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Full Length Article

T cell populations in children with autism spectrum disorder and co-morbid gastrointestinal symptoms

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ABSTRACT

Children with ASD are more likely to experience gastrointestinal (GI) symptoms than typically-developed children. Numerous studies have reported immune abnormalities and inflammatory profiles in the majority of individuals with ASD. Immune dysfunction is often hypothesized as a driving factor in many GI diseases and it has been suggested that it is more apparent in children with ASD that exhibit GI symptoms. In this study we sought to characterize peripheral T cell subsets in children with and without GI symptoms, compared to healthy typically-developing children. Peripheral blood mononuclear cells were isolated from participants, who were categorized into three groups: children with ASD who experience GI symptoms (n = 14), children with ASD who do not experience GI symptoms (n = 10) and typically-developing children who do not experience GI symptoms (n = 15). In order to be included in the GI group, GI symptoms such as diarrhea, constipation, and/or pain while defecating, had to be present in the child regularly for the past 6 months; likewise, in order to be placed in the no GI groups, bowel movements could not include the above symptoms present throughout development. Cells were assessed for surface markers and intracellular cytokines to identify T cell populations. Children with ASD and GI symptoms displayed elevated T_H17 populations (0.757% ± 0.313% compared to 0.297% ± 0.197), while children with ASD who did not experience GI symptoms showed increased frequency of T_H2 populations (2.02% ± 1.08% compared to 1.01% ± 0.58%). Both ASD groups showed evidence of reduced gut homing regulatory T cell populations compared to typically developing children (ASD^{GI}:1.93% ± 0.75% and ASD^{NoGI}:1.85% ± 0.89 compared to 2.93% ± 1.16%). Children with ASD may have deficits in immune regulation that lead to differential inflammatory T cell subsets that could be linked to associated co-morbidities.

1. Introduction

Neurodevelopmental disorders, such as autism spectrum disorders (ASD) are often considered disorders of the brain, particularly since they are defined by behavioral traits including repetitive and stereotyped behaviors and by impairments in communication and social interactions (Association, 2013). However, many individuals with ASD also suffer from one or more medical comorbidities including epilepsy, sleep disorders, asthma and gastrointestinal (GI) dysfunction (Levisohn, 2007; Bauman, 2010; Kohane et al., 2012; Maenner et al., 2012; Chen et al., 2013; Zerbo et al., 2015b; Mannion and Leader, 2016). Reports of GI related symptoms in autism have occurred since the disorder was first described in 1943 (Kanner, 1943) and studies since have continued to report symptoms of constipation, diarrhea, vomiting, abdominal pain, gas and bloating associated with ASD. The full extent of GI symptoms among individuals with ASD has been debated, with differences in study designs, report biases, or population studies vs. clinic based studies, all leading to a wide range (20–90%) in the reported prevalence rates (Horvath and Perman, 2002; Molloy and Manning-Courtney, 2003; Niehus and Lord, 2006; Valicenti-McDermott et al., 2006; Xue et al., 2008; Buie et al., 2010; Coury et al., 2012). One large cohort study including close to 1000 participants reported children with ASD are 6–8

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times more likely to suffer from GI symptoms compared to age-matched typically-developing children; in addition, GI symptoms were associated with poorer behavioral assessment scores (Chaidez et al., 2014).

Immune dysfunction has been reported in approximately 60% of children with ASD (Careaga et al., 2017). Although immune abnormalities in ASD have been reported since the late 1970's (Stubbs and Crawford, 1977) a consensus for differences in cellular activation has not yet been reached (Rose and Ashwood, 2014). In mainly mixed cultures of peripheral blood mononuclear cells (PBMC), upon stimulation some studies have observed elevated T-helper (T_H)1 cytokines (Singh, 1996; Croonenberghs et al., 2002a; Ashwood et al., 2011b; Ricci et al., 2013; Careaga et al., 2017) while others found increased T_H2 cell associated cytokines (Molloy et al., 2006; Ashwood et al., 2011c; Goines et al., 2011b; Careaga et al., 2017; Krakowiak et al., 2017); in addition to elevated innate cytokines (Jyonouchi et al. 2001, 2005, 2014; Croonenberghs et al., 2002a; Enstrom et al., 2010; Ashwood et al., 2011b; Malik et al., 2011; Careaga et al., 2017). A number of studies have found altered frequencies or altered activation status of specific immune cells or cell subsets (Gupta et al., 1998; Sweeten et al., 2003; Enstrom et al. 2009b, 2010; Mostafa et al., 2010; Ashwood et al., 2011a; Lopez-Cacho et al., 2016; Ahmad et al., 2017), elevated chemokines concentrations (Ashwood et al., 2011d; Manzardo et al., 2012; Al-Ayadhi and Mostafa, 2013; Zerbo et al., 2014), decreased soluble TNF receptors (Ashwood, 2018), altered immunoglobulin titers (Croonenberghs et al., 2002b; Heuer et al., 2008; Enstrom et al., 2009a) and the presence of autoantibodies to brain or CNS proteins (Singh et al. 1993, 1997; Singer et al., 2006; Cabanlit et al., 2007; Braunschweig et al., 2008; Wills et al., 2009; Goines et al., 2011a; Frye et al., 2016). A recent study seeking to better characterize immune endophenotypes in ASD identified a subgroup of individuals within the ASD population who exhibited elevated production of inflammatory cytokines from stimulated immune cells. Using cluster analyses, those with enhanced immune activation following immune challenge displayed more severe core ASD features and impaired behaviors (Careaga et al., 2017). This was true for both children with ASD who displayed increased T_H1 cytokine profiles and those with increased T_H2 cytokine profiles.

