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TITLE: The role of the gut microbiome in colorectal cancer

PRINCIPAL INVESTIGATOR: Ana Gamero

CONTRACTING ORGANIZATION: Temple University- Of the Commonwealth System of Higher Education

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Fort Detrick, Maryland 21702-5012

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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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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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:** *Provide a brief list of keywords (limit to 20 words).*

Colorectal, cancer, microbiota, dysbiosis, colitis, chronic, IBD, STAT2 and Il10

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The primary goal as described 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*^{-/-}, *Stat2*^{-/-} and *Il10;Stat2* double knockout (dKO). In year 2, *Stat2*^{-/-} mice were successfully rederived and maintained under SPF conditions to generate dKO mice. In Aim 1, Major Task 1 (Subtask2), the goal was to start monitoring colitis and collection of fecal pellets starting at 4-weeks of age from the various mouse strains. Execution of this task, however, was met with an unforeseen problem caused by our *Il10*^{-/-} mouse colony that stopped producing litters and did not permit the subsequent generation of dKO mice. After several failed attempts, we have recently been able to generate heterozygote *Il10;Stat2* mice. Multiple breeders of *Il10;Stat2*^{+/-} mice have been set up to obtain the desired genotypes. This effort completes Major Task1/Subtask 1. However, due to halt of research operations caused by the Covid19 pandemic and the recent establishment of breeding colonies, we do not anticipate completion of all Major Tasks as proposed for Aims 1 and 2. Given these unexpected circumstances, we resorted to the use of the chemically-induced colitis model of dextran sodium sulfates (DSS) to execute some of subtasks of the study.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

- 1) Major Activities: In Year 2, the goal was to establish a specific-pathogen free (SPF) mouse breeding colonies consisting of wild type, single and double *Il10* and *Stat2* KO mice to be used for Specific Aims 1-2 (Major Task1/Subtask 1 in both aims). Completion of this Major Task was met with unexpected challenges and took months to resolve as our *Il10*^{-/-} 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.
- 2) Specific objectives: Our backup plan while we were resolving issues with breeding and generating dKO mice was to employ the well-established DSS model of colitis. We adhered to the SOW to execute work proposed in Specific Aim 1/Major Task 1 and Major Task 2. We must stress that the use of the DSS model did not change the direction of the project. It is our goal to validate findings obtained from the DSS model with *il10/Stat2* dKO mice; time and budget permitting.
- 3) Significant results: The animal protocol we used involved chemical induction of colitis by administering 3% DSS in the drinking water for 7 days that was switched to water for 3 days at which time, mice were sacrificed on day 10. Fecal pellets and blood samples were collected prior to DSS administration, during (day 5) and at the end of study.

Specific Aim 1

Major Task1/Subtask 2 and 4: 75% completed

Major Task 2/Subtask 2: 30% completed

Major Task 3/Subtask 1-3: 80% completed

Stat2 deficiency confers protection against DSS-induced colitis. 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). Co-housing wild type and Stat2KO mice after weaning did not cause Stat2KO mice to succumb to colitis or wild type mice to be protected from colitis (Fig. 1B). These phenotypes were seen in both male and female mice.

