Award Number: W81XWH-15-1-0155

TITLE: Novel Listeria Vectors Secreting Gut Flora-Altering Agents to Prevent Colon Cancer and Treat Colitis

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REPORT DATE: NOVEMBER 2018

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED *(From - To)*
NOVEMBER 2018	Final	15AUG2015 - 14AUG2018

4. TITLE AND SUBTITLE
Novel Listeria Vectors Secreting Gut Flora-Altering Agents to Prevent Colon Cancer and Treat Colitis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0155

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We defined effects of the immune co-signaling molecule B7-H1 on colitis and cancer development, and effects on immune events in colon. *Specific objectives.* 1. Define the effects of colon B7-H1 on colitis and development of colon cancer. 2. Test if altering colon B7-H1 signals using recombinant *Listeria* could affect colitis and subsequent colon cancer. *Significant results or key outcomes.* We have shown that host B7-H1 and gut B7-H1 contribute to colitis and colon cancer development. These data provide the proof of concept that targeting this molecule can help reduce colon cancer or treat it. We showed that conventional T cells can induce colon cancer and unconventional γδ T cells can inhibit colon cancer. We showed that B7-H1 alters the gut microbiome to alter colitis and cancer. These data help understand colon cancer immunopathogenesis for future prevention/treatment approaches. We identified specific immune molecules and cells participating in colitis and colon cancer to inform further studies.

15. SUBJECT TERMS

NONE LISTED

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Gut dysbiosis contributes to colitis that increases colorectal cancer (CRC). Mechanisms are obscure but dysbiosis-driven alterations of metabolism, colon epithelial integrity and immunity are likely. Mechanisms regulating dysbiosis are likewise obscure. The immune co-signaling molecule B7-H1 regulates colon inflammation by regulating gut dysbiosis that increases CRC risk. We will use a well-validated mouse model of colitis and related CRC to test if manipulating colon epithelium B7-H1 can mitigate gut dysbiosis that leads to colitis and to related CRC risk, using recombinant bacterial vectors to reduce B7-H1 effects in colon.

2.

Colon, cancer, microbiome, B7-H1, PD-L1, immunity, gamma delta T cells

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Generate recombinant *Listeria* strains. 100% accomplished by December 2016. Generate LH1169 inlA^m *Listeria* strains 100% accomplished by December 2016. Screen scFv library for anti-B7-H1 100% completed by December 2017. Generate LH1169 and LH1169 inlA^m expressing anti-B7-H1 scFv 0% accomplished by October 2018. Generate LH1169 and LH1169 inlA^m strains expressing B7-H1 0% accomplished by October 2018. Because we failed to generate recombinant *Listeria* bacteria as planned, we focused on colitis and host B7-H1 effects in discussions with CDMRP staff. Assess host B7-H1 effects on colitis 100% accomplished by August 2017. Assess host B7-H1 effects on colon cancer 100% accomplished by August 2018. Assess host B7-H1 effects on colon immunity 100% accomplished by August 2018.

What was accomplished under these goals?

1) Major activities. We defined effects of the immune co-signaling molecule B7-H1 on colitis and colon cancer development, and effects on immune events in colon.

2) Specific objectives. 1. Define the effects of colon B7-H1 on colitis and development of colon cancer. Test if altering colon B7-H1 signals using recombinant *Listeria* could affect colitis and subsequent colon cancer. 3. Test if any colitis or cancer effects from B7-H1 were due to altered colon microbiome.

3) Significant results or key outcomes. 1. We clearly showed that colon B7-H1 protected from colitis and colon cancer. 2. We clearly showed that colon B7-H1 altered the colon microbiome to affect colitis. 3. We clearly found that unconventional γδ T cells, not conventional T cells contributed to B7-H1 effects on colitis and colon cancer risk.

