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PRINCIPAL INVESTIGATOR: Paul Ashwood

CONTRACTING ORGANIZATION: University of California, Davis, CA

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Paul Ashwood

E-Mail:pashwood@ucdavis.edu

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University of California Davis, One Shields Avenue, Davis, CA 95616

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14. ABSTRACT

Nearly half of all children with autism spectrum disorders (ASD) also have co morbid gastrointestinal (GI) symptoms that can affect behavior through a brain-immune gut feedback loop. The primary GI condition is due to uncontrolled inflammation. The main objectives of this project are (1) to characterize defects in Tregs function and ability to suppress responses; (2) to identify epigenetic mechanisms that control Tregs lineage commitment; and (3) to determine the stability of Tregs under inflammatory conditions and the Tregs- T helper (TH)17 balance, in children with ASD and GI symptoms. Our main hypothesis is that Tregs dysfunction causes a lack of immune control in children with ASD who experience GI co-morbidities.

15. SUBJECT TERMS
None listed.

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Introduction

The most recent Interagency Autism Coordinating Committee concluded that over half of all children with autism spectrum disorders (ASD) experience gastrointestinal (GI) dysfunction, yet few receive treatment for this. GI problems occur 6-8 times more frequently in ASD than in typically developing (TD) children but the mechanisms underlying GI dysfunction in ASD remain unknown. Regulatory T cells (Tregs) are key mediators of immune tolerance that prevent inappropriate GI inflammation in response to bacteria and other luminal antigens/ components. Our main hypothesis is that Tregs dysfunction causes a lack of immune control in children with ASD who experience GI co-morbidities. The main objectives of this proposal are to characterize defects in Tregs function and ability to suppress responses; to identify epigenetic mechanisms that control Tregs lineage commitment; to determine the stability of Tregs under inflammatory conditions and the Tregs- T helper (Th)17 balance.

Specific Aims/Study Design: Specific Aim 1: To test the hypothesis that mechanisms of action of Tregs are defective in children with ASD who exhibit GI symptoms. Tregs will be isolated from the blood and intestinal mucosal tissue of ASD and TD children with GI symptoms. Tissue samples will be collected during clinically indicated colonoscopy. These Tregs will be analyzed for their ability to suppress immune responses in cocultures, to produce immunosuppressive cytokines, and express intracellular and cell surface molecules associated with regulatory function.

Specific Aim 2: To test the hypothesis that mechanisms determining commitment and/or stability of Tregs in children with ASD who exhibit GI symptoms are altered. Tregs lineage commitment requires the transcription factor forkhead box P3 (FoxP3) and demethylation of the Tregs-specific demethylated region (TSDR). To assess the commitment and stability of Tregs, DNA methylation will be characterized at the TSDR. Tregs lineage instability can cause Tregs plasticity leading to impaired immune tolerance and the development of TH17 cells that produce interleukin (IL)-17, a cytokine recently shown to alter neurodevelopment and lead to ASD-relevant behaviors. The plasticity of Tregs to convert to TH17 cell lineages under inflammatory conditions will be assessed.

Keywords

Autism spectrum disorders (ASD), Gastrointestinal (GI), co morbidities, inflammation, immunology, T cells, Lymphocytes, regulatory T cells (Tregs), regulation, immune control.

Accomplishments

Autism spectrum disorders (ASD) are behaviorally defined and affects 1 in 54 children in the United States; however, little is known about its etiology and pathophysiology. GI problems occur 6-8 times more frequently in ASD than in typically developing (TD) children and are associated with more exacerbated behavioral impairments. Although very little is known about the pathophysiological pathways underlying GI problems in ASD. Increased immune activation, pro- inflammatory cytokine production and autoantibodies directed to gut epithelium have been reported in children with ASD and GI symptoms. Regulatory T cells (T_{regs}) are key mediators of peripheral tolerance that maintain their lineage commitment and function through epigenetic regulation. These T_{regs} are key players that help prevent inappropriate mucosal inflammation in response to bacteria and other luminal antigens/components.