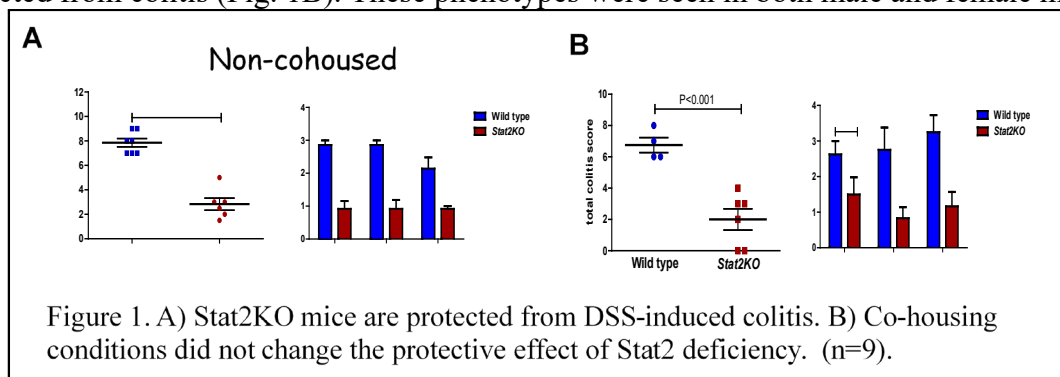
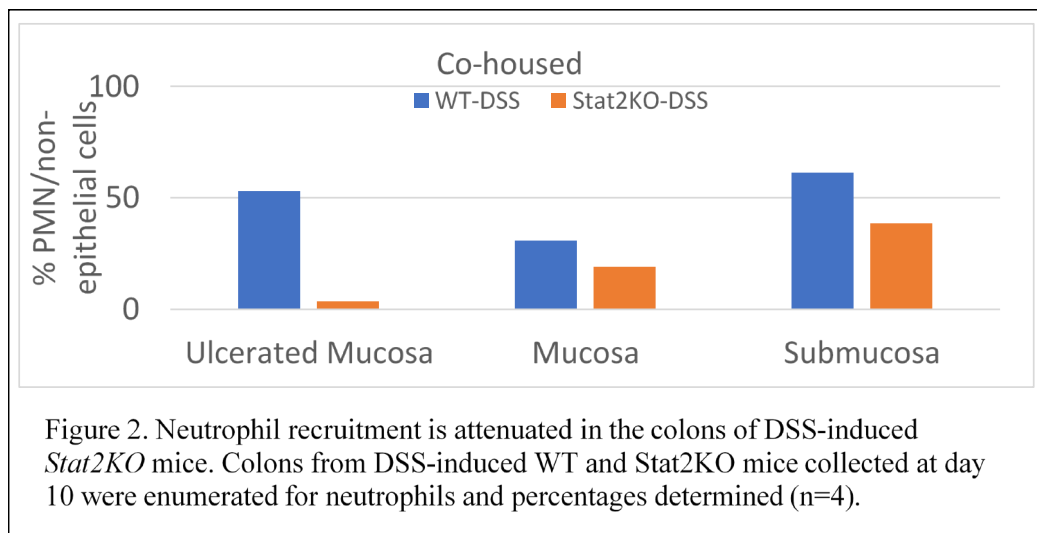


Figure 1. A) Stat2KO mice are protected from DSS-induced colitis. B) Co-housing conditions did not change the protective effect of Stat2 deficiency. (n=9).

Stat2 deficiency 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).



Stat2 deficiency restricts expansion of bacterial species belonging to the genus Enterobacteriaceae 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 did not change the outcome, indicating that the colitis effects were driven by host STAT2. Based on these findings, our subsequent studies were performed under co-housing conditions to minimize the role of the gut microbiome.

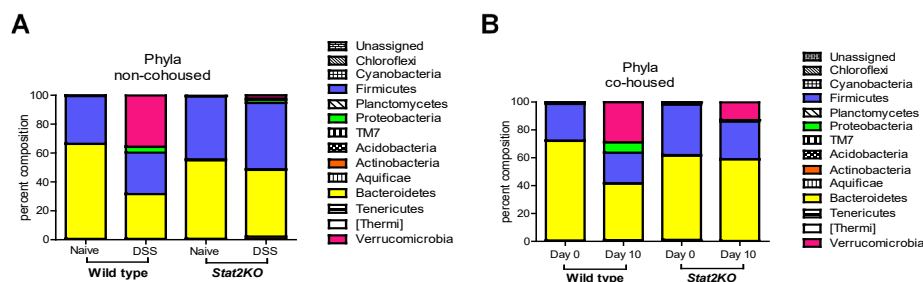


Figure 3. Stat2 deficiency restricts the expansion of microbial species associated with severity of colitis. A) Non-cohoused and B) Co-housed conditions minimally altered the microbiome composition of *Stat2KO* mice after DSS-induced colitis (n=3).

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*.

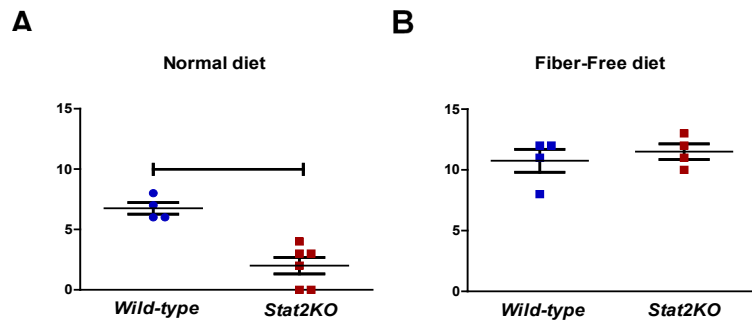


Figure 4. A fiber free diet abolishes the protective effect of Stat2 deficiency. A) Normal diet (n=4-6) and B) fiber free diet (n=4).