4) Other achievements. 1. We defined a number of colon B7-H1-regulated immune molecules and immune cells involved in colitis and possibly colon cancer that could be key mediators of colitis, colon cancer or microbiome effects, setting the stage for follow up studies of manipulating the colon B7-H1 axis to reduce colitis and help prevent or treat colon cancer. 2. We failed to generate recombinant *Listeria* expressing molecules that could affect the colon B7-H1 signaling axis. We speculate that we need an alternate *Listeria* construct with different genetic alterations or a different scFv library to screen. Nonetheless, our findings justify further work to attempt to make such constructs to treat colitis and be used to help prevent or treat colon cancer.

SPCIFIC RESULTS

We screened an scFv library for anti-B7-H1. A *S. cerevisiae* library containing >10^{10} scFv clones expressed on its surface was screened as detailed in **Figure 1**.

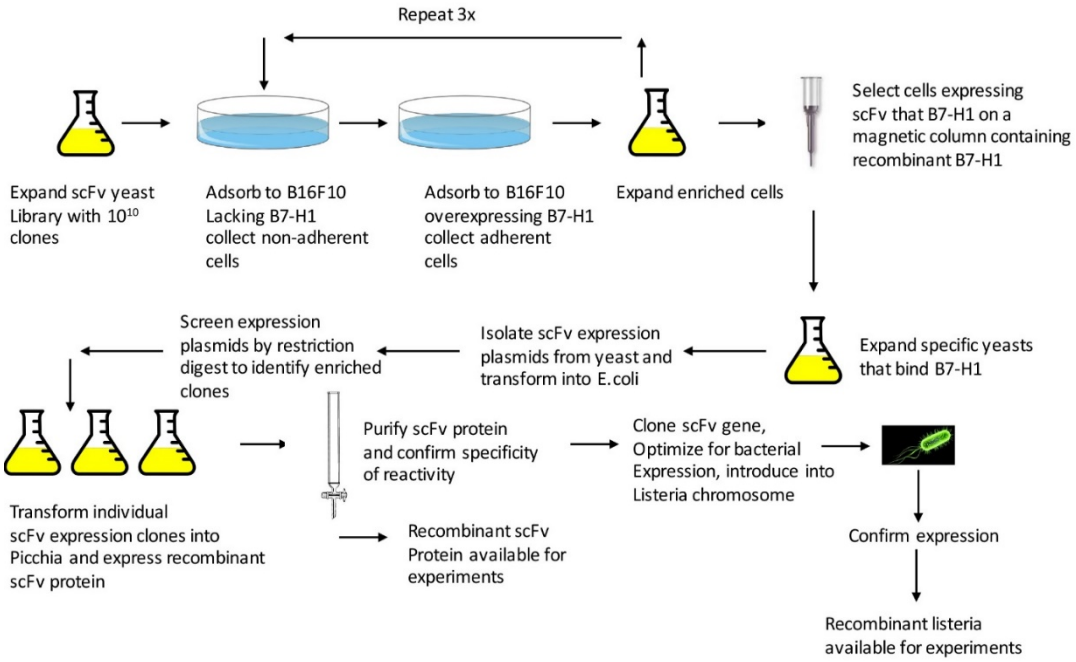

Figure 1 Screen to identify scFv clones for expression in *Listeria*
We screened the scFv library 3 times and identified yeast that bound to the B7-H1 column (**Table 1**).

Table 1 Clones from the scFv screen

Screen	Individual clones after B7-H1 magnetic column	Enriched clones identified by restriction digest	Individual clones for expression in *Picchia*
1	50	12	2
2	66	9	2
3	28	2	1

We were able to identify clones enriched in the screen through restriction length polymorphism analysis and they were cloned into the *Picchia* expression system to produce secreted scFv that could be used in validation assays prior to cloning into *Listeria* for *in vivo* expression. We encountered significant technical difficulties at this stage as all five clones that were selected for expression in *Picchia* were expressed but not secreted. We were able to verify intracellular expression by cellular fractionation and Western blotting. Fractionation experiments confirmed that the scFv while being expressed, was not being secreted. These data confirm that the *Picchia* promoter and the reading frame of the constructs were correct but suggest a problem with the secretion signal peptide or folding of the scFv. We have sequenced all of the constructs and there appears to be no obvious problems with the architecture of the secretion construct.
As detailed in **Table 1** above, the 3 screens identified 144 clones that potentially express scFv proteins that bind B7-H1. However, we ultimately failed to validate than any clones were *bona fide* B7-H1 binding.

