral abnormalities seen in ASD including repetitive behaviors, stereotypies, anxiety, and impaired social behaviors.

during gestation and within the fetal brain may hopefully lead to therapeutics targeting this cytokine.

IMMUNE FINDINGS IN INDIVIDUALS WITH ASD

Immune Mediated Co-morbidities

Among the many immune findings in ASD, several recent large-scale studies have indicated that individuals with ASD have frequent immune-mediated comorbid health issues that may progress or predispose to later-life autoimmune conditions (**Figure 2**). Zerbo and colleagues found that allergies and autoimmunity diagnoses were significantly more common in children with ASD, with odds ratios of 1.22 and 1.36, respectively (Zerbo et al., 2015). Children with ASD, as surveyed in the National Health Interview Survey, require higher health care use and have a higher prevalence of most medical conditions defined in autoimmune areas, compared to those without developmental disabilities (Schieve et al., 2012). However, as many autoimmune conditions do not manifest until adulthood, the young age of most study populations is a limitation to finding associations between ASD and autoimmunity. To determine significant pathogenic components of ASD, cluster analysis found immune dysfunction to be the best-defined cluster for ASD. This study noted that immune dysfunction underlined the majority of comorbidities observed in ASD (Sacco et al., 2012). Allergic diseases are overrepresented in ASD, and in some individuals may influence behaviors and severity of core behavioral deficits (Mostafa et al., 2008; Shibata et al., 2013). A large epidemiological study found that asthma was 35% more common in children with ASD compared to typically developing children (Kotey et al., 2014). This supports a previous study that found a significantly increased risk of asthma in ASD subjects, with an odds ratio of 1.74 (Chen et al., 2013). Increased risk of type 1 diabetes, allergic

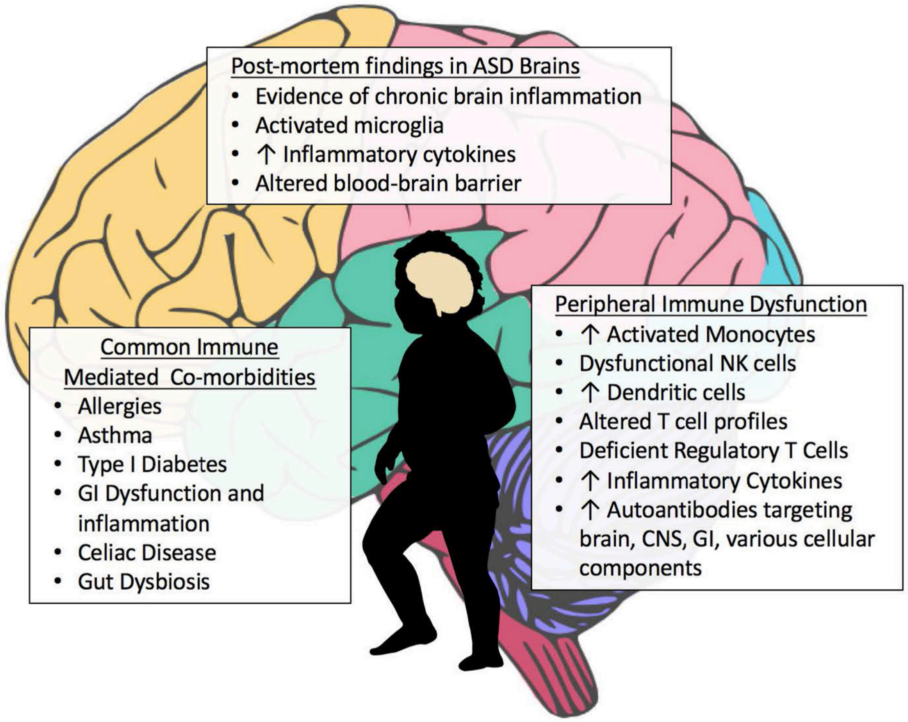

FIGURE 2 | Aberrant Immune Findings in Individuals with ASD. Evidence of immune dysfunction in ASD has grown substantially in recent years. Individuals with ASD commonly have immune-mediated comorbidities such as allergies and gastrointestinal (GI) dysfunction that may be contributing to the aberrant behaviors seen in ASD. Although research in this area is occasionally contradictory, the vast majority of immune studies in individuals with ASD have shown immune dysfunction and dysregulation. Several studies have found elevations in inflammatory cells and cytokines, both peripheral as well as within post-mortem brain tissue. A variety of autoantibodies targeting various tissues and cellular components throughout the body have been identified in subsets of subjects with ASD. Individuals with ASD also have fewer regulatory T cells.

rhinitis, atopic dermatitis, urticaria and a trend toward increasing comorbidity with Crohn's disease are also observed in subjects with ASD (Chen et al., 2013). The same group looked specifically at asthma in a nationwide population-based prospective study over 8 years and found children with asthma in early life had an increased risk of developing ASD (adjusted hazard ratio: 2.01, 95% confidence interval: 1.19–3.40) (Tsai et al., 2014). An analysis of over 1500 adults with ASD showed significantly increased rates of medical conditions in individuals with ASD compared to non-ASD controls, including but not limited to immune co-morbidities, gastrointestinal (GI) disorders, diabetes, obesity, seizures, and sleep disorders (Croen et al., 2015).

One of the most commonly reported comorbidities in ASD is the incidence of GI dysfunction and inflammation in the GI tract (**Figure 2**). Assessment for GI dysfunction is often challenging in individuals with ASD due to communication deficits. Reported prevalence of GI disturbances varies widely, with ranges from 9 to over 90% in ASD subjects; however, the 2013 Interagency Autism Coordinating Committee concluded that at least 50% of children with ASD had GI issues (discussed in Mcelhanon et al., 2014). A 2014 assessment of 960 children enrolled in the Childhood Autism Risks from Genetics and the Environment (CHARGE) study found that children with ASD had significantly increased odds of having at least one GI symptom compared to typically developing controls (OR 7.92 [4.89–12.85]) (Chaidez et al., 2014). Discrepancies in prevalence rates of GI comorbidities in ASD are likely due to differences in defining criteria for symptoms, referral bias, variations in samples sizes and sources of data, as well as the timing in which symptoms were reported (Buie et al., 2010; Coury et al., 2012). Early studies examining intestinal dysfunction in ASD showed increased monocytes, lymphocytes, NK cells, eosinophils, and intraepithelial lymphocytes in duodenal biopsies, and autoantibody IgG and co-localized C1q complement bound to the basal membrane of GI epithelial cells (Torrente et al., 2002). This autoimmune phenomena—directed against the gut lumen barrier—could be responsible for the increased intestinal permeability seen in individuals with ASD (de Magistris et al., 2010). Inflammatory immune cells were found to infiltrate the epithelium and lamina propria of the GI tract (Furlano et al., 2001; Torrente et al., 2002; Ashwood et al., 2003). Further, studies showing increased production of inflammatory cytokines and decreased regulatory IL-10 production by mucosal Cluster of Differentiation(CD)3^+ T cells suggested the presence of increased inflammation and dysregulation of mucosal immune responses that could be contributing to disruption of the intestinal barrier (Ashwood et al., 2004; Ashwood and Wakefield, 2006). Mucosal gene profiling adds further support to these findings, with upregulation of cytokine production, including IL-17 and increased immune activation in children with ASD who have comorbid GI issues (Walker et al., 2013).

