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CONTRACTING ORGANIZATION: Yale University

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<b>14. ABSTRACT</b>  The proposed project relates to the FY17 PRMRP topic area on pancreatitis. The project will explore a previously undescribed mechanism of acute pancreatitis in which pathological hemodynamic changes in the pancreas could induce acute pancreatitis responses. Two Aims are proposed. Aim 1 will determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis. In Aim 2, we will determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis. During the reporting period, we have established tissue clearing method with a immune-labeling technique for 3-dimensional imaging of lymphatic vessels in the pancreas. The method will be useful for analysis of not only lymphatic vessels, but also other cells in the pancreas. We also showed that macrophages are a key player for acute pancreatitis and pancreatic lymphangiogenesis in mice with portal hypertension.					
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## Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	5
3. Accomplishments.....	6
4. Impact.....	10
5. Changes/Problems.....	11
6. Products, Inventions, Patent Applications, and/or Licenses.....	12
7. Participants & Other Collaborating Organizations.....	13
8. Special Reporting Requirement.....	14
9. Appendices.....	15

## 1. INTRODUCTION:

The proposed project relates to the FY17 PRMRP topic area on pancreatitis. The causes of acute pancreatitis are not fully elucidated. Further, the role of the lymphatic system is little understood in acute pancreatitis in particular and in the study of the pancreas in general. The development of simple and reproducible experimental models of acute pancreatitis that are relevant to human disease are urgently needed. This project addresses these critical problems with innovative ideas. First, the project will define a new etiology of acute pancreatitis and explore a previously undescribed mechanism in which pathological blood flow changes in the pancreas could induce acute pancreatitis. Second, it will examine the role of the pancreatic lymphatic system in acute pancreatitis, representing the first step toward understanding biology of the pancreatic lymphatic system. Third, a new and simple experimental model of acute pancreatitis, which can also be used for the study of the pancreatic lymphatic system, will be established. Addressing a new etiology and a new area of study, the project will significantly contribute to our understanding of the etiology and mechanism of acute pancreatitis and could lead to the identification of new risk factors as well as new therapeutic strategies for this disease.

**2. KEYWORDS:** *(limit to 20 words).*

Acute pancreatitis, lymphatic system, lymphangiogenesis, etiology, blood flow, experimental model, risk factors, macrophages, T-cells, pancreatic stellate cells, inflammation, edema, VEGF-C

### 3. ACCOMPLISHMENTS:

- **What were the major goals (Specific Aims) of the project?**

**Specific Aims 1:** Determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis.

1. Determine the role of mechano- signaling in the development of acute pancreatitis (AP) (Exp 1-1). **In progress**
2. Determine the role of immune cells in the development of AP (Exp 1-2 to 1-4).  
**Exp 1-2 completed**
3. Assess a sensitizing effect of pancreatic hemodynamic changes on AP (Exp 1-5).  
**In progress**

**Specific Aim 2:** Determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis.

4. Establish a 3-D imaging method for lymphatic vessels in the pancreas (Exp 2-1).  
**Completed**
5. Determine the role of lymphangiogenesis in the development of AP (Exp 2-2 & 2-3).  
**Exp 2-2 completed**
6. Meeting presentation and manuscript preparation.  
**Two abstracts related to this project were presented during the 1<sup>st</sup> year of funding. Currently, two manuscripts are in preparation and expected to be submitted/accepted before 12/31/2020.**

