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14. ABSTRACT Pancreatitis (inflammation of the pancreas) leads to hundreds of thousands of hospital admissions each year in the United States. We studied the role of a noncoding RNA gene in pancreas inflammation using mouse cells. Gene deletion of this RNA reduces the pro-inflammatory response of mouse cells. These data support that this RNA is a critical promoter to pancreatitis.					
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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Pancreatitis (inflammation of the pancreas) leads to hundreds of thousands of hospital admissions each year in the United States. NF- κ B is a master regulator of inflammation.¹ MicroRNAs (miRNAs) are short (20-25 nucleotide) RNA molecules that negatively regulate gene expression. A number of miRNAs are dysregulated in pancreatitis and activate NF- κ B.² However, the underlying molecular mechanisms of pancreatitis remain elusive. Thus, there is an urgent need to investigate inflammation-related miRNAs using animal models for a comprehensive understanding of the biology and pathology of pancreatitis. miR-301a is identified as the most potent NF- κ B activator² and is also upregulated in pancreas tissues from patients with chronic pancreatitis compared to that from healthy donors.³ In this study, we propose two specific aims to ascertain the role of miR-301a in NF- κ B activation and pancreas inflammation using cells and mouse models.

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

MicroRNA, Pancreatitis, NF-kappaB, Mice

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

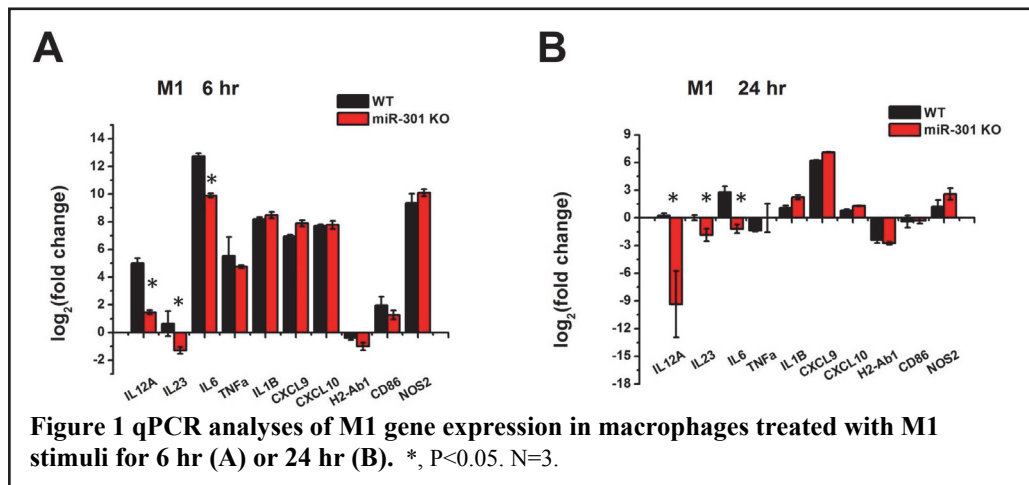
We present here progresses we have made in the funding period while the PI was at University of Louisville and after he moved to Cleveland Clinic Foundation (CCF). The objectives during this study period are to obtain regulatory approval on animal studies and determine the role of miR-301a in NF-kappaB signaling and macrophage activation using isolated macrophages from mice. We crossed miR-301a \pm mice to generate wild-type (WT) and miR-301a-null (KO) littermates for isolation of bone marrow-derived macrophages. Macrophages display remarkable plasticity and follow two distinct states of polarized activation: the classically activated (M1) phenotype and the alternatively activated (M2) phenotype. Under M1 activation, macrophages secrete cytokines including TNF α , IL-1 β , and IL-6, and enzymes such as inducible nitric oxide synthase (iNOS, a.k.a., NOS2). M2 polarization of macrophages was discovered as a response to the T helper type 2 (T_H2) cytokines IL-4 and IL-13.

We treated isolated macrophages with M1 [LPS (100ng/ml, TLRgrade, Enzo Life Sciences) and IFN- γ (10ng/ml, R&D Systems)] and M2 stimuli [mouse recombinant IL-4 (10ng/ml) and IL-13 (10ng/ml), obtained from R&D Systems], respectively. Quantitative real-time PCR (qPCR) was performed to determine whether miR-301a deficiency affects NF-kappaB activation and macrophage polarization. In addition, we performed Western blotting analyses to determine NF-kappaB activation and Stat3 activation in immune cells. We found that miR-301a KO macrophages have weaker M1 response than WT controls.

