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TITLE: Macrophage Polarization and Utility of in Vivo Therapy with a Brain-Permeable Anti-TNF Agent in Models of Autism

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14. ABSTRACT
Human studies and animal models suggest that maternal immune activation (MIA) can have lasting effects on the offspring's neurodevelopment, immune set points, and behavior. The overall goal of this proposal is to define relevant signaling pathways and identify molecular targets and developmental windows for intervention and treatment.
During this period, we ordered and expanded the TNFR1R2 DKO mice from Jackson labs on site. The mice are breeding and we plan to colonize them with stool of the Taconic C57Bl/6N mice so that they will be responsive to the viral mimetic we plan to use. We began work on Aim 4 because it did not involve use of transgenic mouse colonies and pharmacological studies are underway to interrogate the role of soluble TNF in the MIA model of ASD.

15. SUBJECT TERMS
MIA, MIF, TNF, Gut, Neurodevopment, neuroinflammation, macrophage, IL-17, fetal insult, ASD

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1. INTRODUCTION:

Human studies and animal models suggest that maternal immune activation (MIA) can have lasting effects on the offspring's neurodevelopment, immune set points, and behavior. The overall goal of this proposal is to define relevant signaling pathways and identify molecular targets and developmental windows for intervention and treatment.

2. KEYWORDS:

MIA, MIF, TNF, Gut, Neurodevelopment, neuroinflammation, macrophage, IL-17, fetal insult, ASD

3. ACCOMPLISHMENTS

What were the major goals of the project?

Perform MIA model in mice deficient in TNF or MIF signaling

Assess role of TNF and MIF signaling in neuroinflammation and behavioral dysfunction in the MIA model of ASD

Assess role of TNF and MIF signaling in modifying expression of immune- and ASD-related genes in the MIA model of ASD

Investigate the therapeutic efficacy of XPro1595 to attenuate neuroinflammation and ASD-like features in the MIA model of ASD.

What was accomplished under these goals

SPECIFIC AIM 1

Specific Objectives: *This aim has the goal to use mice deficient in TNF receptors (TNFR1/2-/-) to interrogate the relationship between MIF, TNF, and the inflammatory M1 macrophage phenotype in pups whose mothers were subjected to MI*A.

Major Activities: Despite having expanded a breeding colony of TNFR1/2 -/- and +/+ mice on a C57BL/6J genetic background from Jackson Labs that have been used by our group in previous published studies, these mice were suspected not to have the SFB gut microbiome reported by Dan Littman's group required to develop the MIA phenotype compared to C57Bl/6NTac mice from Taconic.

Protocol steps:

1. Co-housing was undertaken: Because the mice deficient in TNF receptors (TNFR1/2-/-) on a C57BL/6J background were obtained from JAX, we decided to co-house our female C57BL/6J with the C57BL/6NTac (Taconic) female mice for 4 weeks in order to increase the likelihood of maternal gut bacteria to promote neurodevelopmental abnormalities in the offspring of the MIA model [1]This procedure would allow the mouse intestine to be colonized with segmented filamentous bacterium or SFB (present in the Taconic mice) required for induction of Th17 cells in the intestinal lamina propria which produce IL-17 and IL-22 which then act on the brain. In pregnant mice, interleukin-17a produced by T helper 17 (TH17) cells induces behavioral and cortical abnormalities in the offspring exposed to MIA [1].

2. Breeding

Following this co-housing period, the female with C57BL/6J background were matched with age related males for breeding. Mice were mated overnight and the presence of a vaginal plug was designated as E0.5.

3. MIA
Each pregnant dam was weighed and administered 20 mg/kg PolyI:C potassium salt (Millipore Sigma) or saline by i.p. injections on both E11.5 and E12.5 [2]

Significant Results and Key Outcomes/Accomplishments:
A large number of pregnant dams injected with saline or Poly:IC had either resorbed their litters in utero or cannibalize them once born. This way we could not reach a reasonable N to perform the studies proposed in aim for receptors (TNFR1/2-/-) mice.

The listed measures below were taken to control stressful conditions in an attempt to improve breeding performance; however we could not reach a sufficiently high N to complete this aim 1 despite the following:

a. Avoiding frequently opening the cage,
b. Avoiding over-handling,
c. Use of enrichment in the cages (nestlets and igloos),
d. Having the same caretaker condcut all animal procedures, always working gently, slowly, and quietly when handling the mice.