We validated that our AOM/DSS model generated colitis and colon cancers.

Sample number	**Histopathological findings and comment**
1	Adenocarcinoma in situ (X2), Adenomatous polyp with severe dysplasia (X2), Inflammation 2+ lamina propria and submucosa, (polys, plasma cells, lymphocytes), crypts normal
2	Adenocarcinoma in situ (X1), inflammation 1+, submucosa (lymphocytes and plasma cells), crypts normal
3	Adenomatous polyps (X2), inflammation 1+, submucosa, dysplastic crypts
4	Adenomatous polyps (X4) mild to moderate dysplasia, inflammation 1+ (plasma cells lymphocytes, macrophages), submucosa
5	No slide
6	Adenomatous polyps X2, mild to moderate dysplasia, 1+ inflammation, lamina propria, crypts normal
7	Adenomatous polyp X1, moderate dysplasia, inflammation 1+ lamina propria (plasma cells, lymphocytes), crypts normal,
8	No polyps, focal dysplastic crypts, inflammation 1-2+ (lymphocytes)
9	Adenomatous polyp X1, inflammation 1+ lamina propria, crypts normal

10	Adenomatous polyps X2, inflammation 1+ (plasma cells, polys, lymphocytes), lamina propria and submucosal, crypts normal
11	Adenocarcinoma in situ X1, adenomatous polyps X4, inflammation 3+, focally transmural (plasma cells, few polys, lymphocytes), crypts normal
12	No polyps present, inflammation 2+, submucosa (lymphocytes, plasma cells, histiocytes), reactive crypts
13	No slide
14	No slide
15	Multiple sites of insitu adenocarcinoma and adenomatous polyps, inflammation 3+ (plasma cells, lymphocytes, polys), transmural, crypts normal
16	Adenomatous polyps X2, Adenocarcinoma X2, inflammation 3+ (plasma cells, lymphocytes, polys), transmural, crypts normal
17	Adenomatous polyps X3, mild to moderate dysplasia, inflammation 3+ (plasma cells, lymphocytes, polys), transmural, crypts normal
18	Adenomatous polyps X3 (mild to moderate dysplastic changes), inflammation 1-2+ (lymphocytes, polys), submucosa, crypts normal
19	Adenomatous polyps X3 (mild dysplasia), inflammation 1-2+, submucosa (plasma cells, polys), crypts normal
20	Adenomatous polyps X8 (moderate to severe dysplastic changes), inflammation 3+ (transmural - plasma cells, lymphocytes, polys), crypts normal

Table 2. Histology in 20 mice after the carcinogen azoxymethane plus three cycles of dextran sodium sulfate as described in our proposal, and sacrificed 4 months later.

We tested B7-H1 modulation in models of colitis and colon cancer, in the above DSS/AOM colorectal cancer model. Groups of 5 mice each (WT versus B7-H1 KO, and also young (3-6 months) versus old [19-22 months]) were given 2.5% of the inflammatory agent dextran sodium sulfate (DSS) in drinking water and colon cytokines were assessed 11 days later using a multiplex Luminex kit per the manufacturer's directions. Several important immune leads emerged. For example, lack of colon B7-H1 blunted the IFN-γ response (**Fig. 2**). As IFN-γ plays a role in colitis and in colon cancer, B7-H1 bacterial constructs affecting colon IFN-γ could be useful for any clinical effects observed if and when such constructs are able to be made.

