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14. ABSTRACT

Inflammatory bowel disease (IBD) is a chronic condition of the gastrointestinal tract that predisposes individuals to develop CRC. Chronic inflammation is one of the key hallmarks of cancer and dysbiosis of the gut microbiome is proposed to promote CRC. The prevalence of IBD has increased 2- to 3-fold among veterans. The objective of this proposal is to utilize Il10-/- mice, a model of human IBD, together with Stat2-/- mice, which are more resistant to CRC to: 1) identify unique microbial communities in the gut and 2) metabolites of bacterial and host origin that mediate anti-inflammatory and antitumor effects to control inflammation and drastically reduce the risk of CRC development.

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None listed.

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1. INTRODUCTION:

Inflammatory bowel disease (IBD) is a chronic condition of the gastrointestinal tract that predisposes individuals to develop CRC. Chronic inflammation is one of the key hallmarks of cancer and dysbiosis of the gut microbiome is proposed to promote CRC. The prevalence of IBD has increased 2- to 3-fold among veterans. The objective of this proposal is to utilize Il10-/- mice, a model of human IBD, together with Stat2-/- mice, which are more resistant to CRC to: 1) identify unique microbial communities in the gut and 2) metabolites of bacterial and host origin that mediate anti-inflammatory and antitumor effects to control inflammation and drastically reduce the risk of CRC development.

2. KEYWORDS:

Colorectal, cancer, microbiota, dysbiosis, colitis, chronic, IBD, STAT2 and IL10

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The primary and shared objective for Specific Aims 1 and 2 and Major Task1 (Subtask 1), was to establish a breeding colony of specific-pathogen free (SPF) mice of the following genotypes: wild type, *Il10 KO, Stat2 KO* and *Il10;Stat2* double knockout (*dKO*). This milestone was partly achieved in the first quarter of year 2, wherein *Stat2 KO* mice were successfully rederived and maintained under SPF conditions for the purpose of generating dKO mice. In Aim 1, Major Task 1 (Subtask2), the goal was to start monitoring colitis and collecting fecal pellets beginning at 4-weeks of age from the various mouse strains. Because our *Il10 KO* mouse colony stopped producing litters, we were unable to continue with the generation of dKO mice. This hurdle was circumvented by generating heterozygote *Il10;Stat2* mice to obtain dKO mice to complete Major Task1/Subtask 1. However, due to halt of research operations prompted by the Covid19 pandemic, expansion of our mouse breeding colony was suspended temporarily and prevented continuation of Aims 1 and 2 for the remainder of year 2. Amid resolving issues with the *Il10 KO* mouse colony, we continued with the study using the chemically-induced colitis model of dextran sodium sulfate (DSS) to execute some of the subtasks as part of Major Task 1 (Subtask 2-4, 100%) and Major Task 2 (Subtask 2). This effort had led us to collect large amount of data and we are in the process of drafting a manuscript describing our findings. In year 3 of no-cost extension, the heterozygote *Il10;Stat2* mouse colony was successfully expanded and we have completed Major Task1 (Subtasks 2 and 3 and part of Subtask 4). The milestone for Major Task 1 was achieved by establishing STAT2 as a genetic factor that contributes to the pathophysiology of colitis. Despite of this success, objectives for Specific Aim 2 could not be met as funds had depleted. Overall, our data strongly indicate that STAT2 is a detrimental factor. We have confirmed these results by employing two different models of IBD (DSS and *Il10* deficiency). Sufficient data was generated from this study to apply for external funding to continue this project. We submitted an R21 grant application to be reviewed in March 2022 and a R01 application is in preparation.

1) Major Activities: The goal was to generate single *Il10KO* and *Il10*; *Stat2 dKO* mice to conduct Specific Aim 1 (Major Task1/Subtask 2-4). However, difficulties with breeding the desired strains became problematic and was not resolved until year 2. It took months to resolve as our *Il10 KO* mouse colony ceased to produce litters. This problem exacerbated due to the Covid-19 pandemic as plans to expand our breeding colony were suspended because our institution shut down and we had limited access to the research labs and animal facility. While resolving this issue, we used as our alternate plan, the well-established dextran sodium sulfate (DSS) model of colitis. We adhered to the SOW to execute work proposed in Specific Aim 1/Major Task 1 and Major Task 2. The use of the acute DSS model of colitis did not change, in any respect, the direction of the project and afforded us the opportunity to continue with our investigation. In year 3, we successfully expanded our breeding colony of *Il10 KO;Stat2-/+* mice to obtain sufficient mice of the desired genotypes to monitor long-term. Note that this task could not be accomplished in Year 2 due to limited access to the animal facility and suspension of expanding our heterozygote *Il10/Stat2* breeding colonies due to the Covid-19 pandemic.