SPECIFIC AIM 4
Major activities: Due our difficulties in developing the MIA model in TNFR-deficient mice on a C57BL/6J background in our facilities (that could be associated to environmental factors), we decided to move forward with C57BL/6NTac (Taconic) mice in order to secure the impairment associated to maternal immune activation for treating this condition with the selective soluble TNF biologic XPro®1595.

Specific Objectives: The main objective of the **aim 4** is to test the ability of XPro®1595 to interrupt pro-neuroinflammation and gut dysfunction in mouse models of ASD during the early postnatal period, and ameliorate neurological and behavioral deficits in the offspring.

Experimental design
Pregnant C57BL/6N mice were injected i.p. on E11.5 and E12.5 with saline or 20 mg/kg of poly(I:C) [2](Fig. 1). Maternal immune activation and control offspring (males and females) were tested at 6 weeks of age for open field exploration and repetitive behavior (marble burying). Plasma, hippocampus, and cerebellum were assessed for metabolic and immune alterations by qPCR and multiplexed immunoassays. Data were analyzed by one-way ANOVA and Tukey's post hoc comparisons or unpaired t-test where appropriate. $P < 0.05$ was considered statistically significant.

Significant Results and Key Outcomes

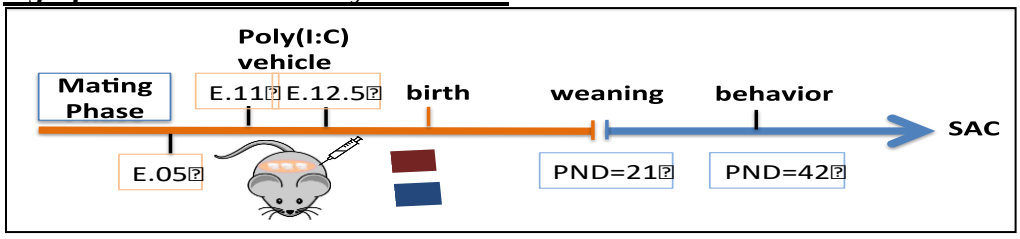

Fig. 1. Experimental design of pilot using C57BL/6NTac mice

Results

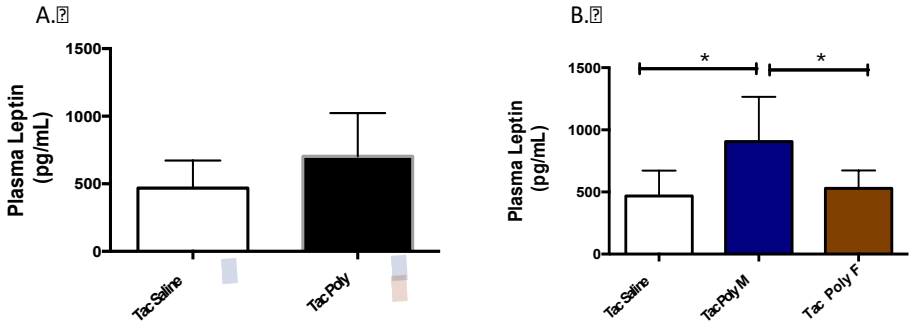

Fig. 2. Maternal immune activation with Poly(I:C) injections increases plasma leptin levels in male offspring mice. Data was collected from *n* = 9-10 mice/group and plotted as mean ± SEM for each group. *P*-values were indicated for unpaired t-test (A) and one-way ANOVA with Tukey's post-hoc test (B) (*$p < 0.05$).