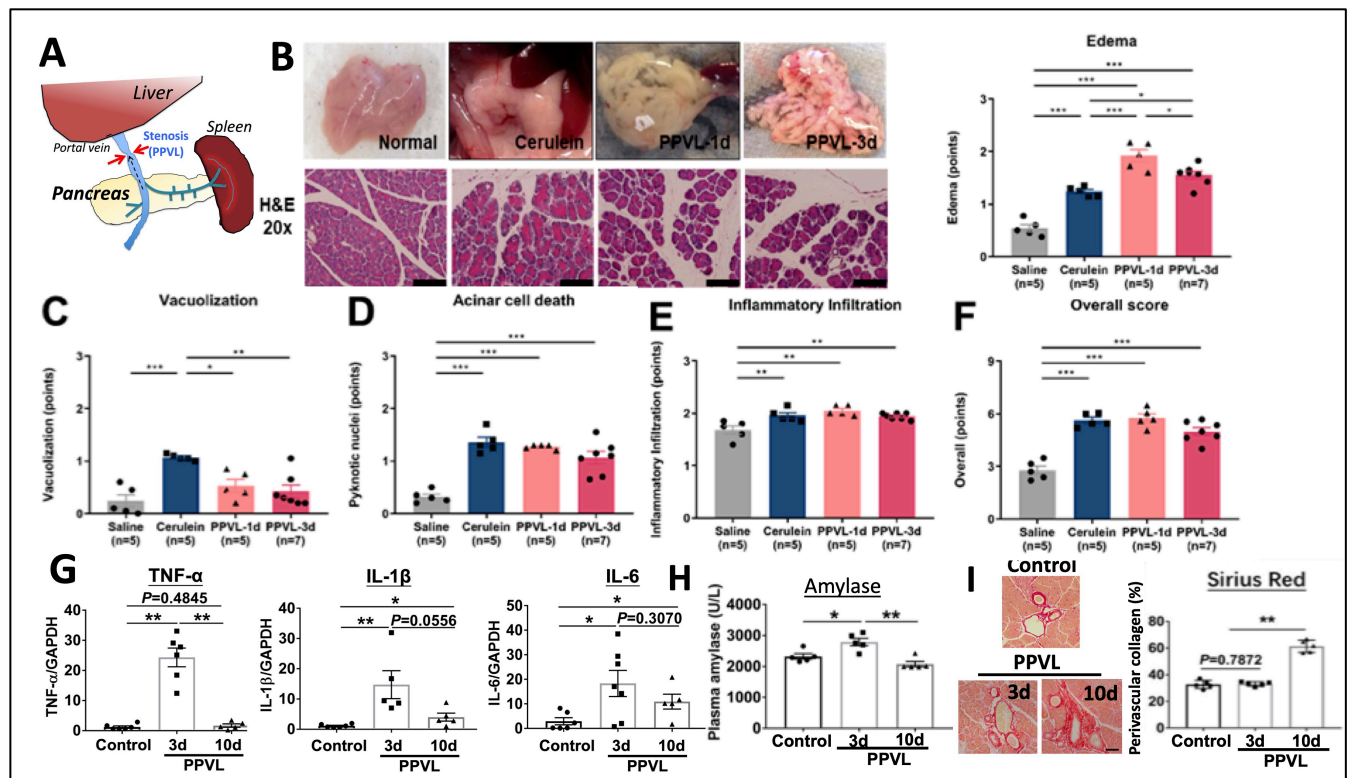
- **What was accomplished under these goals (Specific Aims)?**

Major accomplishments made are listed under each goal above in red. Below are summary of major activities, specific objectives and significant results/key outcomes in each Aim (Goal).

**Specific Aims 1:** Determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis (AP).

We have demonstrated that portal venous congestion, induced by partial portal vein ligation (PPVL) surgery (Fig 1A), leads to pancreatic edema (Fig 1B), inflammation (Fig 1C, D, E, F and G), injury (Fig 1H) and excess collagen deposition (Fig 1I), which are all typical of acute pancreatitis. Thus, pathological hemodynamic changes in the pancreas caused by portal venous congestion represent a new etiology of acute pancreatitis. The PPVL procedure, which has been used for the study of portal hypertension, can be used as a new experimental model for acute pancreatitis driven by hemodynamic changes.

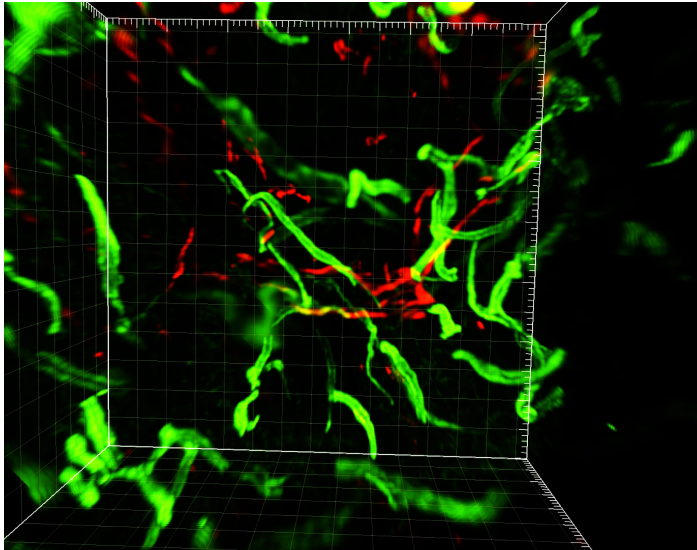
Macrophages started to infiltrate to the pancreas as early as 1 day after PPVL surgery (Fig 3A) and the depletion of these macrophages tended to decrease inflammation as indicated by edema formation. We are currently in the process of determining specific inflammatory cytokines associated with macrophage depletion.