We hypothesize that immune activation involving several arms of the immune system is observed in ASD, and that the unifying denominator among these studies is in fact a decrease in immune regulation and inability to control immune responses. Corroborating this hypothesis, decreased levels of the regulatory cytokines transforming growth factor (TGF)β1 (Okada et al., 2007; Ashwood et al., 2008; Abdallah et al., 2013b), interleukin (IL)-10 (Jyonouchi et al. 2005, 2012, 2014; Abdallah et al., 2012) and IL-35 (Rose and Ashwood, 2019) have been reported from blood components or in stimulated immune cell cultures from individuals with ASD. In addition, decreased frequency of regulatory T cells (T_{regs}) have also been found in individuals with ASD (Ashwood et al., 2004; Mostafa et al., 2010; Ahmad et al., 2017). On a background of decreased regulation, the varying pro-inflammatory immune abnormalities reported in ASD may reflect the heterogeneity of ASD, genetic background or environmental exposures, and illustrate the need to find common subgroups within ASD, that may help define more targeted treatments to benefit more individuals across the spectrum (Critchfield et al., 2011; Coury et al., 2012; Ousley and Cermak, 2014).

We previously reported altered immune responses in children with ASD who experience GI symptoms. Peripheral blood mononuclear cells from children with ASD and GI symptoms produced increased mucosa-related cytokines but decreased TGFβ after stimulation in vitro (Rose et al., 2018), suggesting a net imbalance away from a regulated response. In the current study we seek to expand upon these findings and further characterize the cellular mediators that may be driving these differential profiles. We utilized flow cytometry to identify and characterize T cells in children with ASD who experience GI symptoms, children with ASD who do not experience GI symptoms and in typically-developing children who do not experience GI symptoms. T cells were identified and grouped based on their expression of CD3, CD4, CD8, and CD25 and further characterized by intracellular expression of interferon gamma (IFNγ), IL-10, IL-13, IL-17. Furthermore, we also evaluated populations of T cells expressing the gut-homing integrin $\alpha_4\beta_7$.

2. Methods

2.1. Subjects

Children with ASD and GI symptoms of irregular bowel movements (ASD^{GI}) (n = 14), or children with ASD and no GI symptoms (ASD^{NoGI}) (n = 10), and typically developing children without GI symptoms (TD) (n = 15) were recruited into this study. Participants had previously been enrolled in the Childhood Autism Risk from Genetics and Environment (CHARGE) study (Hertz-Picciotto et al., 2006). A diagnosis of autism spectrum disorder was confirmed at the UC Davis MIND Institute by trained staff using the Autism Diagnostic Interview-Revised (ADI-R), and the Autism Diagnostic Observation Schedule (ADOS). The Social Communication Questionnaire (SCQ) was used to screen for behavioral and developmental characteristics of ASD in the typically-developing group. Participants in the TD groups had to score within the typical range, i.e. below 15, on the SCQ and above 70 on the Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Score (VABS). All participants were assessed using the Aberrant Behavior Checklist (ABC) to assess impairments within the domains of irritability, lethargy, social withdrawal, stereotypic behavior, hyperactivity and inappropriate speech. Participants were randomly recruited from the CHARGE database based on inclusion/exclusion criteria. A telephone interview, along with GI and health questionnaires were used to assess participant eligibility into the study. Medications and/or behavioral therapies used at the time of enrollment or within the previous year were collected and recorded. Participants were excluded if they had a known diagnosis of other GI pathology (e.g. celiac disease or Inflammatory Bowel Disease), use of antibiotics or antifungal medications within the prior month, medications affecting GI transit (stool softeners), and/or recent evidence of a GI infection based on stool laboratory tests. In addition, participants were excluded if there was evidence of a seizure disorder, genetic disorders (i.e. Fragile X syndrome, Tuberous Sclerosis Complex), liver or pancreatic disease, cystic fibrosis, or chronic infection. Children receiving clinically monitored and prescribed dietary interventions under the guidance of trained nutritionists/clinicians, medications, or complementary alternative treatments such as supplements other than a standard daily multivitamin/mineral tablet were also excluded. However, for children who were receiving nutritional modifications that were not overseen by trained nutritionists/clinicians, the dietary changes were documented but the participants not excluded from the study. Parental reports of suspected food sensitivities/intolerances that had not been diagnosed through clinical assessment were also not grounds for exclusion but were documented.