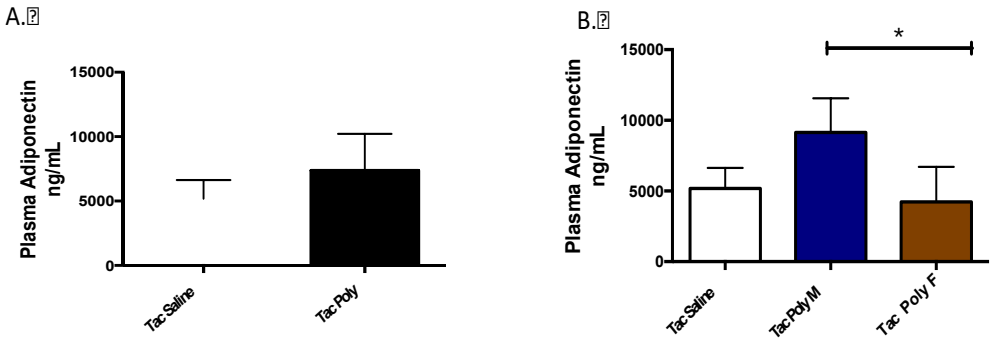

Fig 3. Maternal immune activation promotes increase in adiponectin plasma levels in male offspring mice. Data was collected from n=9-10 mice/group and plotted as mean SEM for each group. p-values were indicated for unpaired t-test (A) and one-way ANOVA with Tukey's post-hoc test (B) (*p < 0.05).

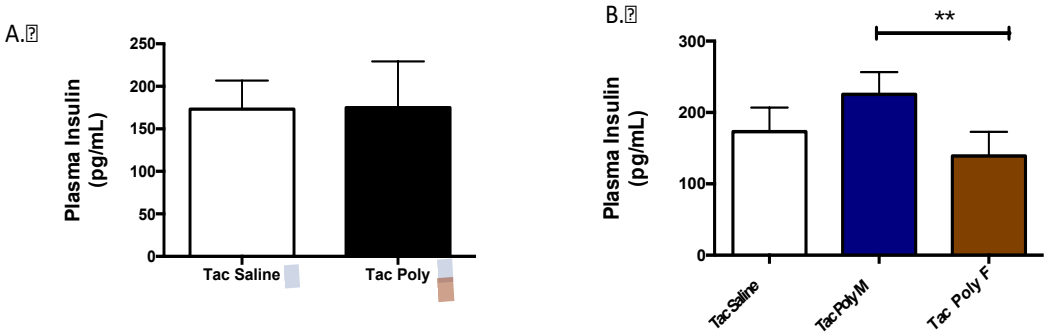

Fig 4. Maternal immune activation increases insulin plasma levels in male offspring mice. Data was collected from *n = 9-10* mice/group and plotted as mean ± SEM for each group. *P*-values were indicated for unpaired t-test (A) and one-way ANOVA with Tukey's post-hoc test (B) (**$p < 0.005$)

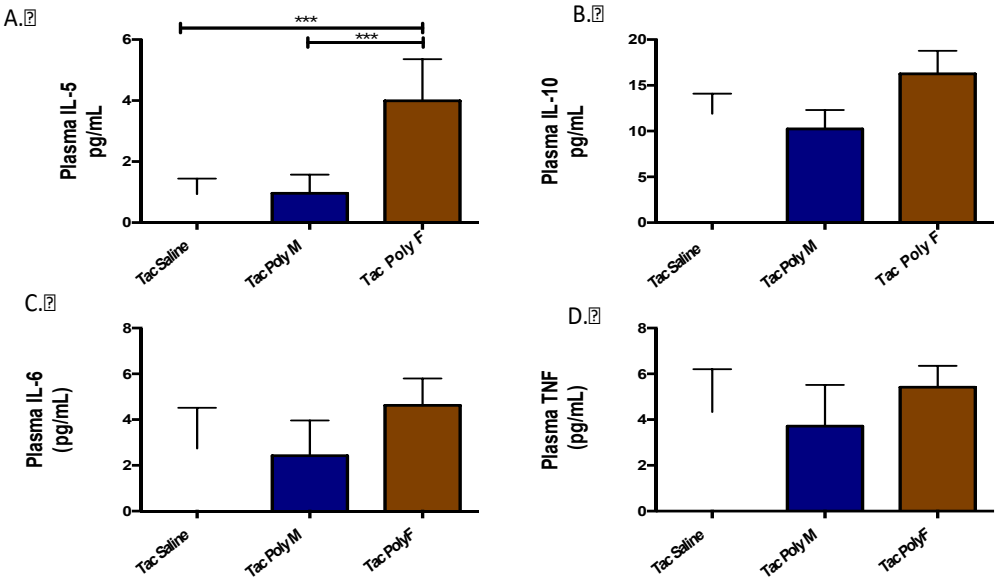

Fig. 5. Cytokines plasma levels in offspring of pregnant mice injected with Poly (I:C) or vehicle. Data was collected from *n* = 9-10 mice/group and plotted as mean ±SEM for each group. *P*-values were indicated for one-way analysis of variance with Turkey's post-hoc test (A-B) (***p < 0.001).