Increased inflammation and dysregulation of the GI tract in ASD is important as this compartment comprises a significant percentage of immune cells in the body, and immune cells educated here participate in immune function throughout the body. Cellular education and the maintenance of regulatory immune cells in the mucosal GI compartment are strongly influenced by the community of microbes that reside here, and this influences systemic immune homeostasis (reviewed in Wu and Wu, 2012). Additionally, if the intestinal barrier is disrupted, antigenic material from the lumen of the gut may enter the periphery and initiate an immune response. Indeed, ASD subjects with GI comorbidities have increased peripheral inflammation and lower production of regulatory cytokines (Jyonouchi et al., 2001, 2005, 2011; Ashwood and Wakefield, 2006; Rose et al., 2018). Circulating antigens and bacterial metabolites could also directly influence the brain if both the intestinal and blood-brain barriers are not sufficiently intact. This is one proposed mechanism of how the microbiota-gut-brain axis may have pathological involvement in neurological disorders (Cryan and Dinan, 2012). Interestingly, recent findings in post-mortem tissue suggest that individuals with ASD have alterations in the blood-brain barrier and deficiencies in gene expression of intestinal tight junction proteins (**Figure 2**) (Fiorentino et al., 2016). Several studies have also indicated that individuals with ASD have dysbiotic alterations in gut flora and altered bacterial metabolites (**Figure 2**) (Finegold et al., 2010; Williams et al., 2011, 2012; Gondalia et al., 2012; Kang et al., 2013; Tomova et al., 2015; Strati et al., 2017; Hughes et al., 2018), including recent findings that children with ASD who exhibit GI symptoms have differences in microbiota compared to children with ASD who have no GI dysfunction. Their microbiota also differed from typically developing children with similar GI symptoms (Rose et al., 2018). These studies suggest an ineffective immune response to bacteria in ASD, or production of inflammatory mediators leading to preferential bacterial growth (Spees et al., 2013). It is unclear whether the dysbiosis seen in ASD is driving the immune dysfunction and altered immune regulation, or is a result of it and much more research is needed in this area.

It has been suggested that food allergy may play a role in GI dysfunction, and some parents of children with ASD have seen behavioral improvements after implementing diets that eliminate suspect antigens such as gluten and casein; however, the role of food allergies remains controversial. Reports of IgE-mediated food allergies correlating with GI symptoms are inconclusive (Buie et al., 2010), although, one group found a high incidence of non-IgE-mediated food allergy in younger children with ASD (Jyonouchi et al., 2008). Celiac disease has also been reported to have an association with ASD (Barcia et al., 2008; Ludvigsson et al., 2013). A recent Italian study looked at a cohort of 382 preschoolers diagnosed with ASD, and found the prevalence of celiac disease among the ASD cohort was significantly increased compared to the general pediatric population, with an overall prevalence of 2.62% (Calderoni et al., 2016). This supports the earlier work by Barcia et al. who found a 3.3% prevalence of celiac disease in ASD by exploring biopsied tissue (Barcia et al., 2008). As GI disturbances may exacerbate behavioral symptoms, better screening techniques that include identification of behaviors associated with GI distress may help to better elucidate the actual prevalence within this population.

Additional research seeking to identify a genetic and molecular basis for the comorbidities plaguing individuals with ASD consistently found dysregulation of multiple innate signaling pathways by utilizing searches of curated gene pathways. Nine Kyoto Encyclopedia of Genes (KEGG) pathways were recently identified to overlap in ASD and other disease comorbidities common in ASD. For example, three pathways involved in regulation of the immune response, the toll-like receptor (TLR), nucleotide-binding and oligomerization domain (NOD) pathways, and chemokine signaling pathways significantly overlapped with asthma and inflammatory bowel disease (Nazeen et al., 2016). Further studies are needed to examine these pathways in ASD. However, it is important to note that not all of these dysregulated pathways may exist in individuals with ASD (Campbell et al., 2013), but may be dysregulated in those with immune comorbidities and help account for the wide heterogeneity and conflicting results seen in some studies in ASD.

Autoantibodies in Individuals With ASD

In addition to antibodies targeting the GI epithelium, autoantibodies specific to self-proteins in the brain, CNS and cellular components have been frequently reported in individuals with ASD (**Figure 2**, **Table 4**). Autoantibodies are a common feature in autoimmunity, and their presence may be predictive of the development of certain autoimmune disorders (Anaya et al., 2007; Lleo et al., 2010). Presence of autoantibodies that react to components of the brain and CNS in individuals with ASD have been identified since as early as 1988, when antibodies to neuron-axon filament proteins (NAFP) were found in 10 out of 15 children with ASD (Singh et al., 1988). A year later, researchers identified IgG and IgM antibodies that target cerebellar neurofilaments (Plioplys et al., 1989). Anti-myelin basic protein (MBP) antibodies were identified in individuals with ASD in 1993 (Singh et al., 1993) and later supported by additional studies (Singh et al., 1998; Connolly et al., 2006). The anti-MBP results have been replicated in additional studies, including a 2013 investigation that linked these autoantibodies to both severity of ASD as well as allergic manifestations (Mostafa and Al-Ayadhi, 2013). However, other studies have refuted these findings which underscores the wide variations of immune phenotypes seen in ASD (Libbey et al., 2008).

More recent studies have found autoantibodies to the prefrontal cortex, caudate, putamen, cerebellum and cingulate gyrus regions of the brain (Singer et al., 2006) and hypothalamus (Cabanlit et al., 2007) in children with ASD. In 2009, researchers found that 21% of plasma samples from children with ASD had intense immunoreactivity to Golgi neurons in primate cerebellum, with no reactivity occurring in controls. These ASD autoantibodies reacted to a protein of a molecular weight of 52kDa in human cerebellum (Wills et al., 2009). A follow-up study identified reactivity to interneurons in other regions

TABLE 4 | Studies identifying presence of autoantibodies in individuals with ASD.