2.2. GI symptom evaluation

CHARGE GI history (GIH) survey and GI symptom survey (GISS), based upon Rome III Diagnostic Questionnaire for the Pediatric Functional GI Disorders (Walker et al., 2006) were obtained from parent/legal guardians. The GIH (Chaidez et al., 2014) and GISS (Rose et al., 2018) assessments have been reported previously. The GIH scores the frequency; abdominal pain, blood in stool/vomit, constipation, diarrhea, difficulty swallowing, gaseousness/bloating, pain on stooling, sensitivity to foods, vomiting, on a Likert scale [*(0) = never, (1) = rarely, (2) = sometimes, (3) = frequently and (4) = always)*]. The GIH assessed any allergies to foods, if any foods caused or worsened symptoms, reported dietary restrictions, by whom (child, parent or doctor) and for what reason; food aversions and what they were, and; if a clinical GI diagnosis had ever been given. The assessment was for both current (within the past three months) and previous experiences. The GISS consisted of 7 sections, each section had 1 to 6 questions to determine if the participant

met the criteria for constipation, diarrhea or irritable bowel syndrome (IBS) (see (Chaidez et al., 2014) and (Rose et al., 2018) for more information about the GIH and GISS).

Participants who met the criteria for constipation, diarrhea or IBS on the GI history and symptom surveys were placed in their corresponding GI group), those that did not meet criteria for irregular bowel movements, GI symptoms and had consistent stooling patterns for the past 6 months were placed in the corresponding no GI group. Participants who did not meet the criteria for constipation, diarrhea or IBS, but had inconsistent stooling patterns during the last 6 months were excluded from this study.

This study was approved by institutional review boards for the State of California and the University of California, Davis. Both written and informed consent was obtained from a legal guardian for all study participants prior to data collection in accordance with the UC Davis IRB protocol.

2.3. Blood collection and cellular assays

Peripheral blood was collected from each participant in acid-citrate dextrose Vacutainers (BD Biosciences; San Jose, Ca). Each tube was centrifuged at 2100 rpm for 10 min, plasma was removed and the remaining blood components were layered onto lymphocyte separation medium (Corning; Manassas, VA), and centrifuged at 1700 rpm for 30 min. PBMC from the buffy layer were collected and washed with Hanks balanced salt solution (Corning; Manassas, VA). After isolation, PBMC were allowed to rest overnight in complete media (RPMI 1640 (Invitrogen; Carlsbad, CA) with 10% Fetal Bovine Serum (FBS) (Corning; Manassas, VA), 100 IU/ml penicillin (Invitrogen; Carlsbad, CA) and 100 IU/ml streptomycin (Invitrogen; Carlsbad, CA)). PMBC were then divided and plated in complete media containing 10.6 μM Brefeldin A and 2 μM Monesin (protein transport inhibitor cocktail (ebioscience; San Diego,CA)) or in complete media containing 10.6 μM Brefeldin A, 2 μM Monesin, 81 nM Phorbol 12-Myristate 13-Acetate (PMA) and 1.34 μM Ionomycin (Cell Stimulation Cocktail (plus protein transport inhibitors) (ebioscience; San Diego, CA)) for 4 h. Following stimulation, PBMC were washed with PBS (Corning; Manassas, VA), 1×10^6 cells per well were plated for each condition and stained with 100 μL of Live/dead amine dye (LIVE/DEAD Fixable Aqua Dead Cell Strain Kit, for 405 nm excitation (Invitrogen; Carlsbad, CA) for 20 min in the dark. PBMC were washed with PBS, then reconstituted in 100 μL of PBMC wash (PBS (Corning), FBS (Corning), sodium azide (Sigma-Aldrich; St. Louis, MO)) containing 10% FcR block (Miltenyi Biotec; San Diego, CA). Cells were incubated in the dark for 10 min at 4 °C, antibody cocktails (see below) were added and cells were incubated in the dark for another 30 min at 4 °C. Following staining, cells were washed 3 times with PBMC wash and resuspended in 100 μL of 1x Fix/Perm (BD Cytofix/Cytoperm solution; BD Bioscience, San Jose, CA)) and incubated in the dark for 20 min at 4 °C. Cells were washed twice with 1x perm wash buffer (BD Perm/Wash buffer (BD bioscience)) and resuspended in 50 μL of perm wash buffer and intracellular antibody cocktail, followed by a 30 min incubation in the dark at room temperature. Cells were subsequently washed twice with 1x Perm wash buffer then resuspended in 100 μL 1% paraformaldehyde (Sigma-Aldrich). Cells were stored in the dark at 4 °C until analysis. Flow cytometric acquisition was performed on a LSR II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences) and 100,000 acquired events were captured for each staining tube. Flow cytometry data was analyzed with Flowjo software (Tree Star, Inc; Ashland, OR). Lymphocytes were gated using forward scatter and side scatter parameters. Dead cells were excluded and live cells were gated for presence of CD3 (T cells).

2.3.1. Antibodies

Antibodies were purchased from BioLegend: anti-human CD3 (clone UCHT1)-brilliant Violet 421; anti-human integrin β7 (clone FIB27); anti-human FoxP3 (clone 206D)-PE. The following antibodies were purchased from eBioscience: anti-human CD8α (clone OKT8)-Alexa Fluor 700; anti-human IL-17A (clone eBio64DEC17)-FITC; anti-human IL-13 (clone 85BRD)-FITC; anti-human CD25 (clone BC96)-Alexa Fluor 488; anti-human IL-10 (clone JES3-9D7)-PE. Anti-human IFNγ (clone B27)-PE was purchased from BD Bioscience.