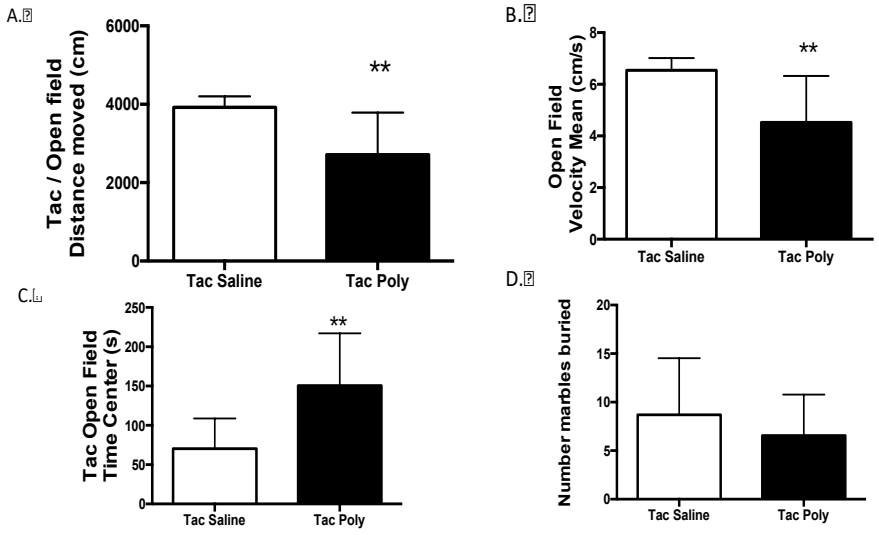

Fig 6. Maternal immune activation decreases distance moved (A), velocity (B) and time in center (C) in the open field test. There was no statistical difference in the marble burying test between the experimental groups. Data was collected from *n* = 9-10 mice/group and plotted as mean ±SEM for each group. p-values were indicated for unpaired t-test (**p < 0.005). Animal behavior activity was assessed using EthoVision XT Software.