Subject details	Methods	Summarized findings	References
48 autism (5.9 ± 3.9 years) 19 CDD (7.0 ± 2.4 years) 14 PDD-NOS (4.8 ± 3.9 years) 9 LKS (7.4 ± 2.3 years) 37 epilepsy (5.9 ± 3.8 years) 29 HC (4.3 ± 2.0 years) 21 NNI (4.2 ± 2.6 years)	Serum ELISA measurements of BDNF, IgG/IgM auto-Abs to BDNF, endothelial cells, MBP, and histones *Note: Subject numbers indicate total included in study. Actual numbers varied slightly depending on assay*	↑ BDNF in ASD, CDD compared to HC and NNI ↑ anti-BDNF IgM and IgG in autism, CDD and epilepsy compared to HC ↑ IgM to endothelial cells in autism, CDD, PDD-NOS, and epilepsy compared to HC and NNI ↑ IgG to endothelial cells in autism and PDD-NOS compared to HC ↑ IgM and IgG to MBP in autism, CDD, PDD-NOS, and epilepsy compared to both HC and NNI, LKS not elevated	Connolly et al., 2006
29 ASD (3–12 years) 9 SIB (4–8 years) 13 TD (9–17 years)	Serum ELISA and Western blot reactivity to human brain	↑ reactivity to 100 kDa epitope in caudate putamen and prefrontal cortex in ASD ↑reactivity to 73 kDa epitope in cerebellum and cingulate gyrus in ASD and SIB	Singer et al., 2006
63 ASD (2–15 years) 63 TD (2–14 years) 25 SIB (1–13 years) 21 DD (2–5 years)	Western blot of plasma reactivity to adult human hypothalamus and thalamus protein extracts	↑ reactivity to 52 kDA thalamus and hypothalamus proteins in ASD ↑ reactivity to 3 hypothalamus proteins (42–48 kDa MW)	Cabanlit et al., 2007
11 ASD 9 SIB (>6 years)	72-h neuronal culture analyzed for effect of ASD sera on differentiation of NPCs by immunoblotting, morphometry, and immunocytochemistry	Treatment with ASD sera: ↓ NPC proliferation ↑ cell migration ↑small cells with processes ↑ length and number of processes ↑ synaptogenesis	Mazur-Kolecka et al., 2007
33 ASD (7.3 ± 3.0 years) 26 regressive autism (6.7 ± 2.7 years) 25 TD (8.9 ± 3.4 years) 24 Tourette syndrome (10.0 ± 2.6 years)	Plasma ELISA and Western blot reactivity to MBP	↑ auto-Abs to MBP found in regressive autism compared to classic (infantile) autism and Tourette syndrome subjects.	Libbey et al., 2008
63 ASD (2–15 years) 63 TD (2–14 years) 25 SIB (1–13 years) 21 DD (2–5 years)	Western blot of plasma reactivity to human cerebellar protein extracts. Cerebellar-specific auto-Abs detected by IHC of *Macaca fascicularis* monkey cerebellum.	↑auto-Abs to 52 kDa cerebellar protein in ASD ↑"intense immunoreactivity" to Golgi cells of the cerebellum in ASD, associated with auto-Abs to 52 kDa cerebellar protein	Wills et al., 2009
37 ASD (1–12 years) 37 TD (1–14 years)	Measured effect of ASD sera on cell response to oxidative stress via immunoblotting, morphology, immunofluorescence, apoptosis, and proliferation assays.	Oxidative stress reduced proliferation in differentiating NPCs treated with TD sera. Effect was not as prominent with ASD sera, indicating an altered response to oxidative stress.	Mazur-Kolecka et al., 2009
20 ASD (3.0 ± 0.4 years) 12 TD (3.0 ± 1.2 years)	Taqman Real time PCR to detect serum mtDNA. Serum ELISA analysis to detect mtDNA antibodies	↑ extracellular mtDNA in ASD ↑ anti-mtDNA auto-Abs (type 2) in ASD	Zhang et al., 2010
277 ASD (70 BAP) 189 TD (2–5 years)	Western blot for child plasma reactivity to Rhesus macaque cerebellum protein medley	↑auto-Abs to 45 kDa protein in ASD ↑auto-Abs to 62 kDa protein in BAP Increases in either auto-Ab was associated with lower adaptive and cognitive scores, increased aberrant behaviors.	Goines P. et al., 2011
54 ASD 54 TD (4–11 years)	Serum anti-ganglioside M1 Abs were measured by ELISA	↑ antiganglioside M1 auto-Abs in ASD, especially in severe compared to mild or moderate autism.	Mostafa and Al-Ayadhi, 2011
86 ASD (2.0–5.6 years) 43 TD (2.3–4.7 years)	IHC for plasma reactivity to sections of macaque monkey brain (methods similar to Wills et al., 2009), results compared to behavioral assessments.	No differences in rate of plasma immunoreactivity to cerebellar Golgi neurons and other neural elements in ASD vs. TD, however immunoreactivity associated with worsening behavior and higher multiple CBCL scores.	Rossi et al., 2011

(Continued)

TABLE 4 | Continued

Subject details	Methods	Summarized findings	References
7 ASD with reactivity (2.5 to 7 years) 7 ASD with no reactivity 6 TD with no reactivity (2.5 to 8 years)	IHC: follow up of subgroup of ASD children from previous study (Wills et al., 2009) with reactivity to cerebellar 52-kDa protein and to Golgi cell region of the cerebellum. IHC to detect plasma immunoreactivity in the maqaque and male mouse brains.	Reactivity seen in previous study identified as GABAergic interneurons (based on co-localization of staining to calcium-binding proteins). Reactivity extended to other regions of the brain with slight preponderance to superficial layers of the cortex.	Wills et al., 2011
80 ASD 80 TD (6–12 years)	Indirect immunofluorescence used to measure serum anti-neuronal antibodies	↑ anti-neuronal auto-Abs in ASD, associated with increased severity of autism and seen more frequently in females ASD (90 vs. 53.3%, $P = 0.001$).	Mostafa and Al-Ayadhi, 2012b
50 ASD 30 TD (5–12 years)	Serum ELISA measurements of 25-hydroxy vitamin D and anti-MAG autoAbs	↓ 25-hydroxy vitamin D in ASD ↑ anti-MAG auto-Abs in ASD 25-hydroxy vitamin D levels negatively correlated with CARS scores and anti-MAG auto-Abs	Mostafa and Al-Ayadhi, 2012a
54 ASD 22 DD 33 TD (2–5 years)	Plasma ELISA measurements of anti-cardiolipin, anti-phosphoserine, and anti-β-glycoprotein 1 auto-Abs	↑ auto-Abs to cardiolipin, phosphoserine, and β-glycoprotein 1 in ASD compared to TD and DD controls, significantly associated with worsening behaviors.	Careaga et al., 2013
42 ASD 42 TD (6–11 years)	Serum ELISA measurement of human anti-MBP Abs. Severity of ASD and manifestation of allergic/asthma symptoms compared to results.	↑ auto-Abs to MBP and MAG in ASD, regardless of allergies. Severity of autism was also found to be associated with increased allergies.	Mostafa and Al-Ayadhi, 2013
93 ASD (2.9–17.4 years)	Patented process of identifying FRA: incubated serum with folate receptors then added radio-labeled folic acid. HPLC measurement of 5-MTHF in the CSF.	↑ prevalence of FRA in ASD sera. Blocking FRA correlated with CSF 5-MTHF concentrations in 16 children. Treatment with folinic acid improved attention, language and communication, and repetitive behaviors, with "moderate to much" improvement seen in 1/3 of children treated.	Frye et al., 2013
75 ASD (2–22 years) 30 DD (1–18 years)	Patented process of identifying FRA: incubated serum with folate receptors then added radio-labeled folic acid.	↑ prevalence of FRA in ASD vs. DD ↑ prevalence of FRA in parents of ASD vs. DD, suggesting familial autoimmune component to ASD	Ramaekers et al., 2013
20 ASD (1.4–5 years) 18 TD (1.4–4.4 years)	Immunoblotting and immunocytochemistry to detect serum auto-Abs against differentiating NPCs	↑ auto-Abs against human neuronal progenitor cell proteins of 55, 105, 150, and 210 kDa molecular weights in ASD subjects compared to controls. Strongest reactivity noted in NPCs expressing Tuj1.	Mazur-Kolecka et al., 2014
100 ASD 100 TD (4–11 years)	ELISA measurement of serum anti-ds-DNA Abs. Immunofluorescence measurement of serum antinuclear Abs.	↑ anti-ds-DNA and anti-nuclear auto-Abs in ASD Presence of anti-ds-DNA auto-Abs positively associated with a family history of autoimmunity.	Mostafa et al., 2014
355 ASD 142 SIB (2–47 years, mean age: 9.06)	Western blot plasma reactivity to homogenized Rhesus macaque brain tissue and human adult cerebellum	Plasma reactivity at 45 and 62 kDa brain proteins associated with autism severity and larger head circumference. 45 kDa reactivity associated with cognitive impairment/lower VABS scores while 62 kDa reactivity associated with stereotypies.	Piras et al., 2014
60 ASD 60 TD (3–12 years)	ELISA measurement of serum anti-nucleosome-specific antibodies.	↑ anti-nucleosome-specific auto-Abs in ASD, associated with family history of autoimmunity.	Al-Ayadhi and Mostafa, 2014
55 ASD (3–12 years) 25 TD (4–12 years)	ELISA measurement of plasma levels of anti-endothelial cell antibodies	↑anti-endothelial cell auto-Abs in children with autism compared to healthy controls, associated with autism severity.	Bashir and Al-Ayadhi, 2015