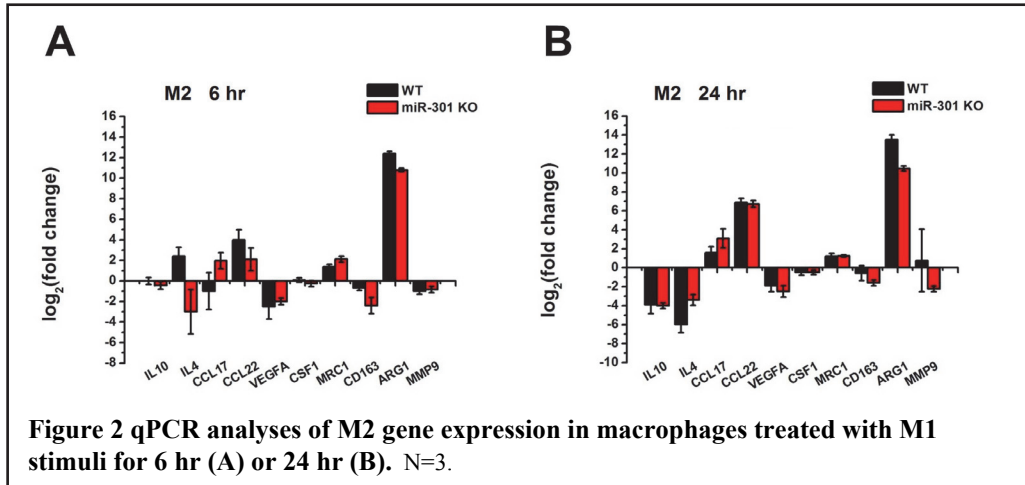
There are no changes to originally proposed methods.

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.

First, we determined M1 and M2 gene expression in wild-type (WT) and miR-301a-null (KO) bone marrow-derived macrophages. Mice (8 weeks old) were sacrificed and hind legs were removed. All muscle tissues from the bones were removed using scissors. The femur was separated and flushed with PBS using a 3-mL syringe and a 23-gauge needle. Bone marrow was passed through a pipette several times to make a single-cell suspension. Cells were washed with PBS twice before being plated onto a culture dish. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and 10 ng/mL murine granulocyte macrophage colony stimulating factor (M-CSF, R&D systems, Minneapolis, MN) and incubated at 37°C with 5% CO₂. We treated isolated macrophages with M1 [LPS (100ng/ml, TLRgrade, Enzo Life Sciences) and IFN- γ (10ng/ml, R&D Systems)] and M2 stimuli [mouse recombinant IL-4 (10ng/ml) and IL-13 (10ng/ml), obtained from R&D Systems], respectively. RNA was reverse-transcribed with the iScript™ Reverse Transcription Supermix kit (BIO-RAD, Hercules, CA).



qPCR was performed using the iQTM SYBR Green Supermix (BIO-RAD) and CFX96TM Real-Time PCR system (BIO-RAD) with primers designed based on Universal Probe Library (Roche) with *ACTB* as the reference. As shown in Figure 1, there were significant down-regulation of IL12A, IL23, and IL6 in KO macrophages treated with M1 stimuli compared with WT controls. However, the expression of M2 genes was not significantly altered (Figure 2).



Second, we determined NF-kappaB and Stat3 activation in isolated WT and miR-301a KO cells as miR-301a is activator to both NF-kappaB and Stat3.^{2,4} We first isolated CD11b⁺ monocytes/macrophages and CD11c⁺ dendritic cells from mouse spleen using flow cytometry. In mouse, the CD11b antigen is expressed on monocytes or macrophages with the later derived from the former. We treated cells with IL6 (50ng/ml), an Stat3 activator or TNF α (20ng/ml), an NF-kappaB activator for 30 min.