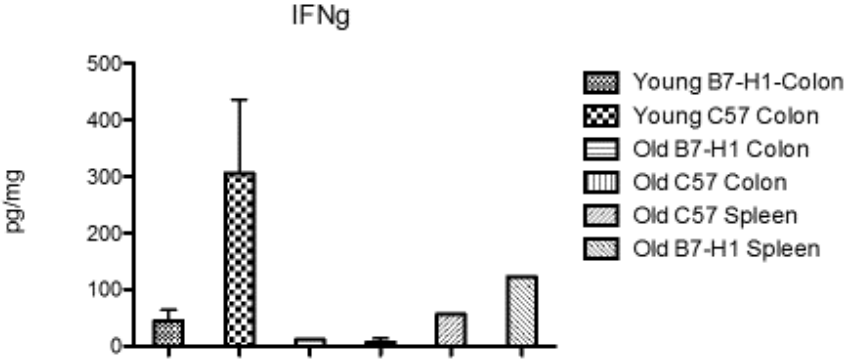

Fig. 2 The IFN-γ response in DSS colitis is blunted in colons of B7-H1 KO mice. Luminex data averaged from 5 colons in each group from mice given 2.5% DSS in drinking water for 5 days and sacrificed on day 11.

By contrast, IL-12p70, a major inducer of IFN-γ, was not different between WT and B 7-H1 KO (**Fig. 3**) suggesting a distinct mechanism for this IFN-γ control.

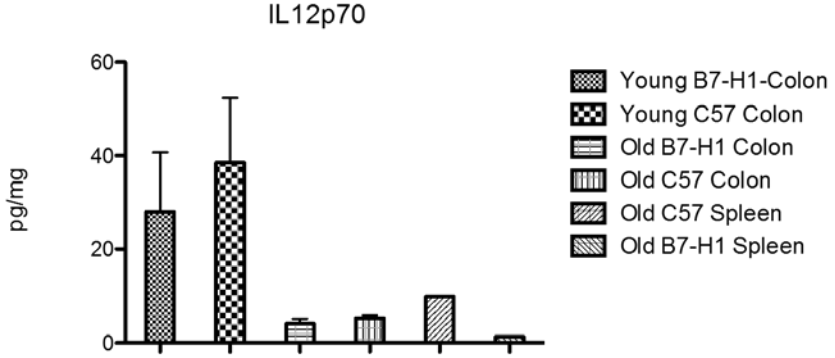

Fig. 3 The IL-12p70 response in DSS colitis is unaffected in colons of B7-H1 KO versus WT mice. Luminex data averaged from 5 colons in each group from mice given 2.5% DSS in drinking water for 5 days and sacrificed on day 11.

Mice lacking all T cells do not lose weight in DSS challenge, but experience colitis as evidenced by colon shrinkage (**Fig. 4**).

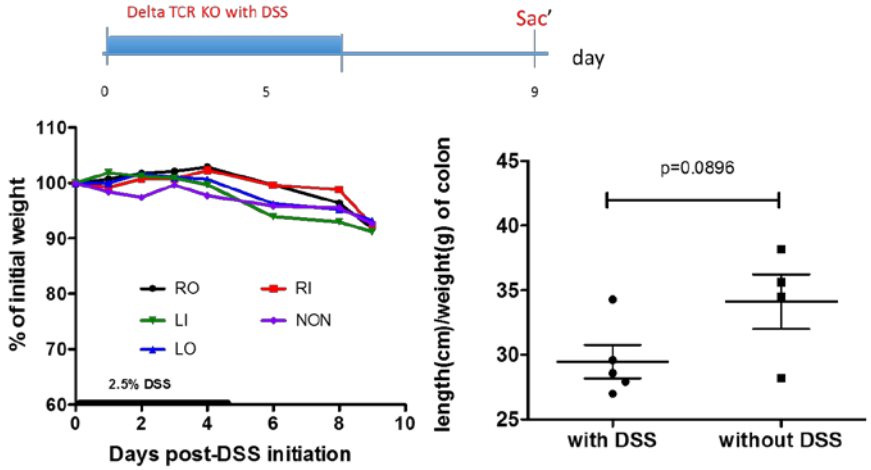

Figure 4 Mice lacking all T cells do not lose weight in DSS challenge, but experience colitis as evidenced by colon shrinkage, on the right. N=5 βδ TCR mice versus 4 control mice. Individual weight loss data for the 5 βδ TCR mice is on the left. The cartoon for experimental design is at the top.