4) Conclusions: Our data indicates that STAT2 is a genetic factor that promotes colitis by yet, identified mechanisms. In the onset of colitis, STAT2 alters the composition of the microbiome giving rise to the expansion of two bacterial phyla associated with severity of colitis. Co-housing conditions was not enough to transfer colitis from wild type mice to Stat2KO mice and the converse effect also did not occur as Stat2KO mice remained protected from DSS-induced colitis. However, changing the mice from a regular diet to fiber free diet (rich in carbohydrates) appeared to abolish the protective effect conferred by STAT2 deficiency. This preliminary observation highlights the importance of diet in controlling colitis. The next phase of the study is to validate these findings with the *Il10^{-/-}* mouse model of IBD and identify metabolites that are protective against colitis.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

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

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will adhere to the SOW in trying to complete Major task 1 and 2 of Specific Aim 1 as outlined in the proposal with the remaining funds.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

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

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an 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 will provide insight of how these may apply to other chronic inflammatory diseases.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We encountered problems in generating the pertinent mouse strains for the study. We believe to have resolved this problem that took nearly 8 months to accomplish. In the interim, we opted to employ the DSS model of colitis and this did not change the direction of the project.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

An anticipated problem was with the generation of dKO mice. Our *Il10*^{-/-} mouse colony stopped producing offspring and different strategies were used to recover the line. We eventually generated *Il10/Stat2*^{+/-} mice, but they could not be used for breeding as our institution shut down in March due to the Covid-19 pandemic. The mice that we managed to obtain for the study had to be destroyed. Prior to Covid-19, we devised a plan of action to use the DSS model of colitis while we resolved issues with breeding *Il10*^{-/-} mice. Although this was not the intended model to use, nonetheless, we have obtained significant amount of data that we foresee validating with the *Il10*^{-/-} mouse model.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Continuation of our study was severely impacted by the Covid-19 pandemic. After overcoming a hurdle with recovery of the *Il10*^{-/-} animal colony, we were not allowed to continue breeding animals and access to the animal facility and labs was drastically reduced. All these factors impacted our budget and productivity as new mice will now have to be generated.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

An unanticipated and major drawback to our study was the inability to generate *Il10;Stat2* dKO mice. Our *Il10*^{-/-} mice stopped producing offspring and we were unable to use them for breeding with our newly rederived *Stat2*^{-/-} mice. We employed different strategies to recover the *Il10*^{-/-} mice and one of them involved generating heterozygote *Il10*^{+/-} mice. We were successful in generating *Il10*^{+/-} mice after housing conditions were changed from SPF to conventional animal housing. This detrimental setback caused severe delays in our progress that was further compounded with the shutting down of our institution due Covid-19. We were prohibited from setting up new breeders until the end of June. We recently obtained *Il10/Stat2* ^{+/-} mice that are now being used to generate *Il10*^{-/-} and *Il10;Stat2* dKO mice. In the interim, we resorted to the use of the DSS model of colitis to achieve some of the goals proposed in the SOW. We are well-versed with this model that was already approved in our animal protocol since December 2017. Our preliminary studies showed that *Stat2*^{-/-} mice developed mild DSS-induced colitis. This observation provided to platform to conduct initial experiments that were proposed to be done with *Il10;Stat2* dKO mice.

Significant changes in use or care of vertebrate animals

While we wait to generate Il10/Stat2 dko mice, we have been using the DSS model of colitis to perform some of the subtasks associated with Specific Aim 1.

Significant changes in use of biohazards and/or select agents

The model of colitis we are currently using employs dextran sodium sulfate (DSS).

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

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

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

An abstract has been submitted for consideration to be presented at the upcoming International Cytokine and Interferon Society virtual meeting to be held November 1-4, 2020

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Ana Gamero (PI): No change

Cagla Tukul (Collaborating PI): No change

Tess Cremers

Role: Lab Technician

Nearest person month worked: 9

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: 6

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?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

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: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

Not applicable

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Nothing to report