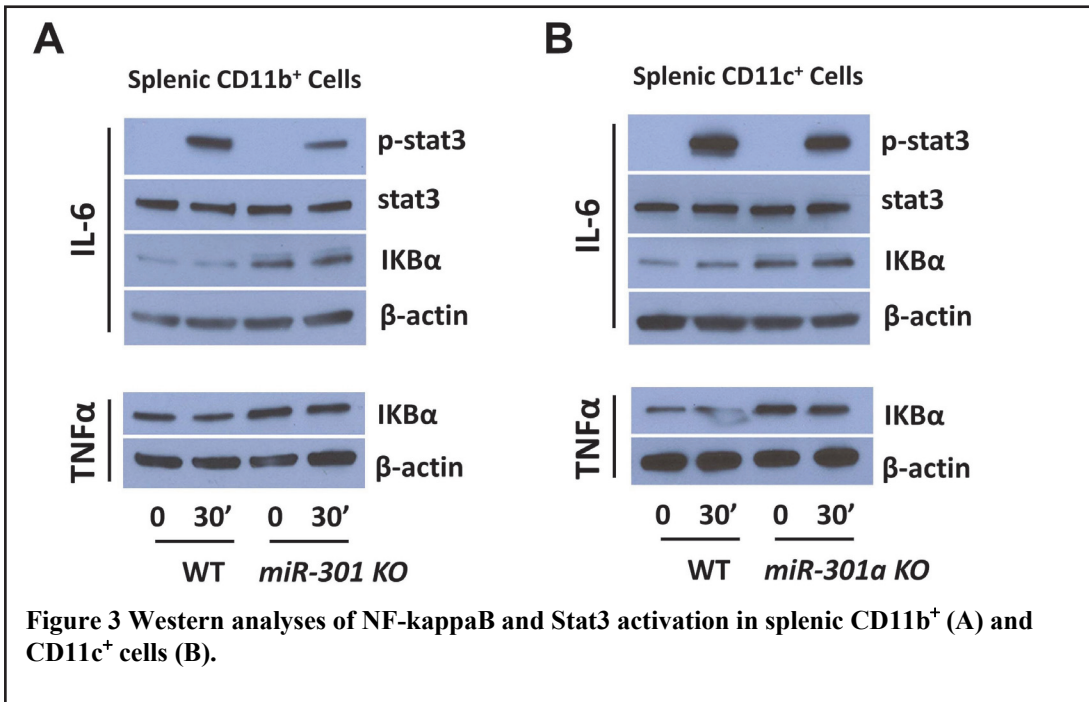
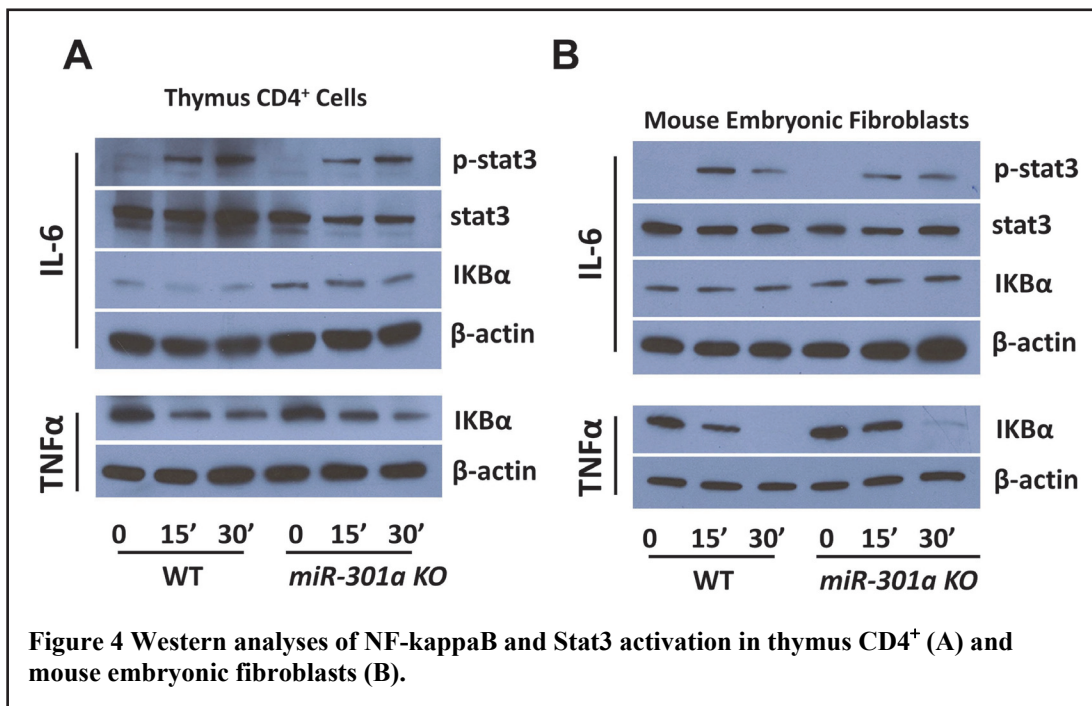


Figure 3 Western analyses of NF-kappaB and Stat3 activation in splenic CD11b⁺ (A) and CD11c⁺ cells (B).