(Continued)

TABLE 4 | Continued

Subject details	Methods	Summarized findings	References
62 ASD (4–11 years) 62 TD (5–12 years)	ELISA measurement of serum ENA-78 and anti-neuronal auto-antibodies	↑ anti-neuronal auto-Abs in ASD ↑ENA-78 (neutrophil-recruiting chemokine CXCL5) associated with increases in anti-neuronal auto-Abs	Mostafa and Al-Ayadhi, 2015
40 ASD/FRAA-(7.0 ± 3.3 years) 16 ASD/FRAA blocking + (6.4 ± 3.0 years) 48 ASD/FRAA binding + (7.3 ± 3.1 years)	Measured redox, methylation, vitamins and immune biomarkers using various assays and compared to behavioral assessments	↓ 3-Chlorotyrosine (a marker of inflammation) was in those positive for blocking FRAs Presence of blocking FRAs in ASD associated with less severe ASD symptoms compared to ASD negative for these FRAs	Frye et al., 2016

ASD, autism spectrum disorders; CDD, childhood disintegrative disorder with regression after age 2 years; PDD-NOS, pervasive developmental disorder-not otherwise specified; LKS, Landau-Kleffner syndrome; HC, healthy children; NNI, children with non-neurologic illnesses; ELISA, enzyme-linked immunosorbent assay; BDNF, brain-derived neurotrophic factor; MBP, myelin basic protein; SIB, typically-developing sibling; TD, typically developing child; kDa, kilodalton; DD, non-ASD developmentally delayed; NPCs, human neuronal progenitor cells; MEF, myelin-enriched fraction of the brain; AEF, axolemma-enriched fraction of the brain; IHC, immunohistochemistry; auto-Abs, autoantibodies; PCR, polymerase chain reaction; mtDNA, mitochondrial DNA; BAP, broader diagnosis of autism spectrum disorder; CBCL, The Child Behavior Checklist; MAG, myelin-associated glycoprotein; FRA, folate receptor antibodies; 5-MTHF, 5-methyltetrahydrofolate; CSF, cerebrospinal fluid; Tuj1, neuron-specific Class III β-tubulin; ds-DNA, double-stranded DNA; VABS, Vineland Adaptive Behavior Scales; ENA-78, Epithelial cell-derived neutrophil-activating peptide-78; CXCL5, C-X-C motif chemokine 5.

of the brain, including those in the superficial layers of the cortex. The target neurons were identified as specifically GABAergic. GABAergic Golgi neurons and interneurons are inhibitory, utilizing the neurotransmitter gamma-aminobutyric acid (GABA) to modulate nearby excitatory synapses. It is unknown whether these antibodies are able to cross the blood-brain barrier (Wills et al., 2011). However, if they are able to enter the brain and reach their target antigens, this could potentially alter numbers or activity of inhibitory neurons, and contribute to the imbalance in excitatory/inhibitory activity that has long been suggested to contribute to certain aspects of ASD (Rubenstein and Merzenich, 2003).

Eighty-six children with ASD and forty-three typically developing controls from the Autism Phenome Project, a large multidisciplinary study conducted at the MIND Institute, were further assessed for these neuronal autoantibodies. Similar reactivity to cerebellar Golgi neurons and interneurons was found throughout the brain in some children with ASD; however, the results were not significantly different than controls, and in contrast to (Wills et al., 2009), some typically developing children also exhibited positive staining. Although these results did not support previous findings that these autoantibodies occur solely in ASD, this group did find a correlation between immunoreactivity and increased scores on the Child Behavior Checklist (CBCL), indicating worsening behaviors with immunoreactivity (Rossi et al., 2011). This finding that the autoantibodies are also present in typically developing children suggests that in ASD, there may be some other pathological mechanism that is allowing the autoantibodies to enter the typically "immune-privileged" brain, contributing to ASD behaviors (Rossi et al., 2011). Using human protein extracts as antigenic targets the same researchers found antibody reactivity to CNS proteins at two separate molecular weights (45 and 62 kDa) that correlated with worsening behaviors in children with ASD (Goines P. et al., 2011). Autoantibodies specific for a 45 kDa cerebellar protein were associated with a diagnosis of autism disorder (Goines P. et al., 2011) and cognitive impairment (Piras et al., 2014), while autoantibodies directed toward the 62 kDa protein were associated with the broader diagnosis of ASD (Goines P. et al., 2011) and motor stereotypies (Piras et al., 2014). A Saudi Arabian cohort of children with ASD also showed high levels of autoantibody reactivity to cerebellar neurons, the presence of which was positively associated with ASD severity (Mostafa and Al-Ayadhi, 2012b). Increased serum autoantibodies against human neuronal progenitor cell (NPC) proteins of 55, 105, 150 and 210 kDa molecular weights in ASD have also been identified, with the strongest reactivity noted in neuronal progenitor cells expressing the mature neuronal marker Tuj1, as opposed to astrocytes expressing Glial fibrillary acidic protein (GFAP) (Mazur-Kolecka et al., 2014). This group had previously found that sera from ASD subjects suppressed differentiation and maturation of NPCs in culture and provided a potential mechanism for aberrant neurodevelopment in ASD (Mazur-Kolecka et al., 2007, 2009, 2014).

Serum antibodies to ganglioside M1, the most abundant sialylated glycosphingolipid component of neuronal membranes, were found to be significantly higher in children with ASD compared to controls, with highest levels seen in the most severe cases of ASD (Mostafa and Al-Ayadhi, 2011). Serum autoantibodies to gangliosides are frequently seen in autoimmune disorders associated with neurological impairment, such as SLE and Guillain-Barré syndrome (Mostafa et al., 2010b; Kusunoki and Kaida, 2011). Additional autoantibodies identified in individuals with ASD include those reactive to cardiolipin, phosphoserine, and β2-glycoprotein 1 (Careaga et al., 2013), endothelial cells (Zhang et al., 2010; Bashir and Al-Ayadhi, 2015), myelin-associated glycoprotein (Mostafa and Al-Ayadhi, 2012a, 2013), double stranded DNA, nucleus and nucleosomes (Al-Ayadhi and Mostafa, 2014; Mostafa et al., 2014) and mitochondrial DNA (Zhang et al., 2010). Recently, folate receptor autoantibodies (FRA) have come to the forefront of autoantibody studies in children with ASD. In 2013, Frye et al. found FRAs to be prevalent in children with ASD (75%), including blocking and binding FRAs, with 29% being positive for both FRAs (Frye et al., 2013). This study additionally looked at supplementation of folinic acid, as FRA may be interfering with folate transport across the blood-brain barrier, and found improvement in communication, language, attention and stereotypic behaviors in treated children compared with non-supplemented ASD controls (Frye et al., 2013). In support of these findings, a Belgium study found significantly higher prevalence of blocking FRA in ASD compared to non-autistic individuals with developmental delays (Ramaekers et al., 2013). Both studies also found a statistically significant increase in FRAs among the parents of individuals with ASD, suggesting a relationship with familial autoimmunity. However, not all parents harbored these autoantibodies, and this suggests that in some instances there is postnatal acquisition of FRA (Frye et al., 2013; Ramaekers et al., 2013). 3-Chlorotyrosine, a marker of myeloperoxidase protein damage and inflammation, was significantly lower in those positive for blocking FRAs, suggesting that this group may have less inflammation than their counterparts positive for binding FRAs. Moreover, the presence of blocking FRAs in children with ASD was associated with less severe ASD symptoms compared to those who were negative for blocking FRA (Frye et al., 2016). Further studies characterizing immune activation in the different FRA groups could help clarify this relationship.