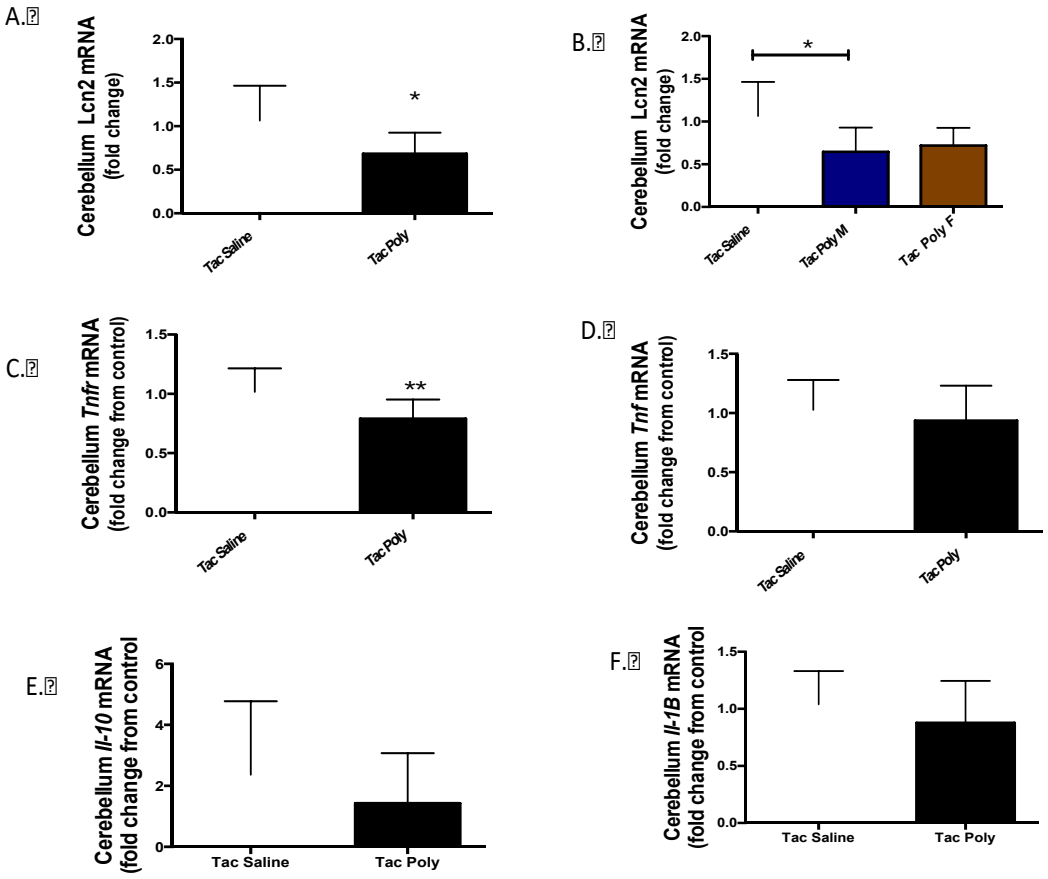

Fig 7 . mRNA gene expression of inflammatory markers in the cerebellum demonstrating decreased *Lcn2* and *Tnfr* expression in MIA offspring. No differences in mRNA expression were observed in the hippocampus between groups (data not shown). For each animal, the Ct values were normalized to the Ct values for *Gapdh* and *Ppia*. The relative expression level of the target gene (fold change) was expressed as $2^{-\Delta\Delta Ct}$, when compared with the mean DCt (threshold cycle) of the control group, $*p < 0.01$, $**p = 0.005$.

Summary of Results/Key Outcomes:

Our results indicate that maternal immune activation in C57BL/6NTac mice can promote long lasting behavioral, metabolic and cerebellar immune alterations in offspring as a result of maternal immune dysregulation during gestation. These results are relevant because recent studies associate metabolic impairment (Alterations in adipokines such as leptin, and insulin) with autism spectrum disorder [3-6]. Additionally, the cerebellar changes observed in the offspring of females submitted to MIA (alterations in TNF receptor and lipocalin-2 mRNA expression/ a specific downstream marker of TNF signaling) re-enforce our hypothesis that solTNF is an important player in in ASD and that it's a suitable target for treating central MIA alterations.

ACCOMPLISHMENTS UNDER AIM 4:

Specific Objectives: Establish the MIA model in C57BL/6Nt mice from Taconic. Treat young offspring (starting at PND 4[1], n= 20/group divided equally by sex) with XPro1595 at 10 mg/kg

s.c. (or saline) for 6 weeks to determine the extent to which soluble TNF inhibition can ameliorate neuroinflammation, neuronal survival, and behavioral deficits.[20/group x 1 genotypes (C57BL/6NTac[2]) x 2 treatment conditions (Poly:IC, saline) x 2 drug treatments (XPro1595, saline) = 80 mice for this aim].

Changes made in the experimental design of this aim:

[1] Use the C57BL/6NTac for MIA protocol

Rationale: In addition to our difficulties involved in performing MIA using C57BL/6J mice in our animal facility, our studies under Aim 1 informed us that C57BL/6NTac offspring from MIA displayed central, immune, metabolic and behavioral alterations consistent with ASD features.

[2] XPro 1595 or Saline injections were administered at PND=4 to decrease the likelihood of mother's cannibalism of pups.