Despite serious limitations to recruitment and laboratory procedures due to the COVID-19 crises we have been able to make progress in several areas. We performed flow cytometry analysis, to examine T_{regs} frequencies and intracellular production of cytokines in T cells. For characterization, isolated T cells were treated with the protein transport inhibitor Brefeldin A for 4 hours in media alone or with the addition of phorbol 12-myristate 13-acetate (PMA) to induce T cell cytokine production. A live/dead stain was used to exclude dead cells. We stained for the T_{regs} markers, CD25 and FoxP3, the gut homing integrin $\beta7$, as well as intracellular cytokines IL-10, an immunosuppressive cytokine, and IL-17, a proinflammatory, T_H17-associated cytokine. We found children with ASD and GI symptoms (ASD^{GI}) have decreased regulatory T cell populations. Decreased frequencies of $\beta7^{+}CD25^{+}FoxP3^{+}$ $CD4^{+}$ T cells and $IL\text{-}10^{+}CD4^{+}$ T cells were observed in children with ASD and GI symptoms compared to ASD with no GI issues (ASD^{NoGI}) and TD ($p < 0.05$). Taken together, these data are suggestive of immune regulation dysfunction in children with ASD with GI problems both systemically as well

as in the mucosal compartments. We also found increased populations of $CD4^+IL\text{-}17^+$ and $CD4^+IL17^+IFN\gamma^+$ T cells in ASD^{GI} compared to ASD^{NoGI} and TD ($p < 0.05$). IL-17 is the signature cytokine of the T_H17 lineage of T cells. In addition to IL-17, T cells that differentiate into the T_H17 lineage also produce IL-22 and are important for defense against mucosal pathogens but have also been implicated in autoimmune disorders and inflammatory bowel disease illustrating the need for balance and regulation. Taken together, these data suggest an immune imbalance, most likely centered on lack of immune regulation. Moreover, our finding of increased T_H17 T cells in the presence of reduced immune regulation may highlight the delicate balance of immune regulation and the plasticity of regulatory T cells.

We further characterized T_{regs} in ASD by analyzing several novel functional markers. Our preliminary data shows children with ASD had fewer gut homing ($\beta7^+$) T_{regs} that expressed the regulatory marker T cell immunoreceptor with Ig and ITIM domains (TIGIT) compared to typically developing children **(Figure 1a).** TIGIT is a relatively newly identified coinhibitory receptor that is thought to identify T_{regs} that specifically control T_H1 and T_H17 immune responses. TIGIT binds to CD155 on dendritic cells resulting in the suppression of inflammatory IL-12p40 and increases the secretion of IL-10. In ASD, IL-12p40 levels have been shown to be increased, a finding that suggests a loss of negative signals through TIGIT. There is also evidence that TIGIT expression on T_{regs} is associated with lineage stability and suppressive capacity and supports our hypothesis that T_{regs} in ASD may have decreased stability of FoxP3 expression. CD39 is another marker of suppressive capability on T_{regs}. CD39 is part of an enzymatic cascade that breaks ATP down into adenosine. Extracellular ATP is normally found in low concentrations under homeostatic conditions, however, ATP can flood in from cellular sources when tissue is under stress, such as from inflammation, and is required for neutrophil activation. Adenosine, on the other hand is a strong immune suppressor that can bind to receptors on lymphocytes. By reducing ATP/ADP to AMP and finally adenosine, CD39 plays an important role in regulating the immune response. Studies have also found that $CD39^{Hi}$ T_{regs} demonstrate lineage stability while under inflammatory conditions. Although, we do not yet have significance differences between groups for CD39 expression on all T cells, gut homing T_{regs} that express both TIGIT and CD39 and, therefore, should represent highly stable and suppressive T_{regs} are decreased in ASD^{GI} and ASD^{NoGI} groups compared to TD **(Figure 1b)**.

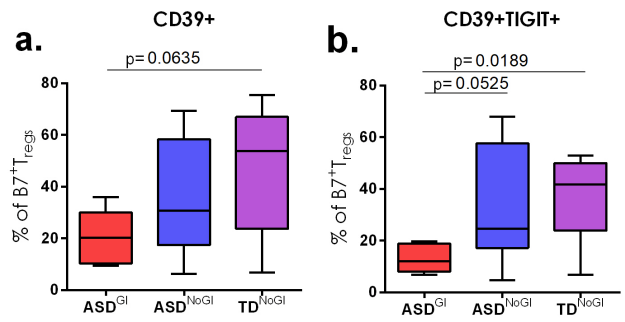

Figure 1. Decreased gut homing T_{reg} $TIGIT^+$ populations in ASD groups with and without GI issues compared to typically developing (TD) children. (a) Frequency of $TIGIT^+$, $\beta7^{Hi}$ expressing T_{regs}, (b) frequency of $TIGIT^+CD39^+$, $\beta7^{Hi}$ expressing T_{regs}. Data depicted as box and whisker graphs. T_{regs} defined as $CD4^+CD25^+CD127^{lo}FoxP3^+$

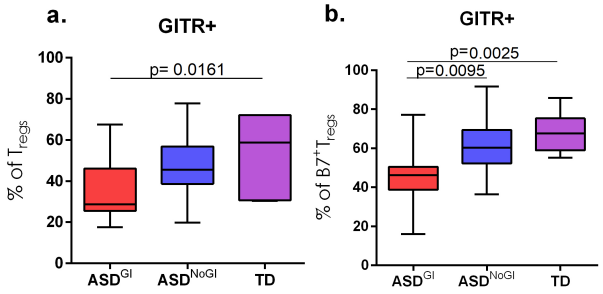

Figure 2. Decreased frequency of $GITR^+$ $CD4^+CD25^+CD127^{lo}FoxP3^+$ (T_{regs}) and $B7^+$ T_{regs} T cell populations. (a) Frequency of $GITR^+$ T_{regs}, (b) frequency of $GITR^+$, $\beta7^+$ expressing T_{regs}. Data depicted as box and whisker graphs.