2.4. Statistical analysis

One-way ANOVA were used to determine statistical significance of cell population frequencies. Multiple comparisons were corrected for using Tukey's multiple comparison test; the adjusted probability (p)-values were reported, except where noted, p-values < 0.05 were considered statistically significant. Outliers were removed in Prism using ROUT with the standard of Q = 1%. Behavioral data (ABC subscales) correlations to cell frequencies were analyzed utilizing using Spearman's correlation, p-values < 0.05 were considered statistically significant.

3. Results

PBMC were cultured with either a stimulation cocktail or a control cocktail for 4 h prior to staining. Cell surface markers and intracellular cytokines or transcription markers were assessed to identify and characterize T cell populations. We did not find any significant differences between live/dead cell populations among groups, nor did we observe any differences among the frequencies of total T cell ($CD3^+$) population, CD4 T cell population, or CD8 T cell sub-population. The frequencies of α4β7 (as assessed by $\beta7^+$) T cell also did not differ between groups.

3.1. Intracellular cytokine profiles

In children with ASD who experience GI symptoms (ASD^{GI}), there was over a 2-fold increased frequency of IL-17^+ CD4 T cells (mean: 0.757%, Standard Deviation: (0.313%)) (Fig. 1a) compared to both children with ASD^{NoGI} (0.39% (0.34%); adjusted p-value = 0.0134) or to TD children (0.297% (0.197%): $p = 0.0004$). In cells that were $\beta7^{Hi}$, elevated frequencies of IL-17^+ CD4 T cells were also observed (Fig. 1b) in ASD^{GI} (1.85% (1.42%)) compared to TD (0.70% (0.52%) $p = 0.0384$). In ASD^{GI} there were also elevated populations of IL-17.1 $CD4^+$ T cells that were double positive for both IL-17 and IFNγ (0.20% (0.12%)) (Fig. 1c) compared to both ASD^{NoGI} (0.09% (0.09%); p = 0.0162) and TD (0.09% (0.05%); $p = 0.0062$). In addition to elevated IL-17^+CD4^+ populations, we observed increased IL-17^+ CD8 T cell populations in ASD^{GI} (0.05% (0.04%)) compared to TD (0.01% (0.01%); $p = 0.0040$). ASD^{NoGI} (0.04% (0.02%)) $CD8^+$ IL-17^+ cells were similarly elevated compared to TD, however, significance was lost after correcting for multiple comparisons (adjusted p-value = 0.0632, uncorrected p-value = 0.0251) (Fig. 1d).

We observed a 2-fold increased frequency of IL-13^+ CD4 T in children with ASD^{NoGI} (2.02% (1.08%)) compared to TD (1.01% (0.58%); $p = 0.0147$) (Fig. 2a). The differences in frequency of IL-13^+ CD4 T cells were not observed for ASD^{GI} compared to TD (p = ns). Interestingly, no differences in the frequency of canonically-defined T_H1 ($CD4^+IFN\gamma^+$) were identified between groups; however, we did observe an increase in median fluorescent intensity (MFI) for IFNγ for ASD^{NoGI} (3112 (1017)) (Fig. 2b) compared to ASD^{GI} (2241 (637); $p = 0.0266$) and there was a trend in TD that did not reach significance after correction (2398 (518); adjusted $p = 0.0580$; uncorrected p-value = 0.0229).

3.2. Regulatory T cells

To determine the frequency of regulatory T cells we used CD25 (IL-2 receptor) and Forkhead P3 (FoxP3) transcription factor. These are thought to define one of the major subsets of T cells with regulatory function in humans; other cells with regulatory function do exist but were not assessed here. We found significantly decreased frequencies of $\beta7^{hi}CD25^+FoxP3^+$ CD4 T cells in both ASD^{GI} (1.93% (0.75%); $p = 0.0473$) and ASD^{NoGI} (1.85% (0.89%); $p = 0.0448$) compared to TD