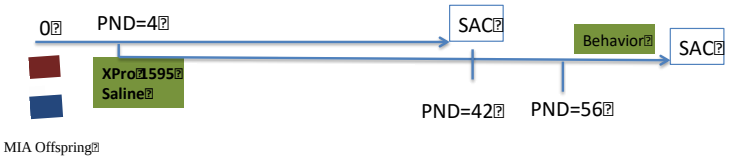

Fig. 1. Experimental design of aim 4 using C57BL/6NTac mice

N of mice sacrificed so far by groups for tissue collection or behavior assessment*:

	PND 42 Male	PND 42 Female	After Behavior Male	After Behavior Female
Mother Saline Offspring Saline	2	1	-	1
Mother Saline Offspring XPro	3	2	-	2
Mother Poly:IC Offspring Saline	8	1	2	2
Mother Poly:IC Offspring XPro	1	5	-	2

* From each litter, offspring are randomly assigned for tissue collection or behavioral tests, this way having representative animals for each litters will minimize cohort effects

Significant Results and Key Outcomes

Measurements of immune and Inflammation-related gene and protein expression, including cytokines in plasma, ileum, duodenal, and colonic tissue homogenates; QPCR for TNF, MIF, CD78, LCN2, CD74, IL-6, MMP9, TNFRSF1B, PTEN, MET, GRIN2B, MECP2, CTNNB1, CUL3, CHD8, TBR1, and SCN2A; neurohistology to measure Reelin, GAD 67, neurogenesis (doublecortin 2), GFAP, and standard apoptotic markers for this aim are ongoing. We are currently breeding 10 more mice to reach the desirable N of offspring/group for performing theses assays.

Behavioral tests were performed according with the number of offspring from each litter and will continue until the desirable N has been reached for each experimental group.

Statistical Analysis: We will place each of the ASD-related genes and inflammatory biomarkers individually into ANOVA models, with mouse type and Poly I:C or saline to examine the relationship to dependent variables (biomarker level).

Acknowledgments
We thank Xencor and INmune Bio for providing XPro1595 for these studies.

References

1. Kim, S., *et al.* Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. *Nature* **549**, 528-532 (2017).
2. Lammert, C.R., *et al.* Cutting Edge: Critical Roles for Microbiota-Mediated Regulation of the Immune System in a Prenatal Immune Activation Model of Autism. *J Immunol* **201**, 845-850 (2018).
3. Steinmetz, A.B., Stern, S.A., Kohtz, A.S., Descalzi, G. & Alberini, C.M. Insulin-Like Growth Factor II Targets the mTOR Pathway to Reverse Autism-Like Phenotypes in Mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **38**, 1015-1029 (2018).
4. Lo, F.S. & Erzurumlu, R.S. Insulin receptor sensitization restores neocortical excitation/inhibition balance in a mouse model of autism. *Molecular autism* **9**, 13 (2018).
5. Kirsten, T.B., Casarin, R.C., Bernardi, M.M. & Felicio, L.F. Pioglitazone abolishes autistic-like behaviors via the IL-6 pathway. *PloS one* **13**, e0197060 (2018).
6. Rodrigues, D.H., *et al.* Changes in adipokine levels in autism spectrum disorders. *Neuropsychobiology* **69**, 6-10 (2014).

What opportunities for training and professional development has the project provided? .
Maria Elizabeth Rodrigues received training opportunities through this award and will be able to submit a future application to the NIH for funding. She learned the MIA model, biochemical/molecular and cellular analysis of metabolism and inflammation and new behavioral tests.

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?
Nothing to report/Not Applicable

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
The impact of our project is several fold. First, we confirmed that the MIA model in C57BL/6NTac mice can promote long lasting behavioral alterations in offspring as a result of maternal immune dysregulation during gestation as published by other groups. Second, we found that MIA induces metabolic and cerebellar immune dysregulation in the offspring and these results are relevant because recent studies associate metabolic impairment such as alterations in adipokines such as

leptin, and insulin with autism spectrum disorder 3-6. Additionally, the cerebellar changes observed in the offspring of females submitted to MIA (alterations in TNF receptor and lipocalin-2 mRNA expression/ a specific downstream marker of TNF signaling) re-enforce our hypothesis that solTNF is an important player in in ASD and that it's a suitable target for treating central MIA alterations. Completion of the remaining analyses will either support or refute this hypothesis and will lay the foundation for future grant applications.

What was the impact on other disciplines?
The findings of this project may be of relevance to other neurological conditions where there is immune activation that leads to disturbances in brain function and behavior.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
As described and explained under Aim 1, despite co-housing the JAX C57BL/6J mice with C57BL/6NTac to colonize them with the correct gut bacteria (SFB) required to elicit a Th17 cell response in the gut and trigger production of IL-17 to establish the MIA model (in preparation to use the TNFR-deficient mice from JAX labs in the MIA model in Aim 1), it did not work. We therefore focused our efforts on interrogating TNF signaling by using the soluble TNF-specific biologic inhibitor XPro1595 and final analyses of experimental results are forthcoming.