2) Specific objectives: The Major Task in Aim 1 was to establish STAT2 as a genetic factor that contributes to the pathophysiology of colitis.
3) Significant results: Specific Aim 1
   Major Task1/Subtask 1: 100% completed
   Major Task 2/Subtask 2: 100% completed
   Major Task 3/Subtask 3: 100% completed
   Major Task 4/Subtask 4: 75% completed

<u>Stat2 loss confers protection against DSS-induced colitis</u>. Our data shows that Stat2 deficiency conferred protection against DSS-induced colitis. Colitis scores were low compared to wild type mice with intact Stat2 function (**Fig. 1A**). Long-term co-housing of wild type with *Stat2KO* mice after weaning did not cause *Stat2KO* mice to succumb to severe colitis or wild type mice to be protected from colitis (**Fig. 1B**). However, we noted that the inflammation individual score in *Stat2KO* mice was not statistically significant from WT mice. These phenotypes were seen in both male and female mice.

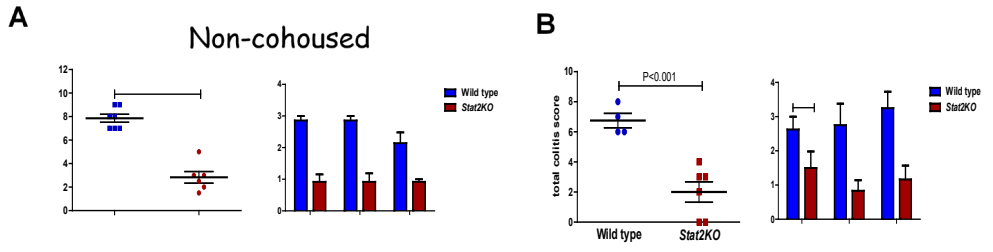

Figure 1. A) Stat2KO mice are protected from DSS-induced colitis. B) Co-housing conditions did not change the protective effect of Stat2 deficiency. (n=9).

Stat2 loss restricts neutrophil recruitment. *Stat2KO mice* showed reduced colonic inflammation after induction with DSS. Neutrophils are a major population of immune cells that are recruited to the site of inflammation. DSS-induced wild type (WT) mice show increased neutrophil presence in the ulcerated mucosa and submucosa. In contrast, the percentage of neutrophils in the colons of Stat2KO mice is low indicating that loss of STAT2 attenuates inflammation (**Fig. 2**).

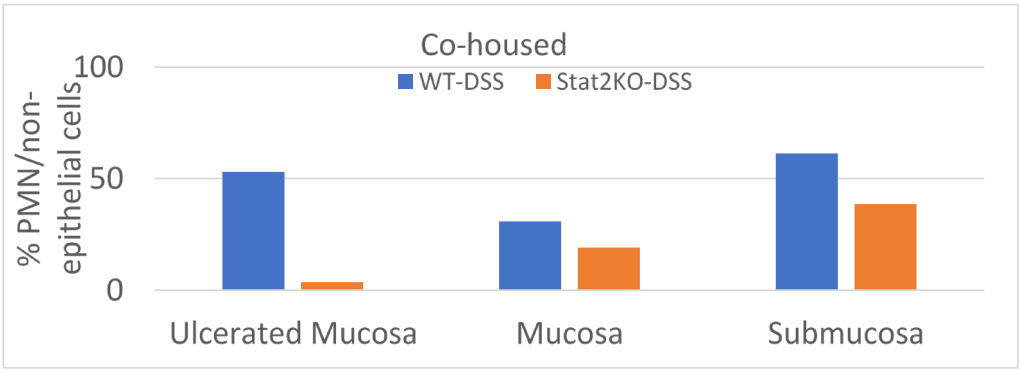

Figure 2. Neutrophil recruitment is attenuated in the colons of DSS-induced *Stat2KO* mice. Colons from DSS-induced WT and Stat2KO mice collected at day 10 were enumerated for neutrophils and percentages determined (n=4).