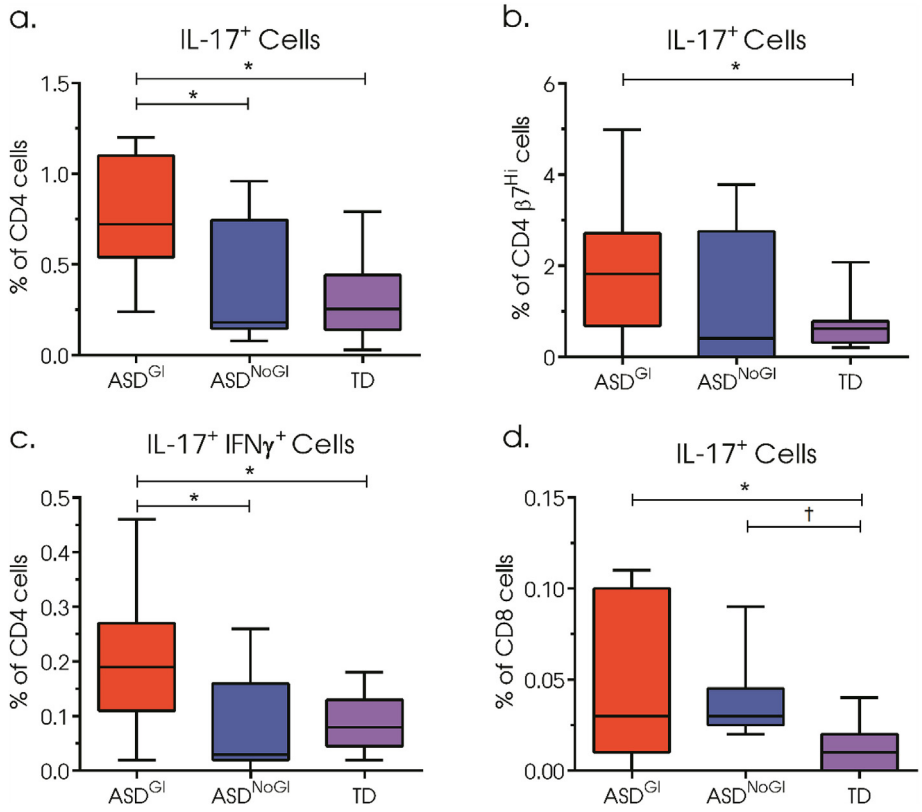

Fig. 1. Frequency of cytokine positive CD4 T cells after 4h stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin and the protein transport inhibitor, brefeldin A. (a) Frequency of IL-17 positive CD4 T cells, (b) frequency of IL-17 positive β7Hi CD4 T cells, (c) frequency of double-positive IL-17 and IFNγ CD4 T cells. (d) frequency of IL-17 positive CD8 T cells. Data depicted as box and whisker graphs. *denotes adjusted p-value < 0.05, † denotes uncorrected p-value < 0.05.

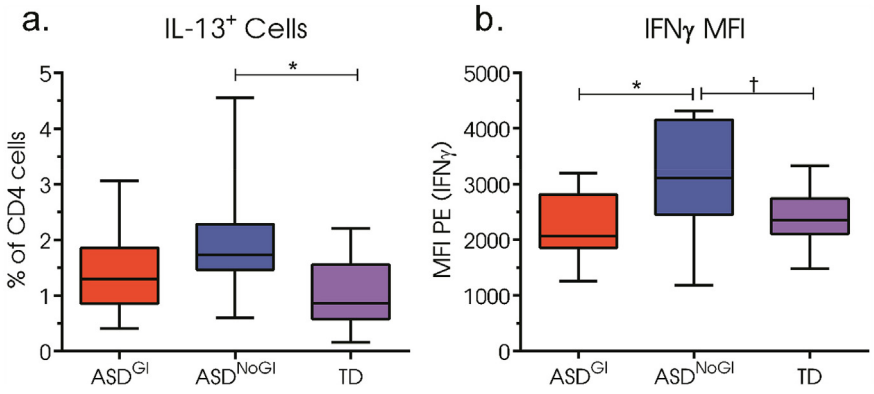

Fig. 2. T cell frequencies and median fluorescent intensities (MFI) of CD4 T cells after 4 h stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin and the protein transport inhibitor, brefeldin A. (a) IL-13 positive CD4 T cells (b) MFI of phycoerythrin (PE) conjugated to IFNγ. Data depicted as box and whisker graphs. *denotes p-value < 0.05, † denotes uncorrected p-value < 0.05.

(2.93% (1.16%)) (Fig. 3a). In addition, there were trends for decreased frequencies of IL-10$^+$ CD4 T cells in children with ASD^{GI} compared to TD, however, significance was lost after correcting for multiple comparisons (adjusted p-value = 0.0946; uncorrected p-value = 0.0386) (Fig. 3b). Considering the increased frequency of T_H17 cells we identify in ASD^{GI} we examined the ratio of T_{reg}/T_H17 (CD4$^+$CD25$^+$FoxP3$^+$)/(CD4$^+$IL-17$^+$) populations among our groups. ASD^{GI} had the lowest ratio of T_{reg} to T_H17 cells (4.28, 1.96) compared to TD (15.89, 12.63; p = 0.0230) (Fig. 3c).

3.3. Behavior correlations

Regulatory T cell populations were negatively correlated with the aberrant behavior checklist (ABC) subscale stereotypy (Spearman r = −0.336, $p = 0.0072$) suggesting the fewer the numbers of T_{regs} the worse the behavior. CD8$^+$IL17$^+$ cell populations positively correlated with increased stereotypy (0.245, $p = 0.0454$) (Table 1).

4. Discussion

In this study we provide evidence of altered immune populations in children with ASD who experience GI symptoms and in children with ASD without GI symptoms compared to typically-developing children. In the ASD^{GI} group we found elevated frequencies of T_H17 and $T_H17.1$ populations, and coupled to this were decreased populations of IL-10 producing CD4 T cells, reductions in regulatory T cells, and decreased