It is important to note that although the presence of autoantibodies are commonly found in autoimmunity, they may not be specific to any single disorder and can be present to some degree in healthy individuals, therefore they are not diagnostic without direct or indirect evidence (Rose and Bona, 1993; Lacroix-Desmazes et al., 1998). It is currently unknown whether these autoantibodies found in individuals with ASD play a causal role in the etiology of the disorder. The lack of consistency in target antigens and wide heterogeneity of type and presence of these autoantibodies suggest they may in fact be epiphenomenon in at least some cases of ASD due to general immune dysregulation (Wills et al., 2009). Collateral damage can occur from unregulated or excessive inflammatory responses, causing subsequent epitope spreading which leads to the development of autoantibodies characteristically seen in autoimmunity (Vanderlugt and Miller, 1996).

Aberrant Innate Immune Responses

Neuroinflammation

One of the major advancements in ASD research in the last 10 years is evidence that active neuroinflammation is a significant component of ASD, including chronically activated microglia (**Figure 2**). Findings of increased microglial and astroglial activation in the cerebellum and various regions of the cortices, specifically increased HLA-DR and GFAP via immunostaining (Vargas et al., 2005) has prompted additional research in this area. These early findings included increases in proinflammatory cytokines in the CSF and within several regions of the brain such as macrophage chemoattractant protein (MCP)−1, a cytokine important for monocyte recruitment. Findings also included marked increases in CD68+ perivascular macrophages and monocytes, suggesting the possibility of monocyte infiltration, which is one of the markers considered when autoimmunity is suspected in MS and EAE models (van Der Valk and De Groot, 2000; Vogel et al., 2013). Additionally, a significant loss of Purkinje neurons in the cerebellum was noted in ASD subjects compared with controls, and anti-inflammatory cytokines were associated with degenerative Purkinje cells and cerebellar astroglia, suggesting an attempt to modulate inflammation in the presence of damaged tissue (Vargas et al., 2005).

Microglia were later characterized in post-mortem brain samples of ASD subjects, revealing alterations indicative of an activated microglia phenotype including increased somal volume, increased density, and amoeboid presentation in 9 of 13 ASD cases (Morgan et al., 2010). These alterations were not correlated with age and researchers found no co-localization of Interleukin 1 receptor, type I (IL-1R1) with the monocyte/microglia marker ionized calcium binding adapter molecule 1 (Iba-1). IL-1R1 is upregulated rapidly during acute inflammation, therefore this lack of increased co-localization suggests this is not an acute inflammatory event, rather a long-standing alteration in the brains of ASD subjects (Morgan et al., 2010). This group later found increases in spatial clustering of microglia to neurons in these brain samples, suggesting neuron-directed recruitment of microglia in ASD subjects (Morgan et al., 2012). Considered the resident innate immune cells of the brain, microglia colonize the brain during the early embryonic period and are essential to neurodevelopment, including involvement in angiogenesis (Fantin et al., 2010; Rymo et al., 2011), regulation of astrocytic differentiation from neuronal precursor cells (Nakanishi et al., 2007), synaptic pruning (Paolicelli et al., 2011) and clearance of newborn neuronal precursors destined for apoptosis (Sierra et al., 2010). When activated to an inflammatory phenotype, microglia secrete inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), IL-1β and IL-6 and produce nitric oxide synthase (iNOS) (Reviewed in Smith et al., 2012). Although some microglia activation is required for productive neurodevelopment (Cunningham et al., 2013), chronic activation is associated with disease states (Smith et al., 2012). Furthermore, excessive activation can lead to cell death and abnormal or reduced connectivity (Rodriguez and Kern, 2011).

To gain a more specific picture of the pro-inflammatory cytokine milieu in the brains of ASD subjects, Li et al. (2009) further investigated cytokines associated with inflammatory responses in post-mortem tissue and found significantly increased pro-inflammatory cytokines including interferon gamma (IFNγ) associated with NK cells and T helper (T_H)-1 activation (Li et al., 2009). In support of altered immune regulation and function in the brains of ASD subjects, recent transcriptome analyses of the superior temporal gyrus and cerebral cortex of postmortem samples indicated upregulation of genes involved in immunity and inflammation, including markers of activated microglia and pathways of innate immunity (Garbett et al., 2008; Voineagu et al., 2011) More recent transcriptome analyses of multiple cortical areas of ASD brains support and add to these findings. Large scale RNA sequencing revealed that the dysregulated co-expression module found in the brains of ASD subjects by Voigneau et al. was enriched for activation specific to the microglia, and showed increased expression of "immune-response" genes (Gupta et al., 2014). Correlating well with these findings, methylation studies have identified that immune-response genes in frontal cortex of individuals with ASD have hypomethylated CpG sites, causing increased transcription of inflammatory genes such as TNF-α, integrin and complement genes, and genes that encode transcription factors involved in microglial development (Nardone et al., 2014).

To allow for *in-vivo* study of individuals with ASD, Suzuki and colleagues utilized positron emission tomography (PET) analysis to assess binding values of the $[^{11}C](R)$-PK11195 radiotracer that binds selectively to the mitochondrial 18 kDa translocator protein (TSPO), specifically targeting activated microglia. They found significantly increased binding values in several regions of the brain compared to controls, suggesting increased microglia activation in all regions analyzed compared to controls, including the cerebellum, several regions of the cortex, and the corpus callosum (Suzuki et al., 2013). These *in-vivo* findings support studies of increased microglia activation in post-mortem tissue; however, it is important to note that (1) the sample sizes were small and only included individuals with high-functioning ASD, (2) significant non-specific binding can occur with the $[^{11}C](R)$-PK11195 radiotracer, and (3) researchers were unable to normalize binding values due to lack of a microglia-free reference region (Suzuki et al., 2013). These findings warrant additional *in-vivo* studies with larger sample sizes/additional ASD phenotypes, and ideally a more-specifically binding radiotracer.

Peripheral Innate Immune Dysfunction

Aberrant innate immune responses are not restricted to the brain and CNS in individuals with ASD, alterations in circulating monocytes, dendritic cells and NK cells have also been identified (**Figure 2**). Early studies found an increased number of monocytes in the peripheral blood of children with ASD (Denney et al., 1996; Sweeten et al., 2003b), and elevated production of IFNγ, IL-1RA, and a trend for elevated IL-6 and TNF-α in whole blood cultures, suggesting increased activation of monocytes in individuals with ASD (Croonenberghs et al., 2002a). In support of these findings, a recent study found increased CD95, a marker of activation on monocytes in the peripheral blood of children with ASD (Ashwood et al., 2011a). Sweeten and colleagues also found elevated plasma neopterin, a pyrazinopyrimidine compound produced by monocytes and macrophages in response to IFNγ stimulation, indicating increased cellular immune activation (Murr et al., 2002; Sweeten et al., 2003b). Additionally, after TLR2 and TLR4 stimulation, upregulation of inflammatory cytokines and the HLA-DR activation marker was seen in monocytes from children with ASD versus typically developing children (Enstrom et al., 2010). Dendritic cell numbers were also increased in children with ASD and associated with bigger amygdala size and more aberrant behaviors (Breece et al., 2013).