**Fig 1. Portal venous congestion leads to acute pancreatitis in mice.** **A.** Partial portal vein ligation (PPVL). **B.** Pancreas isolated from sham, 1 and 3 days after PPVL, and the cerulein model as a reference. Scale bar: 100 $\mu$ m. Evaluation of edema. **C.** Vacuolization. **D.** Acinar cell death. **E.** Inflammatory infiltration. **F.** Overall score of C, D and E. **G.** Inflammatory cytokine expression. **H.** Amylase. **I.** Sirius red staining for assessment of collagen deposition. Scale bar: 200 $\mu$ m. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.005.

**Specific Aim 2:** Determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis (AP).

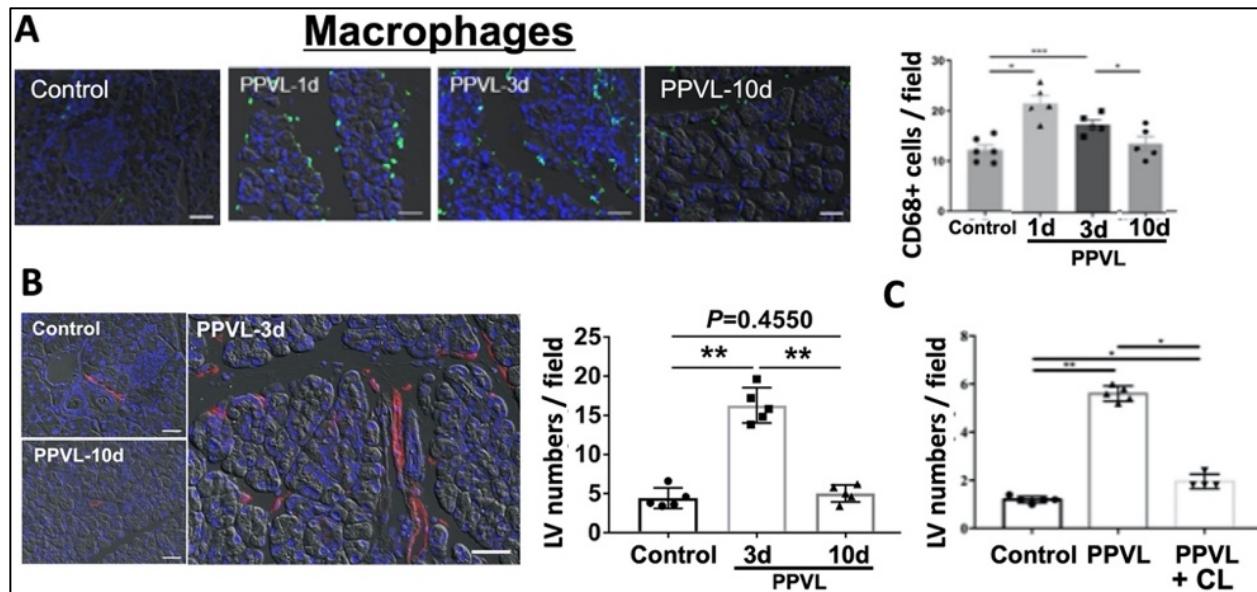
The objective of this Aim is to determine whether lymphangiogenesis facilitates or mitigates AP. We proposed to establish a 3-D imaging method for lymphatic vessels in the pancreas using podoplanin (pdpn)-GFP reporter mice [B6;D2-Tg(Pdpn, -EGFP)16Dobb/J, JAX Stock#:028357, The Jackson Laboratory, Bar Harbor, ME] (Exp 2-1), because the pdpn-GFP mice allow us to visualize lymphatic vessels in the pancreas. We received pdpn-GFP reporter mice recovered from cryopreservation from the Jackson Laboratory and established a colony to use visualization of pancreatic lymphatic vessels as originally planned. However, we found that the mice were not useful for our purpose, because their GFP expression was not strong enough. Instead, to pursue our planned studies we improved an antibody labeling technique and combined that with new tissue clearing methods for visualizing lymphatic vessels as shown in Fig 2.