We performed Western blotting analyses to determine NF-kappaB activation (I κ B α , an inhibitor to NF-kappaB) and Stat3 activation as judged by Stat3 phosphorylation. We found reduction of both NF-kappaB and Stat3 activation in KO CD11b⁺ and CD11c⁺ cells compared with WT controls (Figure 3). We then isolated CD4⁺ cells from mouse thymus and performed similar assays. As shown in Figure 4A, miR-301a deletion causes reduction of Stat3 activation and NF-kappaB activation in CD4⁺ cells treated with IL-6, yet the impact on NF-kappaB activation was marginal when cells were treated with TNF α . Finally, we prepared mouse embryonic fibroblasts, a wide-used surrogate cell model in inflammation studies according to an established protocol^{5,6} and treated cells with IL-6 or TNF α . We observed reduction of both NF-kappaB and Stat3 activation in these fibroblasts (Figure 4B).



In summary, reduction of NF-kappaB activation and M1 activation in primary macrophages and other cells upon loss of miR-301a is an indication that miR-301a is a pro-inflammatory miRNA. Our data suggest that miR-301a is critical for the inflammatory response *in vitro*. We have completed Task 1 (determining the role of miR-301a *in vitro*). These results support that miRNAs are therapeutic targets against pancreatitis.

- CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

Our results indicate that miR-301a deficiency reduces the pro-inflammatory response of macrophages. Given the importance of macrophages in pancreatitis, these studies support the notion that miR-301a is a critical promoter to pancreatitis. The PI has moved from University of Louisville to CCF and is in the process of transferring the grant to CCF. Our investigation will concentrate on Task 2 as outlined in our Statement of Work.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

- a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

- (1) Lay Press:
- (2) Peer-Reviewed Scientific Journals:
- (3) Invited Articles:
- (4) Abstracts:

- b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report.

7. **INVENTIONS, PATENTS AND LICENSES:** List all inventions made and patents and licenses applied for and/or issued. Each entry shall include the inventor(s), invention title, patent application number, filing date, patent number if issued, patent issued date, national, or international.

Nothing to report.

8. **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes that have resulted from this research. 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. This list may include development of prototypes, computer programs and/or software (such as databases and animal models, etc.) or similar products that may be commercialized.

Nothing to report.

9. **OTHER ACHIEVEMENTS:** This list may include degrees obtained that are supported by this award, development of cell lines, tissue or serum repositories, funding applied for based on work supported by this award, and employment or research opportunities applied for and/or received based on experience/training supported by this award.

For each section, 4 through 9, if there is no reportable outcome, state “Nothing to report.”

Nothing to report.

10. REFERENCES: List all references pertinent to the report using a standard journal format (i.e., format used in *Science*, *Military Medicine*, etc.).

1. Steinle AU, Weidenbach H, Wagner M, Adler G, Schmid RM. NF- κ B/Rel activation in cerulein pancreatitis. *Gastroenterology*. 1999;116(2):420-430.
2. Lu Z, Li Y, Takwi A, et al. miR-301a as an NF- κ B activator in pancreatic cancer cells. *EMBO J*. 2011;30(1):57-67.
3. Bloomston M, Frankel WL, Petrocca F, et al. MicroRNA Expression Patterns to Differentiate Pancreatic Adenocarcinoma From Normal Pancreas and Chronic Pancreatitis. *JAMA*. 2007;297(17):1901-1908.
4. Mycko MP, Cichalewska M, Machlanska A, Cwiklinska H, Mariasiewicz M, Selmaj KW. microRNA-301a regulation of a T-helper 17 immune response controls autoimmune demyelination. *Proc Natl Acad Sci U S A*. 2012;109(20):E1248–E1257.
5. Xu C, Inokuma MS, Denham J, et al. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol*. 2001;19(10):971-974.
6. Ma X, Kumar M, Choudhury SN, et al. Loss of the miR-21 allele elevates the expression of its target genes and reduces tumorigenesis. *Proc Natl Acad Sci USA*. 2011;108(25):10144-10149.

11. 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.

None