An early study investigating induced responses of immune cells in children with ASD found increased production of the innate cytokines: TNF-α, IL-1β, and IL-6 after stimulation of peripheral blood mononuclear cells (PBMC) with the TLR-4 ligand lipopolysaccharide (LPS), from children with ASD when compared to typically developing children (Jyonouchi et al., 2001). To improve understanding of differential innate responses to varied TLR stimuli in ASD, investigators measured innate responses to several environmentally relevant pathogen-associated molecular patterns (PAMPs). The outcome of this study demonstrated elevated cytokine production after exposure to several innate immune ligands. Stimulation of isolated monocytes with TLR2 ligand lipoteichoic acid (LTA) produced a significant increase in production of TNF-α, IL-1β, and IL-6 in children with ASD versus typically developing controls, supporting earlier works. TLR4 stimulation with LPS also produced increased IL-1β. Moreover, increased production of IL-1β after LPS stimulation was found to be associated with worsening behaviors (Enstrom et al., 2010). Recently, Nadeem et al. identified increases in the IL-17RA receptor on circulating monocytes in children with ASD (Nadeem et al., 2018). IL-17RA is the receptor for IL-17A, a cytokine associated with autoimmunity and implicated in rodent models of ASD (Choi et al., 2016). Increased expression of the nuclear transcription factor NFkB and inducible nitric oxide synthase (iNOS) were also noted in ASD groups. Cells treated with IL-17 increased expression of iNOS/NFkB and blockade of IL-17 reversed this inflammatory profile (Nadeem et al., 2018). These data may suggest a link between adaptive arm of the immune system and innate immune dysfunction in people with ASD.

Significant increases in cytokines associated with innate inflammation have also been found in phytohemagglutinin (PHA) stimulated PBMC of children with ASD, including TNF-α and GM-CSF (Ashwood et al., 2011b). Excessive production of pro-inflammatory cytokines initiated by the innate immune system could have downstream consequences including over-activation of the adaptive arm, leading to autoimmune sequelae. Abnormal innate cytokines have also been identified in the plasma and sera of individuals with ASD. Significant increases in plasma levels of IL-1β, IL-6, and TNF-α suggest increased activation of the innate arm (Emanuele et al., 2010; Ashwood et al., 2011b; Suzuki et al., 2011; Ricci et al., 2013) and are consistent with the dynamic responses seen previously in stimulated monocytes (Enstrom et al., 2010; Ashwood et al., 2011c). Other innate-associated cytokines reported to be elevated in the plasma or sera of individuals with ASD when compared to typically developing controls include IL-12p40 and the chemokines IL-8, MCP-1, regulated on activation, normal T cell expressed and secreted (RANTES), eotaxin and C-X-C motif chemokine 5 (CXCL5) (Ashwood et al., 2011b; Suzuki et al., 2011; Mostafa and Al-Ayadhi, 2015). While there have been a few contradictory reports regarded plasma/sera cytokine concentrations individuals with ASD, a recent meta-analysis of plasma cytokines showed significant evidence of abnormal cytokine/chemokine profiles in individuals with ASD versus healthy controls, including elevated IL-1β, IL-6, IL-8, IFN-γ, and MCP-1, and reduced concentrations of the anti-inflammatory cytokine transforming growth factor beta 1 (TGFβ1) (Masi et al., 2015). Further characterization of ASD subjects found that circulating plasma levels of pro-inflammatory cytokines associated with increased innate immune activation correlated with worsening behaviors, which suggests that ongoing inflammation likely contributes to the severity of behaviors (Ashwood et al., 2011b). Leukocyte adhesion molecules have also been investigated in children with ASD, and were found to be reduced, indicating dysfunctional immune-endothelial cell interactions that could have implications for the migration of innate immune cells into the CNS (Onore et al., 2012).

NK cells are important early responders of the innate immune system. They specifically target virally infected cells and play important roles in both tumor surveillance and protection of the fetus during pregnancy. As early responders, they can initiate a cascade of immune responses and if dysfunctional these important signals may be missing or altered (Mandal and Viswanathan, 2015). A significant increase of total numbers of NK cells, identified as $CD56^{+}CD3^{-}$, was observed in children with autism with both high and low IQ (Ashwood et al., 2011a). Cytokines produced by NK cells expressing high levels of CD56 can significantly influence the cytokine milieu. Upregulation of mRNA responsible for expression of receptors including killer-cell immunoglobulin-like receptors [KIRs] and increased cytokine, perforin, and granzyme B production was observed at resting levels in NK cells from 2 to 5 year old children with ASD (Enstrom A. M. et al., 2009). The cytolytic function of NK cells is important for immune regulation, as they can remove persistently activated immune cells (Cook et al., 2014). Interestingly, when stimulated, significantly decreased cytotoxicity and lower production of effector molecules (granzyme, perforin, and IFNγ) were seen in children with ASD compared to controls (Enstrom A. M. et al., 2009). Decreased cytolytic activity was seen previously in a large subgroup of children with ASD (Vojdani et al., 2008). This pattern suggested that NK cell activation may be "maxed-out" *in vivo*, and the cells may be unable to respond to further stimuli. Similar patterns of increased $CD56^{+}$ NK cells but impaired cytolytic activity have been seen in the peripheral blood of patients with autoimmune disorders such as MS, T1DM, SLE, and RA (Fogel et al., 2013), again adding evidence of an autoimmune component/lack of immune regulation in individuals with ASD.

Aberrant Adaptive Immune Responses

T Cells

Over the last 10 years, researchers have found significant abnormalities of the adaptive arm of the immune system in people with ASD, including altered numbers of lymphocytes, dysregulation of T and B cell activation, and altered adaptive cytokine production (**Figure 2**). Increased total numbers of T cells and skewed ratios of CD4 to CD8 lymphocytes have been associated with decreased executive function in people with ASD (Han et al., 2011). Several studies have also shown altered cytokine production in T cells. Molloy et al. found a shift to a $T_{H}2$ phenotype with significant increases in IL-4, IL-5, and IL-13 after stimulation *in vitro* (Molloy et al., 2006b). Altered surface markers of T cell activation have also been seen. Specifically, the T cell activation markers HLA-DR and CD26 were found to be increased in children with ASD (Ashwood et al., 2011a). CD5, a transmembrane protein associated with T cells and found to be elevated in autoimmunity (Sigal, 2012), was recently found to be significantly elevated in plasma of ASD subjects, and is associated with worsening severity of ASD (Halepoto et al., 2017). In response to stimulation, T cells from children with ASD also showed increased CD134 and increased cellular proliferation associated with worsening behaviors (Ashwood et al., 2011b). CD134 (also known as OX40) is a co-stimulatory molecule expressed on activated T cells, including memory T cell subsets (Webb et al., 2016) that is required for optimal activation of naïve T cells and is important in survival and maintenance of memory T cells.