**Fig 2. 3D image of lymphatic vessels (red) in relation to all vasculatures (green) in the pancreas.**

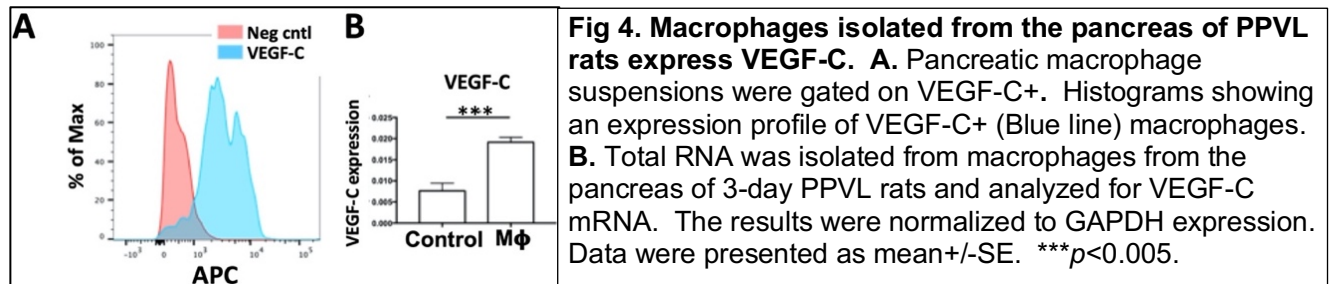
SunJin Lab Optical Clearing Innovation (Cedarlane Corporation, Burlington, NC) was used for tissue clearing. Cdh5-cre mTmG reporter mice, which express GFP in endothelial cells, was used to visualize all endothelial cells (all vasculatures) in the pancreas. Lyve-1 was used for immunolabeling lymphatic endothelial cells.

We have also demonstrated that macrophages facilitate lymphangiogenesis in mice with portal venous congestion (Fig 3B). Macrophages infiltrated the pancreas as early as 1 day after PPVL surgery (Fig 3A), when pancreatic edema was visible (Fig 1B). The presence of macrophages remained high at 3 days after PPVL, but returned to basal levels by 10 days. Pancreatic lymphangiogenesis occurred at 3 days after PPVL (Fig 3B) and after edema and macrophage infiltration increased. Macrophage depletion by chrodronate liposomes significantly reduced PPVL-induced pancreatic lymphangiogenesis in rats (Fig 3C). Further, macrophages isolated from the pancreas



**Fig 3. Macrophages promote pancreatic lymphangiogenesis.** **A.** Macrophage infiltration in the pancreas isolated from sham, 1, 3 and 10-day PPVL mice. Green: CD68 (macrophage), Blue: DAPI (nucleus). Scale bar: 10 $\mu$ m. \* $p$ <0.05, \*\* $p$ <0.01. **B.** Lymphatic vessels (LV). Red: podoplanin (a lymphatic endothelial cell marker). Blue: DAPI. Scale bar: 25 $\mu$ m. \* $p$ <0.05, \*\* $p$ <0.01. **C.** Macrophage depletion decreases lymphatic vessel (LV) numbers. CL: Clodronate liposome. \* $p$ <0.05, \*\* $p$ <0.01.

of 3-day PPVL rats showed VEGF-C expression, the most potent lymphangiogenic factor (Fig 4). Collectively, these observations indicate that macrophages promote pancreatic lymphangiogenesis in the setting of portal venous congestion.



- **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?**

**Specific Aims 1:** Determine the mechanism by which pathological hemodynamic changes cause acute pancreatitis (AP).

1. Determine the role of mechano- signaling in the development of AP (Exp 1-1).  
**Plan:** We will generate a sufficient number of VE-cad-TMD mice and their WT control, perform PPVL surgery and determine effects of mechano-sensing defect on AP and pancreatic fibrosis.
2. Determine the role of immune cells in the development of AP (Exp 1-3 and 1-4).  
**Plan:** First, we assess effects of macrophage depletion on AP and pancreatic fibrosis induced by PPVL. Second, we will perform PPVL surgery on immune deficient mice (nude mice) and assess AP and pancreatic fibrosis. Third, we will isolate macrophages and T-cells from pancreas of mice given PPVL or sham operation and perform RNA sequencing to determine genes responsible for the development of AP and pancreatic fibrosis.
3. Assess a sensitizing effect of pancreatic hemodynamic changes on AP (Exp 1-5).  
**Plan:** We will administer cerulein intraperitoneally to mice with sham or PPVL surgery and AP and pancreatic fibrosis.