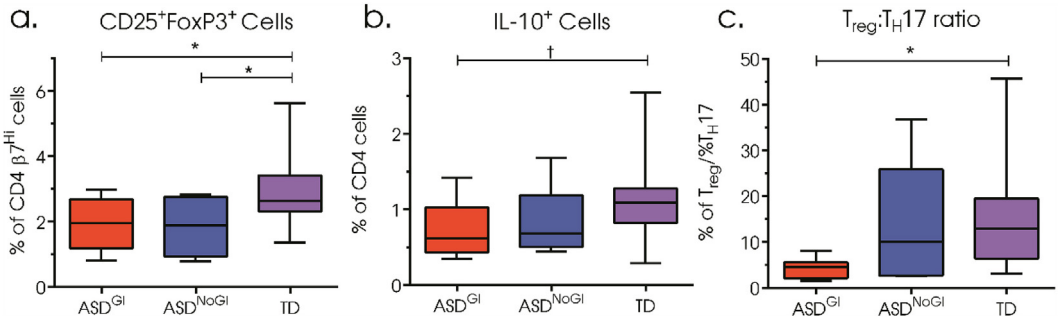

Fig. 3. Frequency of regulatory CD4 T cell populations after 4 h stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin and the protein transport inhibitor, brefeldin A. (a) Frequency of double-positive, CD25 and FoxP3, β7Hi expressing CD4 T cells, (b) frequency of IL-10 positive CD4 T cells, (c) ratio of $CD25^{+}Foxp3^{+}$ cells to $CD4^{+}IL\text{-}17^{+}$ cells. Data depicted as box and whisker graphs. *denotes p-value < 0.05, † denotes uncorrected *p*-value < 0.05.

Table 1
Aberrant behavior checklist (ABC) and cell population frequencies correlations. Spearman's rho (r) value and *p*-values are listed for ABC subscale hyperactivity and stereotypy; *p*-values <0.05 are in bold.

Aberrant behavior Checklist Correlations				
Cell Population	Hyperactivity		Stereotypy	
	Spearman r	p value	Spearman r	p value
CD4 T cell: $\beta7^{Hi}CD25^{+}$	−0.177	0.1528	−0.336	**0.0072**
CD4 T cell $\beta7^{Hi}CD25^{+}Foxp3^{+}$	−0.036	0.7657	−0.114	0.3549
CD4 $IL10^{+}$	−0.149	0.1841	−0.095	0.4037
CD4 $IL\text{-}13^{+}$	0.075	0.5112	−0.090	0.4320
CD8 $IL17^{+}$	0.187	0.1214	0.245	**0.0454**

ratios of regulatory T cells compared to inflammatory T_H17 subsets. Considering our previous findings of increased mucosa-related cytokines and decreased regulatory cytokines, mainly active TGFβ1, in children with ASD who experience GI symptoms (Rose et al., 2018), these data are suggestive of impairments in immune regulation. Alterations in the ASD^{NoGI} group included elevated populations of $IL\text{-}13^{+}$ CD4 T cells that could represent a shift to a T_H2 response. However, an increase in median fluorescent intensity for PE-IFNγ in ASD^{NoGI} children may suggest activation could be different within this group compared to children with ASD^{GI}, or that it is more heterogeneous.

The main aim of this study was to identify differences in inflammatory and regulatory T cell populations in ASD with and without GI symptoms compared to typically-developing children. Children with ASD and GI symptoms displayed the largest number of differences in cell frequencies and our major finding of increased populations of $IL\text{-}17^{+}$ T cells. T_H17 cells play a central role in combating extracellular pathogens, particularly in mucosal tissues (Kleinewietfeld and Hafler, 2013) where they orchestrate immunity to extracellular pathogens by producing cytokines that help to recruit, support, and promote neutrophils, monocytes and other lymphocytes (Kolls and Khader, 2010; Onishi and Gaffen, 2010; Peck and Mellins, 2010). The major cytokine mediators produced by T_H17 cells include its eponymic cytokine, IL-17A, as well as IL-17F, IL-21 and IL-22 (Kolls and Khader, 2010; Onishi and Gaffen, 2010; Peck and Mellins, 2010). These cytokines, IL-17A in particular, act on epithelial, mesenchymal and immune cells to produce an array of inflammatory mediators including neutrophil chemoattractants (CXCL1 CXCL2, CXCL5, CXCL8), monocyte chemoattractants and growth factors (GM-CSF, CCL1, CCL2, CCL20), antimicrobial peptides, and acute phase proteins, among others (Kolls and Khader, 2010; Onishi and Gaffen, 2010; Peck and Mellins, 2010). While these inflammatory mediators help to maintain an effective defense against pathogens, disruption in regulation can lead to excessive inflammation and the T_H17 pathway has been implicated in many autoimmune disorders including those that affect the GI such as Crohn's disease and ulcerative colitis (UC) (Onishi and Gaffen, 2010; Galvez, 2014); as such, balance between T_H17 and regulatory T cell (T_{reg}) responses are particularly crucial for maintaining equilibrium between protection against pathogens and tolerating commensal microbes (Omenetti and Pizarro, 2015).