Significant alterations in cytokines associated with the adaptive arm have been found in children with ASD versus controls, including IL-5, IL-13, IL-17 (Suzuki et al., 2011), IL-23 and IL-12 (Ricci et al., 2013), IL-21 and IL-22 (Ahmad et al., 2017a). Two recent studies found altered cytokine profiles in neonatal blood spots, suggesting early immune dysregulation. Increased IL-4 at birth was associated with increasing severity of ASD, and increased IL-1β with milder versions of ASD (Krakowiak et al., 2017). Zerbo et al. observed increased MCP-1 and decreased RANTES at birth in children with ASD (Zerbo et al., 2014). It is noteworthy to mention that various reports of T cell skewing in ASD does not necessarily implicate a specific polarization associated with the disorder, rather it supports the suggestion that a lack of regulation may be at play. A recent study clustered subjects into immune endophenotypes based on T cell polarization after stimulation with PHA, and found that both $T_{H}1$ and $T_{H}2$ responses were associated with worsening behaviors and increased severity of core ASD symptoms (Careaga et al., 2017). Transcription factors associated with inflammatory T cell activation, and different T cell subsets, namely T-box transcription factor (Tbet), GATA binding protein 3 (GATA3) and retinoid-acid receptor-relat-ed orphan receptor gamma t (RORγT) are all increased in children with ASD (Ahmad et al., 2017b). These studies support the notion of distinct clusters of ASD phenotypes characterized by immune dysfunction (Sacco et al., 2010, 2012).

Aberrant T cell responses or decreased removal of activated T cells can lead to autoimmune pathology (Joller et al., 2012). CD95 is the first apoptosis signal (Fas) receptor, which initiates apoptosis of activated T cells when they are repeatedly exposed to antigen. It has critical importance in tolerance and regulation, and alterations in Fas signaling may play a role in the development of autoimmunity (Siegel and Fleisher, 1999). Reduced CD95 expression on T cells from ASD subjects compared to controls is suggestive of decreased apoptosis of potentially overactive T cells in ASD (Engstrom et al., 2003).

Regulatory T Cells

One of the most important immune components in the prevention of autoimmunity is regulation, and regulatory T cells (T_{regs}) play a key role in immune regulation and homeostasis (Sakaguchi, 2004). Humans that carry mutations in the transcription factor forkhead box P3 (FOXP3) or have depletion in T_{regs} develop severe autoimmunity (Sakaguchi et al., 1995; Miyara et al., 2005; Toubi et al., 2005; Long and Buckner, 2011; Fujio et al., 2012). T_{regs} have been found to be critical for preventing autoimmunity in murine models (Sakaguchi et al., 1995) and a deficiency of T_{regs} may play a role in the development of autoimmune disorders, including RA (Toubi et al., 2005) and SLE (Miyara et al., 2005). Autoimmune pathology can occur when immune regulation breaks down, disrupting tolerance and homeostasis, and leading to an aberrant attack on self (Lourenço and La Cava, 2011). Typically in autoimmunity, a breakdown in tolerance will lead to the production of destructive autoantibodies by plasma cells and self-reactive T cells with a deficit in number or activity of T_{regs} (Sakaguchi, 2004). Notably, several studies have found that T_{regs} or their regulatory effector molecules are decreased in some individuals with ASD (**Figure 2**) (Okada et al., 2007; Ashwood et al., 2008; Mostafa et al., 2010a). For example, plasma TGFβ1 was significantly reduced in adult males with Asperger's syndrome (Okada et al., 2007) and in children with ASD (Ashwood et al., 2008). Reduced TGFβ1 was associated with increased ASD severity and lower adaptive and cognitive behaviors (Ashwood et al., 2008). Furthermore, microRNAs (miRNAs) involved in controlling TGFβ1 signaling pathways show differential expression in the cortex and serum of individuals with ASD (Mundalil Vasu et al., 2014; Ander et al., 2015; Huang et al., 2015). Additionally, circulating $CD4^{+}CD25^{high}$ T_{regs} were found to be significantly decreased in children with ASD, with reduced frequency correlating with severity of the disorder (Mostafa et al., 2010a). Several studies have also shown decreased IL-10 production after stimulation of CD4+ T cells (Jyonouchi et al., 2001, 2005, 2014; Ashwood and Wakefield, 2006).

B Cells

Antibody production of high specificity to various antigens is the primary role of B cells, in order to neutralize and help eliminate pathogens. Despite the growing number of studies identifying autoantibodies in subjects with ASD, the B cells responsible for antibody production have been poorly studied. Irregularities in B cell populations and antibody production have been identified in a small number of ASD studies, although with conflicting results. A 2011 study found increased numbers of total ($CD20^{+}$) and activated ($CD38^{+}$) B cells in children with ASD compared to age-matched controls. No differences were seen in naïve ($CD5^{+}$) B cells, thus the increase in total cells was likely due to increased activated cells, suggesting increased immune activation overall (Ashwood et al., 2011a). Higher numbers of CD19/CD23 B lymphocytes were also found in children age 3–6 recently diagnosed with regressive autism, supporting the previous findings (Wasilewska et al., 2012). However, Heuer et al. found no differences in total numbers of B cells, and B cell responses to stimulation were not different among ASD subjects compared to controls (Heuer et al., 2012). Differences in study design and markers used (CD20 and CD19, respectively) could account for the contradictory results. Neither marker is comprehensive for individual B cell subsets as their expression is decreased as B cells mature and differentiate into antibody-secreting plasma cells (Tedder, 2009). CD38 expression increases significantly upon maturation, therefore this marker may be a better indicator of the population of effector cells that may be responsible the production of specific autoantibodies. Additional research is needed to further characterize these cells, including identifying populations of positive regulators and B-regulatory cells (B_{regs}), as these cells secrete IL-10, have recently been found to play a role in the induction of T_{regs} (Fujio et al., 2013), and play an important role in the acquisition of tolerance during pregnancy (Rolle et al., 2013).

In addition to significant levels of autoantibodies found in individuals with ASD, atypical antibody production has been frequently seen in ASD serum and plasma with correlations to behaviors. Results vary and are often contradictory—these inconsistencies may be due to small sample sizes and improper controls such as "population standard" versus age-matched controls residing in the same locale, and lack of adjusting for seasonality. Comparing immunoglobulin levels across a broad age range can produce inconsistencies, thus it is critical to have age-matched controls. For example, an early report found decreased circulating IgA associated with HLA-DR antigens in a subset of ASD subjects (Warren et al., 1997), and a 2012 study supported these findings (Wasilewska et al., 2012); however, other studies showed no change in IgA (Heuer et al., 2008). Ages varied widely in the Warren study (from ages 5 to 31) and may account for discrepancy because IgA does not reach adult levels until around age 10 (Aksu et al., 2006) whereas the later studies were age-matched. Decreased IgA in children with regressive ASD did not fulfill the criteria for either partial or full IgA deficiency in the study by Wasilewska and colleagues, and likely reflects immune dysfunction in a subset of patients (Wasilewska et al., 2012). Heuer et al. found that decreased plasma IgG/IgM negatively correlated with worsening behaviors as assessed using the Autism Behaviors Checklist (ABC) (Heuer et al., 2008). Researchers also found a significant increase in IgG4 in ASD subjects, with a trending increase in IgG2 subtype (Croonenberghs et al., 2002b; Enstrom A. et al., 2009). IgG4 is a blocking antibody, produced under conditions of chronic antigen exposure and class switch to IgG4 is dependent on T_H2 cytokines (IL-4/IL-13) (Aalberse et al., 2009). This is consistent with the dynamic T cell responses found by later by Ashwood et al. (2011c). These correlations support an association with immune dysfunction and potentially a lack of immune regulation in individuals with ASD.