Azoxymethane (AOM) is a carcinogen that induces colon cancer at 10 mg/kg by IP injection, when combined with DSS to induce colitis. To understand the immune cells mediating colitis and colon cancer in the AOM/DSS model, we used βδ TCR KO that lack all T cells. Strikingly, these mice did not develop colon cancer after AOM/DSS (**Fig. 5**) , although 100% of WT mice developed cancer with this regimen, suggesting that T cells could be targets for cancer prevention by B7-H1 bacterial vectors.

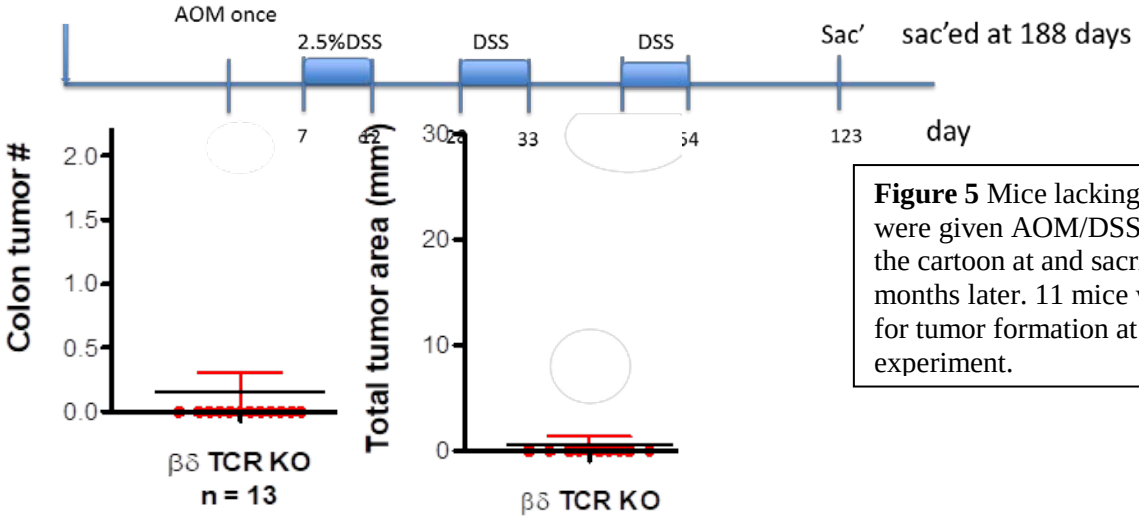

Figure 5 Mice lacking all T cells were given AOM/DSS as shown in the cartoon at and sacrificed 6 months later. 11 mice were studied for tumor formation at the end of the experiment.

We then studied δ (delta) TCR KO mice only lack γδ T cells, but still have conventional (αβ) T cells. We challenged 5 δ TCR KO mice and 4 syngeneic control mice with 2.5% DSS in drinking water for 5 days. We showed that lack of γδ T cells made mice resistant to weight loss in DSS-induced colitis, but that there was evidence for colitis by colon shrinkage (not shown). These data suggested that γδ T cells might not be the top candidates for colitis control by B7-H1 bacterial vectors, as their absence appears to have little impact on colitis.

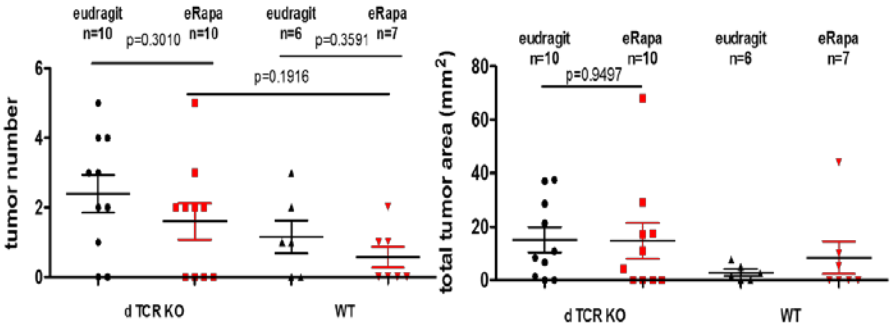

Figure 6 Mice lacking γδ T cells experience increased colon cancer after AOM/DSS versus control WT mice. The cartoon for experimental design is at the top. In this experiment we also included a drug called eRapa that can be ignored for these purposes. Eudragit is the negative control and for these analyses we compare δ TCR KO mice on Eudragit versus WT mice on Eudragit as the relevant comparators. eRapa is an mTOR inhibitor we are testing as mTOR signals are downstream of B7-H1.