**Specific Aim 2:** Determine the role of lymphangiogenesis in the development and/or the resolution of acute pancreatitis (AP).

4. Determine the role of lymphangiogenesis in the development of AP (Exp 2-3).  
**Plan:** We will perform PPVL surgery on mice with control or adoviral-sVEGFR3 delivery to block lymphangiogenesis, then assess AP and pancreatic fibrosis.

I would like to thank the Department of Defense to support our research on pancreatitis. This grant provides us to pursue our research on pancreatitis which was new to us. Recently, we were invited to submit a full application of Discovery Award (PR200215), which is based on the discovery in the current project. We will submit a full application for Discovery Award due on September 3. During the rest of the funding period, our goal is to submit two manuscripts for publication.

#### 4. IMPACT:

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

We have established tissue clearing method and immune-labeling protocol for 3-D imaging of lymphatic vessels in the pancreas. The method can be used for visualizing not only lymphatic vessels, but also other cells in the pancreas. Pathological hemodynamic changes in the pancreas caused by portal venous congestion represent a new etiology of acute pancreatitis. Other conditions, such as transarterial chemoembolization for hepatocellular carcinoma and hypercoagulation states (acquired or inherited), may cause similar hemodynamic changes in the pancreas, indicating broader implications of this study.

- **What was the impact on other disciplines?**

Nothing to report.

- **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:

As described previously, we received podoplanin (pdpn)-GFP reporter mice recovered from cryopreservation from the Jackson Laboratory (Bar Harbor, ME) and established a colony to use visualization of pancreatic lymphatic vessels as originally planned. However, we found that the mice were not useful for our purpose because their GFP expression was not strong enough. Instead, to pursue our planned studies we improved an antibody labeling technique and combined it with new tissue clearing methods for visualizing lymphatic vessels as shown in Fig 2.

Due to the COVID19 pandemic, we had to stop all experiments in mid-March 2020. Included were experiments to investigate the underlying mechanism of acute pancreatitis driven by pathological hemodynamic changes using a transgenic mouse with a mutation in the transmembrane domain (TMD) of VE-cadherin. This domain is important for flow-induced, ligand-independent VEGFR2 signaling (VE-cad-TMD mouse). Also included were experiments to be performed in a T-cell deficient mouse [nude mouse (NU/J, *Foxn1nu*), Jackson Laboratory]. Upon partial re-opening of my lab in early June, we have resumed these experiments with additional postdoc associate to complete the project by the end of the funding period (12/31/2020).

## 6. PRODUCTS:

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

1. **Journal publications.**

Nothing to report.

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

Nothing to report.

3. **Other publications, conference papers, and presentations.**

Nothing to report.

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

Nothing to report.

- **Technologies or techniques**

We have established a tissue clearing method with immune-labeling techniques for 3-D imaging of lymphatic vessels in the pancreas. The method can be used for visualizing not only lymphatic vessels, but also other cells in the pancreas. We will share this technique in our future publication.

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

Nothing to report

- **Other Products**

This project generated a new animal model of acute pancreatitis, namely partial portal vein ligation (PPVL).

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

Name:	Jain Jeong
Project Role:	<i>Postdoc associate</i>
Researcher Identifier (e.g. ORCID ID):	<i>None</i>
Nearest person month worked:	3 months
Contribution to Project:	<i>Dr. Jeong has worked on the experimental protocol to establish immune-labeling and 3-D imaging methods for pancreatic tissues</i>
Funding Support:	DOD

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

R56 DK121511 (Iwakiri)                      09/17/19 – 09/16/20                      1.2 Calendar  
 NIH/NIDDK                                      \$256,685

Lymphatics in the liver

This study investigates the mechanism of hepatic lymphangiogenesis focusing on sympathetic nerve system in the liver and determines the role of lymphatics in the pathogenesis of liver disease. There is no overlap with the current DOD grant.

- What other organizations were involved as partners?

Nothing to report.

## 8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**

Nothing to report.

- **QUAD CHARTS:**

Nothing to report

**9. APPENDICES:**

None.