In addition to evaluating freshly isolated T cells at the "rested" state, we also stimulated T cells with CD2/CD3/CD28 'activation' beads for 24 hrs followed by staining for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor receptor (GITR). While we did not see any striking differences between the groups for CTLA-4, we do see reduced populations of both GITR positive T_{regs} and gut homing ($\beta7^+$) T_{regs} in the ASD^{GI} group **(Figure 2)**. GITR binds to the GITR ligand present on antigen presenting cells and results in proliferation and increased IL-10 production.

Furthermore, for all participants recruited so far we have banked isolated T_{regs} populations for RNAseq and epigenetic characterization for Aim#2.

What opportunities for training and professional development has the project provided?

"Nothing to Report"

How were the results disseminated to communities of interest?

“Nothing to Report”

What do you plan to do during the next reporting period to accomplish the goals?

To continue to recruit participants which are slowly starting to improve as the COVID-19 crisis is more stable. We intend to continue recruitment and basic characterization of T_{regs} cells as outlined in the Aims. We plan to finalize the mechanisms that T_{regs} suppression may be altered in ASD by utilizing the suppression assays and to continue to perform analyses of T_{regs}.

Impact

We seek to fill an important knowledge gap regarding how immune regulation influences GI co-morbidities in ASD. Although we don’t yet know whether children with ASD who experience GI symptoms represent a unique sub-group of ASD, GI symptoms could be used for patient stratification and allow us to fast forward targeted therapeutic interventions. We will identify novel immune profiles that could aid in diagnosis of mechanisms underlying GI pathology, develop cell specific targeted therapies, and monitor and predict outcomes of those treatments. Children with ASD with co-occurring GI symptoms are more likely to experience sleep disturbance, anxiety, sudden irritability, unexplained crying, aggressive behavior and score far worse on behavioral assessments than children with ASD without GI symptoms. Our results will improve our understanding of how immune regulation overlaps with patterns of GI and behavioral symptoms. We will be able to follow up the results and determine critical upstream and downstream parameters, such as the effects of the GI microbiota, metabolites, or dietary intervention that will expand our understanding of GI dysfunction in ASD.

What was the impact on the development of the principal discipline(s) of the project?

“Nothing to report”

What was the impact on other disciplines?

“Nothing to report”

What was the impact on technology transfer?

“Nothing to Report”

What was the impact on society beyond science and technology?

“Nothing to report”

Changes/Problems

Our major and unexpected problem has been the COVID-19 pandemic which has hindered research on several fronts, including but not limited to: 1) limiting access to the laboratory and facilities during lockdown, 2) limited personnel allowed at any one time in the laboratory, 3) closure of core facilities, 4) institutional shut down of research laboratories, 5) limiting clinical hours for assessment and recruitment of human subjects.

Changes in approach and reasons for change

“Nothing to Report”

Actual or anticipated problems or delays and actions or plans to resolve them

We experienced delay due to COVID-19 crisis. As stated above these were in recruitment and access to facilities.

Changes that had a significant impact on expenditures

All personnel are in place for the completion of this project and we do not anticipate any problems with recruiting enough subjects. No changes have occurred that have significantly affected the expenditures.

However, we did defray some personnel costs during the crisis. Costs will be charged in the next reporting period from existing funds.

Significant changes in use or care of human subjects

“Nothing to Report”

Significant changes in use or care of vertebrate animals

“Nothing to Report”

Significant changes in use of biohazards and/or select agents

“Nothing to Report”

Products

“Nothing to Report”

Participants & Other Collaborating Organizations

Name: Paul Ashwood (principal investigator) “No change”

Name Janine LaSalle “No Change”

Name: Daniel Tancredi “No change”
Name: Arthur de Lorimier “No Change”

Post doctoral scholar “No Change”

Has there been a change in the active other support of the PD/PI or senior key personnel since the last reporting period?

“Nothing to Report”

What other organizations were involved as partners?

“Nothing to Report”

Special Reporting requirements

“Nothing to Report”

Appendices

“Nothing to Report”