However, when we challenged δ TCR KO mice versus WT mice with AOM, followed by 3 cycles of DSS to induce colon cancer after colitis, we strikingly found that δ TCR mice had increased cancer versus WT mice 4 months later (**Fig. 6**). These data now suggest that γδ T cells could be targets for our B7-H1 bacterial vectors in cancer prevention and help guide mechanistic studies.

What opportunities for training and professional development has the project provided?
One medical student (Wanjiao (May) Chen) worked on this project that provided an excellent opportunity for her scientific training. Ms. Chen learned mouse husbandry, sterile cell culture, Western blots, animal injections, animal dissection, flow cytometry, pipetting, weighing reagents, keeping lab notes and making oral scientific presentations. Ms. Chen also made oral presentations at scientific conferences, including the international American Association of Immunologists Annual Meeting in 2016.

How were the results disseminated to communities of interest?

Data from this project were presented at the 2016 American Association of Immunologists Annual Meeting, at the UTHSA Research Day Symposium and the UTHSA Chinese Exchange Student Symposium.

What do you plan to do during the next reporting period to accomplish the goals?
Nothing to Report.

4. IMPACT:

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

We have shown that host B7-H1 and gut B7-H1 contribute to colitis and colon cancer development. These data provide the proof of concept that targeting this molecule can help reduce colon cancer or treat it.

We showed that conventional T cells can induce colon cancer and unconventional γδ T cells can inhibit colon cancer. These data help understand colon cancer immunopathogenesis for future prevention and treatment approaches.

We defined immune outcomes of B7-H1 signals in colon that can be used for cancer prevention and treatment approaches.

IFNγ is known to mediate colitis. We showed that colitis IFNγ can be generated in an IL-12-independent manner, suggesting new approaches to reduce IFNγ in colitis.

We showed that unconventional γδ T cells influence colon cancer, and thus could influence other cancers as well, requiring further study.

We showed that our *Listeria* and scFv screening approaches were not optimal for these purposes, and that other approaches should be tested.

Nothing to Report

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

5. CHANGES/PROBLEMS:

The biggest challenge was lack of identifying a clearly B7-H1 binding scFV from our screen. Without it we were never able to make the recombinant *Listeria* to test the hypothesis that we could use the *Listeria* to manipulate colitis. To compensate we did more detailed studies of B7-H1-driven colon immunology and immune cells related to colitis and colon cancer. These were not explicitly stated in the original SOW but were needed data as adjuncts to *Listeria* studies and will inform *Listeria* studies when vectors are successfully made. These changes were discussed with CDMRP during the evolution of studies as we worked to overcome the issues with the scFv screening.

Although we screened the scFv library and developed candidate clones for further work, we could never validate B7-H1 binding. Ultimately, we had to abandon *Listeria* studies in favor of more detailed immune studies as discussed above. These data are highly useful and gave several surprising but highly useful insights into future approaches to target B7-H1 in the gut.

Changes that had a significant impact on expenditures

None. Funds intended for *Listeria* work were used for immune studies with no net changes in total expenditures.

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

None

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

6. PRODUCTS:

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

"Microencapsulated rapamycin prevents carcinogen and inflammation driven colon cancer through immune mechanisms" May Chen… TJ Curiel. *Journal of Immunology* 2016 (meeting abstract)

Other publications, conference papers and presentations.

"Microencapsulated rapamycin prevents carcinogen and inflammation driven colon cancer through immune mechanisms" May Chen… TJ Curiel. Major Block Symposium Oral Presentation Annual Meeting 2016

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

http://gsbs.uthscsa.edu/faculty/tyler-curiel-ph.d

- **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?

No change

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?

None

8. SPECIAL REPORTING REQUIREMENTS

Not applicable