Stat2 deficiency restricts expansion of bacterial species belonging to the genus Enterobacteriacease and Verrucomicrobia. We performed microbiome analysis of fecal pellets collected prior to DSS administration and at the completion of the study. Data indicated that colitic wild type mice underwent changes in their microbiome composition (**Fig. 3A**). Bacteria of the phylum *Proteobacteria* and *Verrucomicrobia* expanded significantly when compared to *Stat2KO* mice. The same observation was made with co-housed mice (**Fig. 3B**). Further analysis indicated that members of the Enterobacteriaceae and Verrucomicrobiaceae genus that fall under the phyla *Proteobacteria* and *Verrucomicrobia Akkermansi*, respectively, were also increased in wild type mice but reduced in *Stat2KO* mice. Of clinical significance is the observation that both *Enterobacteriaceae* and *Akkermansia muciniphila* are implicated on colitis severity. Co-housing conditions changed slightly the outcome, indicating that the colitis effects were driven by host STAT2.

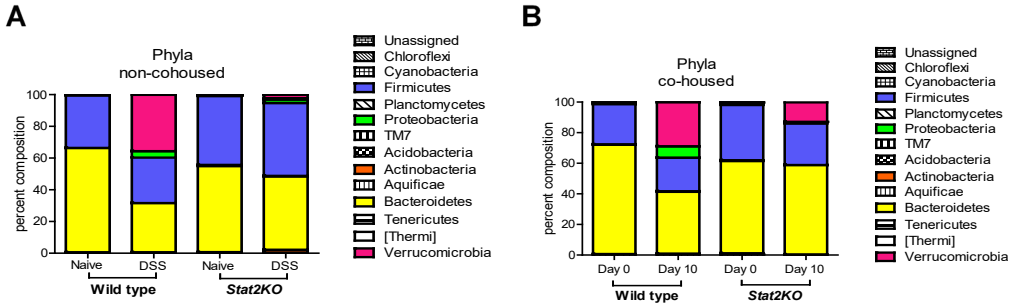

Figure 3. Stat2 deficiency restricts the expansion of microbial species associated with severity of colitis. A) Non-cohoused and B) Co-housed conditions minimally altered the microbiome composition of *Stat2KO* mice after DSS-induced colitis (n=3).

The protective effect of Stat2 deficiency is lost with a fiber free diet. Loss of Stat2 restricted the expansion of *Akkermansia muciniphila,* a mucin degrading bacterium that represents 1-4% of the fecal microbiome. We, therefore, tested whether changing the composition of the diet to one rich in carbohydrates will alter the protective effect of Stat2 deficiency. Both male and female mice placed on a fiber free diet experienced similar body weight gain. This time, Stat2KO mice succumbed to colitis when fed a fiber free diet (**Fig. 4B**) when compared to a regular diet (**Fig. 4A**). This experiment was done with a small number of mice and will have to be repeated before samples can be analyzed for potential changes in the abundance of *Akkermansia muciniphila.*

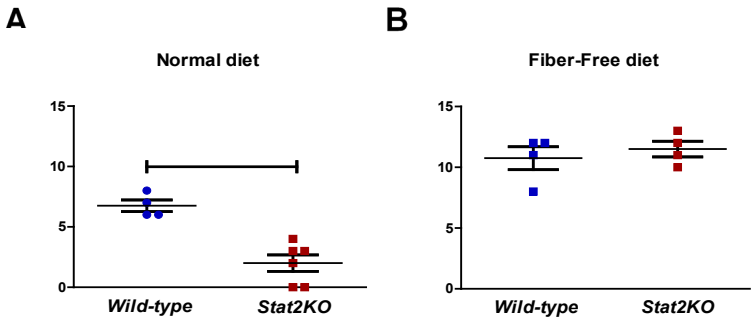

Figure 4. A fiber free diet abolishes the protective effect of Stat2 deficiency. A) Normal diet (n=4-6) and B) fiber free diet (n=4).

Stat2 promotes intestinal inflammation in spontaneous *Il10KO* model of IBD. To assess the role of STAT2 in chronic intestinal inflammation, we generated *Il10/Stat2 dKO* mice and monitored them for 18-20 weeks from birth. Global loss of STAT2 in *Il10KO* mice led to a drastic reduction in colitis scores **(Fig. 5A)**. We also noted that colons of *Il10/Stat2dKO* mice had less immune cell infiltration and reduced inflammation as opposed to what was observed in colons of *Il10KO* mice **(Fig. 5B-C)**. Splenomegaly was only observed in Il10KO mice and not in dKO mice **(Fig. 5D)**. Therefore, Stat2 deficiency attenuated colitis severity that also indicated preservation in the integrity of the gut barrier.