Studies regarding IL-17 producing cells and IL-17 related cytokines in ASD have reported mixed findings. While some studies report elevated levels of IL-17 in serum/plasma (Suzuki et al., 2011; Al-Ayadhi and Mostafa, 2012), others have found no differences in circulating IL-17 or by production of stimulated PBMC (Enstrom et al., 2008; Onore et al., 2009; Jyonouchi et al., 2012). A handful of studies have begun to address frequencies of IL-17 producing cells, particularly $CD4^{+}$ helper T cells. Ahmad et al. reported elevated $CD4^{+}ROR\gamma T^{+}$, $CD4^{+}$ GATA-3^{+}, and $CD4^{+}$T-bet^{+} cells while simultaneously finding decreased $CD4^{+}FoxP3^{+}$ populations (Ahmad et al., 2017). A more recent study also reported elevated T_H17 cells along with increased production of IL-17 but decreased T_{reg} populations and related cytokine production of IL-10 and TGF-β (Moaaz et al., 2019). Another study examined frequencies of T_H1, T_H2, and T_H17 populations and while they did not find differences in total T_H17 frequencies they did report elevated frequencies of activated T_H17 cells, defined as $CD3^{+}CD4^{+}CXCR3^{-}CCR6^{+}HLA\text{-}DR^{+}CD38^{+}$ cells in ASD compared to controls (Basheer et al., 2018). One explanation for the discrepancies concerning IL-17 in ASD may involve the heterogeneity of ASD and the co-morbidities that may accompany individuals with ASD. A 2017 study examining a non-overlapping cohort of the CHARGE study reported elevated production of IL-17 by phytohemagglutinin-stimulated PBMC in ASD compared to TD. Furthermore, when ASD and TD groups where further stratified by a diagnosis of asthma, ASD with asthma produced more IL-17 than any other group, including the TD plus asthma group (Akintunde et al., 2015). This suggest that some groups may find elevated T_H17 cells and IL-17 when the study population is knowingly or unknowingly enriched with co-morbidities that involve T_H17 pathways, such as asthma, autoimmunity, or GI related symptoms, such as we report in the current study. Along these same lines we previously reported finding no differences in T_H17 populations between ASD and TD in an earlier cohort of the CHARGE study (Onore et al., 2009), which was not enriched for any particular co-morbidities but contained more children with autism that had an early onset of symptoms vs. those that undergo regression of autism.

Given the importance of balance between T_H17 and T_{reg} populations, it is noteworthy to mention that in addition to increased T_H17 populations found in children with ASD^{GI}, we also found evidence of decreased T_{reg} populations. Specifically, we found decreased frequencies of $IL\text{-}10^{+}$ CD4 T cells and $\beta7^{+}CD25^{Hi}FoxP3^{+}$ CD4 T cells, suggesting that both peripheral and gut related T_{reg} populations may be reduced in this group. Our previous study also found lower production of active TGFβ1 from PBMC from ASD^{GI} which supports the idea that immune regulation is impaired in ASD^{GI} (Rose et al., 2018). The stability of T_{reg} is complex and likely varies based on a number of factors including type of regulatory T cell. There are 2 main subsets of T_{reg}: natural T_{reg} (nT_{reg}) derived from the thymus and induced T_{reg} (iT_{reg}) that are induced in the

periphery (Lin et al., 2013). While nT_{reg} appear to be very stable, iT_{reg} may be more plastic and may possess the ability to become inflammatory under certain conditions (Kleinewietfeld and Hafler, 2013). Stability of Foxp3 is in part managed by epigenetic regulation and is essential for T_{reg} stability; in particular, demethylation of the T_{reg}-specific demethylated region (TSDR) at *FOXP3* is required for differentiation and results in FOXP3 expression and the suppressive function of T_{reg} (Fontenot et al., 2003; Floess et al., 2007; Toker et al., 2013; Feng et al., 2014). The lack of appropriate demethylation or the gain of methylation at the *FOXP3* TSDR triggers T_{reg} instability, allowing for loss of T_{reg} suppressive functions and gain of effector T cell functions, such as IL-17 production (Hoffmann et al., 2009; Wang et al., 2013). Future studies are needed to further explore the plasticity and stability of T_{reg} in ASD, and how the microbiota and other environmental influences contribute.

Plasticity of T_H17 is not only limited to T_H17/T_{reg} relationships, reports of T_H17 cells gaining a T_H1-like phenotype have also been described (Harrington et al., 2006). While some T_H17 cells that gain T_H1-like features and secrete IFNγ retain expression of IL-17, generally they lose the ability to produce IL-17 and irreversibly transdifferentiate into a T_H1-like cell (Lee et al., 2009; Omenetti and Pizarro, 2015). T_H17 cells that transdifferentiate have been termed ex-T_H17 cells (Hirota et al., 2011). Stability of T_H17 cells is dependent on environmental signals, importantly, TGFβ is required; in the absence of TGFβ and with exposure to IL-12, T_H17 cells can also express IFNγ (Kleinewietfeld and Hafler, 2013). Like T_{reg}, T_H17 stability is also linked to its epigenetic status, which is controlled by both histone and DNA methylation regulation. The T_H1 transcription factor gene promoter, *tbx21*, has a bivalent status in T_H17 cells characterized by dual-positive status of both H3K4me3 and H3K27me3 leading to T_H17 instability and the inclination of T_H17 cells to obtain a T_H1-like phenotype after exposure to IL-12 (Wei et al., 2009). Functionally, T_H1-like, ex-T_H17 cells have been characterized as highly pathogenic, produce more inflammatory cytokines, have a higher proliferative capacity than T_H17 or T_H1 cells, (Basdeo et al., 2017; Gartlan et al., 2017) and have been reported to be elevated in rodent models of autoimmune disorders. In experimental autoimmune encephalomyelitis (EAE), fate-mapping experiments showed that the majority of myelin-specific CD4 T cells in the spinal cord were ex-T_H17 cells (Hirota et al., 2011). Our finding of elevated IL-17^+IFNγ^+ CD4 T cell in children with ASD who experience GI symptoms, lends further evidence to the disruption of T_{reg}/T_H17 balance in ASD^{GI} and is a commonality shared with patients with IBD and other autoimmune disorders in which increased populations of IL-17^+IFNγ^+ CD4 T cells have also been identified (Annunziato et al., 2007; Galvez, 2014; Ueno et al., 2018), and in addition to being described as more inflammatory, they may also be more resistant to suppression by T_{regs}, at least in Crohn's disease (Annunziato et al., 2007). While we know these cells play a role in disease pathogenesis, it is currently unknown if these cells have a role in heathy individuals. One explanation for the flexibility for T_H17 cells to easily transdifferentiate into a T_H1-like phenotype is to enable a quick shift from combating extracellular pathogens to intracellular pathogens (Zhou et al., 2009).