IMPROVEMENT OF SYMPTOMS WITH THE USE OF IMMUNE-MODULATING DRUGS AND SUPPLEMENTS

Several clinical trials have shown the efficacy of immunosuppressive drugs for improving behaviors in individuals with ASD. The first study to show this relationship used corticosteroid treatment in a 6-year-old boy with language regression at 22 months who received a diagnosis of PDD (pervasive developmental disorder) at age 3-1/2. After several weeks of this treatment, the boy experienced significant gains in expressive language and responsiveness to communication to nearly age-appropriate levels, and reduction in stereotypical

FIGURE 3 | Summary of Immune Evidence in ASD–is Immune Dysregulation Causing or Contributing to these Disorders? Immune findings in individuals with ASD have grown from a few scant early studies to a plethora of extensive and varied research showing immune dysfunction that contributes to worsening behaviors. Familial autoimmunity is a common finding within families affected by ASD. In addition, individuals with ASD have significant immune dysregulation that contribute to altered behaviors. These individuals also suffer more so than the general population from immune-mediated comorbidities such as allergies, asthma and gastrointestinal (GI) disturbances. Mechanistically, studies have shown that the gestational immune environment must be delicately balanced, and without such balance neurodevelopment can be altered. Whether these immune characteristics are causal or just sequelae of the overarching disorders remain to be determined; however, the evidence is building that the dysregulated immune response may be pathologically contributing to ASD.

echolalia (Stefanatos et al., 1995). Since then, improvements have been seen in language ability, behaviors and motor development in several case studies and clinical trials using corticosteroids and immunosuppressive drugs (Mott et al., 1996; Chez et al., 1998; Shenoy et al., 2000; Mordekar et al., 2009) including a recent study that showed improvement in language-specific electrophysiological brain function after treatment with corticosteroids (Duffy et al., 2014). The effectiveness of the anti-psychotic medication risperidone in improving stereotyped behaviors and social withdrawal in individuals with ASD was increased with the addition of celecoxib, a cyclooxygenase-2 (COX-2) selective nonsteroidal anti-inflammatory drug (Asadabadi et al., 2013). Human cord blood mononuclear cell transplantation, alone and in combination with umbilical mesenchymal stem cell transplantation significantly improved behaviors in children with ASD compared to controls. These transplanted cell types are known to have profound immune-regulatory capabilities, suggesting that a possible mechanism of improvements seen may be immune-modulation (Lv et al., 2013). These trials and cases studies, although small and specific to select groups of ASD subjects, suggest that in some children with ASD, immune abnormalities may be driving certain behaviors.

Nutritional or supplemental approaches may be helpful in modulating immune function in people with ASD. Several studies have found children with ASD to be deficient in serum levels of vitamin D (25-hydroxycholecalciferol), and serum levels were found to be negatively associated with language and behavioral scores on the ABC and the Childhood Autism Rating Scale (CARS) (Desoky et al., 2017; Saad et al., 2018), (Feng et al., 2017). Serum vitamin D levels also negatively correlated with the presence of anti-myelin-associated glycoprotein autoantibodies (Mostafa and Al-Ayadhi, 2012a). This fat-soluble vitamin has important immunomodulatory and neuroprotective functions (Aranow, 2011; Wrzosek et al., 2013), and deficiency may be contributing to immune and behavioral abnormalities in people with ASD. Daily supplementation with Vitamin D, not to exceed 5,000 IU/day, was found to significantly improve behavioral outcomes and lowered elevation of CD5 expression in children with ASD, supporting a role for Vitamin D in modulating the immune system (Desoky et al., 2017; Feng et al., 2017; Saad et al., 2018). As previously discussed, children with ASD have altered T cell profiles, perhaps due to altered transcriptional activity (Ahmad et al., 2017b). Similar T cell alterations have been seen in the inbred BTBR mouse model of ASD, and a recent study showed modulation of this transcriptional activity through the administration of the antioxidant resveratrol, a nutritional component found in various fruits, legumes, and grape juice. Resveratrol increased mRNA expression of Foxp3 in spleen and brain tissues, and increased the number of $Foxp3^+$ T regulatory cells in the periphery of BTBR and B6 mice. Resveratrol also decreased expression of transcription factors associated with inflammatory T cells, including T_H17 cells. Additionally, the nutritional compound decreased ASD associated repetitive behaviors in BTBR mice (Bakheet et al., 2017). These studies, offer support that immune dysfunction is driving at least some of the pathological outcomes in subsets of people with ASD. As our understanding of how immune dysregulation is contributing to the pathogenesis of ASD grows, more treatments can be targeted specifically to these mechanisms.

OUTSTANDING QUESTIONS AND CONCLUSION

The evidence that immune dysfunction likely plays a role in the etiology/pathophysiology of ASD is becoming substantial. Familial autoimmunity is a common risk factor, and maternal autoantibodies and inflammation during gestation significantly increase the risk of having a child with ASD. Furthermore, individuals with ASD have significant immune dysfunction and inflammation. They also suffer from immune-mediated co-morbidities much more often than the typically developing population, including GI dysfunction and dysbiosis. The presence of autoantibodies in individuals with ASD is increased, and evidence of neuroinflammation has been substantiated both *in vivo* and in post-mortem brain tissue. Although the plethora of evidence identifying a connection between autoimmunity, immune dysfunction, and ASD (summarized in **Figure 3**) is tantalizing, it still leaves many mechanistic questions regarding the impact of immune system dysfunction on the development of ASD.

The most outstanding question remaining from these studies is whether the immune dysfunction is causal or rather sequelae of the larger disorder. The origin of the immune dysfunction seen in many individuals with ASD and the role it plays in the aberrant behaviors is still unknown, although many of these studies discussed throughout this review support an association of worsening behaviors associated with altered immune function. Gestational influences, including maternal immune activation and the presence of maternal autoantibodies may be contributing to altered early neurodevelopment and immune dysfunction in offspring, and these are supported by preclinical animal models of both maternal immune activation and passive transfer of autoantibodies. Interactions between the different immune cells leading to inflammation and altered cytokine production in people with ASD may be directly contributing to abnormal brain development and signaling, and the ever expanding knowledge of neuro-immune cross-talk may eventually elucidate some of the mechanisms involved in the pathogenesis of ASD.

To date, categorizing ASD and immune dysfunction has been a difficult task due to the heterogeneity of the disorder and the changing diagnostic criteria; however, the recent focus on clustering phenotypes may provide a clearer picture to help elucidate the different factors involved in the etiologies of these complex disorders. The immune dysfunction driving the development of autoantibodies and overall immune abnormalities in people with ASD remains unknown, however, recent insights into dysbiosis causing aberrant immune system education could be a plausible mechanism as to the origin of immune dysfunction. Prenatal immune influences could be driving direct and/or epigenetic changes in gene expression responsible for altered neurodevelopment. As future studies improve our understanding of these complex and interconnected systems, it will allow for development of new therapies that target immune dysfunction in ASD. Future research could focus on interventions that improve immune parameters to help identify mechanisms involved in development and exacerbation of ASD symptoms. As our understanding of the involvement of the immune system in ASD grows, it can shape future hypotheses and research to better identify the pathological mechanisms involved.

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HH wrote the first draft of the manuscript. EM, DR, and PA wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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