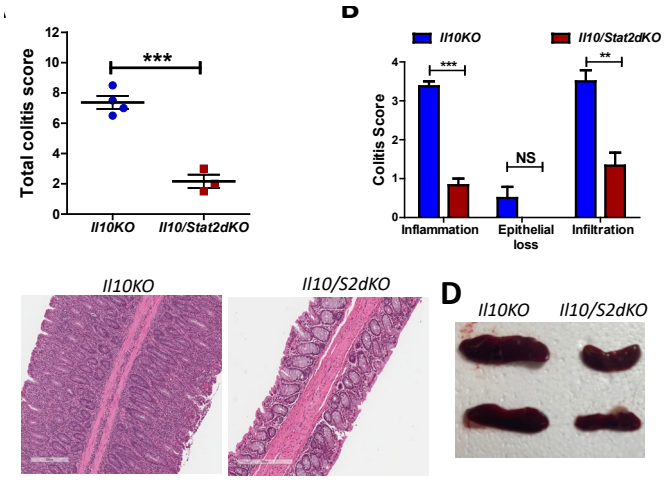

Fig 1. STAT2 loss attenuates inflammation in experimental model of IBD. A) STAT2 deficiency in *Il10KO* mice led to mild colitis. B-C) H&E stains show reduced colonic epithelial wall thickness and immune cell infiltration in *Il10/Stat2 dKO* mice. D) dKO mice show normal size spleens (n=5-7 mice, ***, $p < 0.001$).

4) Conclusions: Our data indicate that STAT2 is a crucial factor in the pathogenesis of IBD. These preliminary observations are first to identify STAT2 as pro-inflammatory and damaging in the gut. With data we have collected, we will seek external funding to continue this project to define mechanistically how STAT2 mediates inflammation and metabolic changes that are conducive to damage in the gastrointestinal tract. Most importantly is to translate these findings by examining samples from IBD patients.

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?

Nothing to Report. This is our Final Report.

4. IMPACT:

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

Our findings stress the importance of diet in intestinal inflammation and how specific changes to the composition of the microbiome could contribute to severity of colitis. Identification of metabolites that are protective or harmful are, therefore, important to keep in check during bouts of colitis.

What was the impact on other disciplines?

IBD is a chronic condition that increases the risk of developing colorectal cancer. A better understanding of the metabolic changes facilitated by STAT2 in coordination with the intestinal microbiome and diet will provide insight of how these changes may apply to other chronic inflammatory diseases.

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

Progress in our work was notably affected by the Covid19 pandemic. This limited our execution of Specific Aim 2 as funds depleted.

Changes that had a significant impact on expenditures

Continuation of our study was severely impacted by the Covid-19 pandemic. In year 3 on no-cost extension, we managed to accomplish what was financially achievable as lab personnel were let go. All these factors impacted our productivity as our mouse colony consumed most of the budget.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of human subjects

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

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Journal publications.

We have one manuscript in preparation describing our findings using the DSS chemical model of acute colitis.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.
Our findings were presented at the following meetings:
International Cytokine and Interferon Society virtual meeting (November 2020)
American Associate of Immunologists Annual meeting (May 2021)

- **Websites.**

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?

Ana Gamero (PI): No change

Cagla Tukel (Collaborating PI): No change

Tess Cremers
Role: Lab Technician
Nearest person month worked: 1
Contribution to Project: Ms. Cremers was instrumental in establishing mouse breeding colonies and instituted protocols for verifying mouse genotypes. She also carried out the experimental colitis model of DSS and molecular characterization.

Dorret Lynch
Role: Senior Lab Manager
Nearest person month worked: 1
Contribution to Project: Ms. Lynch handled the general maintenance of the lab. She performed work in the areas of molecular biology.

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

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

The tasks to be executed at the site of Collaborating PI were not completed in Year 2 of the grant. However, she has been consulted throughout the Year in preparation for completing Major Tasks in grant. She was instrumental in preliminary microbiome analysis performed with DSS colitis model.

QUAD CHARTS:

Not applicable

9. APPENDICES:

Nothing to report