As well as a change in the balance of immune activation in ASD^{GI} we also noted decreased regulation in ASD^{NoGI}. In this context, IL-13^+ CD4 T cells but not IL-17 cells were increased in ASD^{NoGI}. These data may suggest that both ASD^{GI} and ASD^{NoGI} have decreased regulation and increased inflammatory responses but that inflammatory signals may be different and lead to different co-morbidities. IL-4, IL-5 and IL-13 are the classical cytokines associated with T_H2 responses (Nakayama et al., 2017) and are the major cytokines involved with mediating type 2 responses. T_H2 cells are important for humoral responses, protection against extracellular parasites and are drivers of inflammation in asthma and atopy (Paul and Zhu, 2010; Nakayama et al., 2017). ASD are often accompanied with co-morbidities, including an increased proclivity towards allergies and asthma (Mostafa et al., 2008; Sacco et al., 2012; Chen et al., 2013; Kotey et al., 2014; Zerbo et al., 2015a). Epidemiology studies have found associations between maternal asthma and increased risk for developing a neurodevelopmental disorder (Croen et al., 2005; Leonard et al., 2006; May-Benson et al., 2009; Langridge et al., 2013; Lyall et al., 2014; Instanes et al., 2015; Gidaya et al., 2016; Theoharides et al., 2016), furthermore, findings of elevated cytokines associated with a T_H2 response have also been reported from mid-gestational maternal sera, amniotic fluid, and in neonatal blood spots from children who later developed ASD (Goines et al., 2011b; Abdallah et al., 2013a; Krakowiak et al., 2015). Interestingly, studies in mice lacking T cells were shown to have impairments in cognitive function (Kipnis et al., 2004; Brynskikh et al., 2008), and in particular, IL-4 producing T cells in the meninges were shown to be important in learning and memory (Derecki et al., 2010). Mice who had undergone Morris water training had an increase in IL-4 producing meningeal T cells, moreover, mice who were deficient in IL-4 (IL-$4^{-/-}$ mice) displayed learning defects that could be restored upon adoptive transfer of T cells from wildtype mice (Derecki et al., 2010). The impact of IL-4 on neurodevelopment is likely complex, dependent on concentration, stage of development and region.

4.1. Study limitations

Our study does have several limitations, mostly revolving around small sample sizes which restricted how we could stratify our study population. In the future we would like to further stratify the GI populations based on specific symptoms (constipation vs. diarrhea vs. IBS). A fourth group of typically-developing children with GI symptoms of irregular bowel movements (TD^{GI}) were also recruited but due to a statistically lower median of age for the group TD^{GI} as well as low recruitment numbers ($n = 5$) we were not able to include this group in statistical analysis. Furthermore, our population age range was wide and included children 3–12 years of age, larger sample sizes would have made it possible to compare data across age. Lastly, the smaller population size made it difficult to study gender differences as our study is primarily composed of males, which is consistent with the gender ratio of ASD diagnosis, however, resulted in too few females to perform statistical analysis. In order to analyze cytokine profiles of T cells, the cells first required stimulation, for this we choose to use a standard stimulant and timeframe to stimulate with (PMA/Ionomycin for 4 h), however, it is possible that other routes of activation may reveal variation in results or that a longer stimulation may show more dramatic results. IL-10 and IL-17, for example, take longer to reach peak cytokine production. Despite these limitations we feel that this study provides valuable insights on immune regulation in children with ASD and GI symptoms.

5. Conclusions

Considering the heterogeneity of ASD and the varying types of immune dysfunction reported in ASD, we sought to investigate differences in T cell profiles in children with ASD who experience GI symptoms compared to ASD without GI symptoms. We were able to find differences in dominant T cell lineages based on whether or not GI symptoms were present. One commonality among our ASD groups compared to the TD group was evidence of decreased immune regulation; both ASD^{GI} and ASD^{NoGI} displayed lower frequencies of $B7^+CD25^+FoxP3^+$ CD4 T cells. Moreover, this decrease in regulation was accompanied by an increase in differential inflammatory T cell lineages; for ASD^{GI}, this was elevated T_H17 populations and for ASD^{NoGI}, increased T_H2 populations. Elevated T_H17 populations have frequently been identified in autoimmune and GI disorders and may provide a target for future treatments that may help alleviate GI inflammation. The role T_H2 cells play in ASD is currently unknown and should be investigated further.

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Declaration of competing interest

There are no conflicts of interest for any of the authors.

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