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects
Nothing to report/Not Applicable

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/Not Applicable

6. PRODUCTS

- **Publications, conference papers, and presentations**

Journal publications
Nothing to report

Books or other non-periodical, one-time publications.
Nothing to report

Other publications, conference papers and presentations.
Nothing to report

- **Website(s) or other Internet site(s)**
  Nothing to report

- **Technologies or techniques**
  Nothing to report

- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	**MariadeLourdes Tansey, PhD**
Project Role:	**Principal Investigator**
Researcher Identifier (eRA User ID)	**MTANSE**
Nearest person month worked:	**1**
Contribution to Project	**Dr. Tansey performed oversight of personnel, study design, wrote the report.**
Funding Support:	**N/A**
Name:	**Maria Elizabeth de Sousa Rodrigues, PhD**
Project Role:	**Post Doc**
Researcher Identifier (eRA User ID)	**IZABETEL**
Nearest person month worked:	**3**
Contribution to Project	**Dr. de Sousa Rodrigues participated in studies in Aim 4.**
Funding Support:	**N/A**
Name:	Yuan Yang
Project Role:	Research Specialist, Sr.
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	**1**
Contribution to Project	**Yuan Yang participated in studies in Aim 4.**
Funding Support:	**N/A**

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

TANSEY, MALÚ G.

PREVIOUS (funding complete since last reporting period)

Hospital District of Helsinki and Uusimaa (Tansey) **0.12 CM**
Research Agreement 02/01/2017- 01/31/2018 $22,000 Direct Costs
Microbiota and the host immune system in Parkinson's Disease
Goal: Specific Aims
1. Identifying connections between the immune response of the host and microbiota community structure in Parkinson's Disease (PD)
2. Looking for different patterns of the immune response to microbiota in healthy controls and PD patients.
3. Studying interactions between microbiota, immune response and host genotype as well as metabolomics profile. Genotype and metabolomics data will be supplied by other labs.

NEW (since last reporting period)

Accelerating Drug Discovery for Frontotemporal Degeneration (Kukar, Tansey) **0.36 CM**
Alzheimer's Drug Discovery Foundation 05/01/2018 -04/31/2020 **$**75,000 Year 1 Directs
Rescue of lysosomal dysfunction, neuroinflammation, and neurodegeneration by lysosomal GRN-2 in a mouse model of PGRN deficiency and Frontotemporal Dementia.
The overall objective of this proposal is to investigate the role of progranulin replacement in a mouse model of FTD.
Role: Co-PI

1R25GM125598-01 (Corbett/Tansey/Morgan) 06/01/2018-05/31/2023 **0.60 CM**
NIH/NIGMS $288,514 Year 1 Direct Costs
Emory Initiative to maximize Student Development
This new Initiative for Maximizing Student Diversity (IMSD) proposal will continue an established IMSD Program effort to increase the number of those individuals from groups that are under-represented in STEM fields that continue on in research and research-related careers.
Role: MPI Contact

MJFF for Parkinson's Research (PI: Tansey, MG/Dzamko, N) 11/15/2018-11/14/2019 **1.2 CM**
LRRK2 Research Grant 2018 Tansey $139,077 Direct Costs
Assessing LRRK2, GCase and cytokines in cryopreserved monocytes"
The overall goal is to provide a protocol for isolating and cryopreserving blood cells in order to standardize clinical collection of samples for assessing the relationship between LRRK2 and GCase in PD patient monocytes. Our results will be helpful for informing clinical trial design of LRRK2 inhibitors and/or GCase activators.
Role: Co-PI

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
Dr. Bradley Pearce (partnering PI) will be submitting his Final Technical Report separately.
AR150035P1, Award W81XWH-16-1-0721
"Macrophage Polarization and Utility of in Vivo Therapy with a Brain-Permeable Anti-TNF Agent in Models of Autism"

QUAD CHARTS
Not Applicable

9. APPENDICES:
Not Applicable