Microenvironment Changes in the Pancreatic Stroma Induced by Inflammation

2016

Kathryn Cline
University of Central Florida

Find similar works at: https://stars.library.ucf.edu/honorstheses

University of Central Florida Libraries http://library.ucf.edu

Part of the Life Sciences Commons, and the Medicine and Health Sciences Commons

Recommended Citation

Cline, Kathryn, "Microenvironment Changes in the Pancreatic Stroma Induced by Inflammation" (2016). Honors Undergraduate Theses. 104.
https://stars.library.ucf.edu/honorstheses/104

This Open Access is brought to you for free and open access by the UCF Theses and Dissertations at STARS. It has been accepted for inclusion in Honors Undergraduate Theses by an authorized administrator of STARS. For more information, please contact lee.dotson@ucf.edu.
MICROENVIRONMENT CHANGES IN THE PANCREATIC STROMA INDUCED BY INFLAMMATION

by

KATHRYN A. CLINE

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biomedical Science in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, Florida

Spring Term, 2016

Thesis Chair: Dr. Deborah A. Altomare
ABSTRACT

Pancreatic cancer is the product of microenvironment alterations which emerge from inflammatory signaling and progress to more devastating cases such as Pancreatic Ductal Adenocarcinoma (PDAC). PDAC is extremely aggressive with a statistical five-year survival rate of merely 3%-5%, and is more than relevant to cancer research being that it is the fourth leading cause of cancer-related deaths in the US. Unfortunately pancreatic cancer is often unnoticed until reaching its hardly treatable end stages, which perpetuates the low survival rate.

The onset of PDAC may be facilitated by the activation of pancreatic stellate cells (PSCs), which secrete collagen and markedly contribute to tissue fibrosis. Inflammatory factors and activation of PSCs are hallmarks of pancreatitis and could increase occurrence rates of pancreatic cancer.

The purpose of this thesis is to elucidate inflammatory signaling patterns starting with the onset of acute pancreatitis and through future studies of the more damaging states of chronic pancreatitis and cancer progression. Through the induction of acute pancreatitis in oncogenic and wild type mouse models and evaluating cytokine expression levels via RT-PCR a link between inflammatory signaling and disease state progression will be delineated. This model utilizes mice with mutant KRas, a gene activated in nearly all PDAC incidences, and constitutively active Akt, an oncogene activated in nearly all cancers. Preliminary results indicate that when experimentally inducing pancreatitis in mice predisposed to pancreatic cancer tissue remodeling and leukocyte infiltration is observed as a result of cytokine expression. Furthermore, macrophage and neutrophil
stains are positive with one round of cerulein injections proving that acute inflammation is induced by these methods.

Pancreatitis is a risk factor for pancreatic cancer which can be caused by environmental factors including smoking, alcohol consumption, and obesity. By understanding the mechanism by which inflammation occurs and the cytokine signaling involved we can attempt inhibit tumor-promoting signaling pathways in the pancreas stroma.
ACKNOWLEDGEMENTS

I wish to express my thanks to all who helped this thesis come to fruition. Thank you to Dr. Michal Masternak and his lab for donating their time, knowledge, and reagents. Thank you to Aladdin Raid for educating me throughout this process. To my committee, Dr. Eric Hoffman and Dr. Michal Masternak I am grateful for your time and guidance in completing this project. Sarah Gitto, the years spent under your direction and unending support have been greatly enjoyed. You truly have been a guiding influence for me and this project and I will be forever grateful. I am especially grateful to my thesis chair and mentor Dr. Deborah Altomare for giving me the opportunity to volunteer in her lab and always supporting my interests and ventures. Without your guidance and support none of this would have been possible. Finally, my family, thank you for always driving me to be better and work harder each day and always reminding me that I am capable of achieving great things.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>BACKGROUND</td>
<td>4</td>
</tr>
<tr>
<td>Inflammation: Extravasation and Diapedesis</td>
<td>4</td>
</tr>
<tr>
<td>Monocytes: Maturation to Macrophage</td>
<td>6</td>
</tr>
<tr>
<td>Macrophage Identification and Population Profiling</td>
<td>7</td>
</tr>
<tr>
<td>Animal Model</td>
<td>9</td>
</tr>
<tr>
<td>Cytokines and Regulators of Interest</td>
<td>9</td>
</tr>
<tr>
<td>SPECIFIC AIMS</td>
<td>11</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>11</td>
</tr>
<tr>
<td>Specific Aim 1</td>
<td>11</td>
</tr>
<tr>
<td>Specific Aim 2</td>
<td>11</td>
</tr>
<tr>
<td>Specific Aim 3</td>
<td>12</td>
</tr>
<tr>
<td>PRELIMINARY RESULTS</td>
<td>13</td>
</tr>
<tr>
<td>Inducing Inflammation: Cerulein Injections</td>
<td>13</td>
</tr>
<tr>
<td>Attempt at Analyzing Cytokine Expression: mRNA Quantification</td>
<td>16</td>
</tr>
<tr>
<td>METHODS</td>
<td>17</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1: Cerulein Injection Scheme ................................................................. 13
Figure 2: Cerulein Injected Tissue Injury at 24 Hours ........................................ 14
Figure 3: Immune Response in KRas Mice after 1 Injection (H&E, Left, Trichrome, Right) ..... 15
Figure 4: Microenvironment Changes and Increased Inflammatory Response Over Time ........ 15
Figure 5: RNALater Preserved Whole Tissue Agarose Gel with 28S and 18S rRNA Bands ...... 21
Figure 6: Housekeeping Gene Amplification Agarose Gel (QARS and GAPDH) ............... 24
Figure 7: Housekeeping Gene Amplification Agarose Gel (QARS, GAPDH and B2M) ....... 25
Figure 8: cDNA Agarose Gel .............................................................................. 32
Figure 9: IL-6 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression .............................................................................................................. 35
Figure 10: F4/80 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression .............................................................................................................. 35
Figure 11: TNFa Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression .............................................................................................................. 36
Figure 12: Akt1 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression .............................................................................................................. 37
Figure 13: Akt2 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression

Figure 14: NF-κB Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression Indicating ** Significant Variance
LIST OF TABLES

Table 1: 96-Well Plate Organization for RT-PCR Optimization of Housekeeping Genes (QARS, GAPDH and B2M) .................................................................................................................. 26

Table 2: Organization of Mice into Injection Groups............................................................................................................. 29
LIST OF ABBREVIATIONS

AP: Acute Pancreatitis
CP: Chronic Pancreatitis
PanINs: Pancreatic Intraepithelial Neoplasia
PDAC: Pancreatic Ductal Adenocarcinoma
PSCs: Pancreatic Stellate Cells
KRas: Oncogene involved in cell proliferation
Akt: Oncogene involved in cell immortality
ICAM1: Intracellular adhesion molecule 1
TNFα: Tumor Necrosis Factor alpha, pro-inflammatory cytokine
IL-6: Interleukin 6, pro-inflammatory cytokine
MCP-1: Monocyte chemotactic protein 1
NF-κB: Nuclear Factor kappa B, transcription regulator
IFNγ: Interferon γ
F4/80: Mouse macrophage surface antigen
CCK: Cerulein, inflammatory drug
PBS: Phosphate Buffered Saline
RT-PCR: Real Time Polymerase Chain Reaction, quantifiable amplification

H&E: Hematoxylin and Eosin morphology stain

Trichrome: Collagen stain

MPO: Myeloperoxidase

QARS: Glutaminyl-tRNA synthetase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

B2M: Beta 2 Microglobulin

EtBr: Ethidium Bromide

Tm: Melting temperature
INTRODUCTION

Pancreatitis is characterized as inflammation of the pancreas and can be categorized into two main forms, either acute (AP) or chronic (CP). Acute occurs suddenly and with a short duration, while chronic inflammation is prolonged and leads to irreversible scarring or fibrosis of the pancreas. Acute pancreatitis has been shown to be perpetuated by the release of pancreatic digestive enzymes into the pancreatic tissue thus further damaging pancreatic cells and causing production of inflammatory cytokines such as TNF-α and IL-1β which recruit immune cells to the site of trauma. Acute pancreatitis alone causes the most gastrointestinal related hospitalizations in the US, in fact in 2009 it accounted for 275,000 known hospital admissions. Chronic and acute pancreatitis occur annually at instances estimated to be between 5 to 12 cases per 100,000, and 13 to 45 cases per 100,000 respectively. Both conditions are commonly linked to alcohol consumption, smoking, and gallstones and lead to pancreatic cell death as well as irreversible fibrosis, thus decreasing overall function of the pancreas [1]. Chronic pancreatitis is a known risk factor for the development of pancreatic cancer, both baring the same central theme of pancreatic stellate cell activation [1].

Because pancreatic cancer is the product of microenvironment alterations which emerge as pancreatic intraepithelial neoplasia (PanINs) and progress to more devastating manifestations such as Pancreatic Ductal Adenocarcinoma (PDAC), it is of interest to elucidate the connection between repetitive pancreatic trauma and tumor formation. PDAC is extremely aggressive with a statistical five-year survival rate of merely 3%-5%, and is more than relevant to cancer research being that
it is the fourth leading cause of cancer-related deaths in the United States [2]. The onset of PDAC may be facilitated by the activation of pancreatic stellate cells (PSCs), which secrete collagen and markedly contribute to tissue fibrosis. Inflammatory cytokines and activation of PSCs are hallmarks of pancreatitis and could increase occurrence rates of pancreatic cancer.

Unfortunately pancreatic cancer is often unnoticed until reaching its hardly treatable end stages, which perpetuates the low survival rate. Furthermore, methods of screening are nearly nonexistent apart from invasive biopsy and histological evaluation of the pancreas. Thus, prevention by means of understanding and limiting risk factors such as pancreatitis may serve as a new means for decreasing this diseases toll on our population. Eventually we hope that by understanding the mechanism by which inflammation affects tumor formation we can inhibit tumor-promoting changes in the pancreas stroma.

KRas mutations are linked to nearly all cases of ductal proliferation in the pancreas which results in PDAC [3]. While Akt is hyperphosphorylated in nearly all forms of cancer because when constitutively active it prevents apoptosis, thus creating the basis of immortal cancer cells [4]. Cerulein which is a decapeptide analog of the peptide hormone cholecystokinin, is seen to induce an inflammatory response that mimics pancreatitis when injected into mice due to its regulatory effects on the secretion of pancreatic enzymes and bile [5][6].

This project will focus on determining the expression levels of inflammatory and immunosuppressive cytokines induced in transgenic mice that have been engineered with activated KRas, and or Akt1 (Akt1/KRas) oncogenes, which are known to facilitate PDAC. Preliminary studies, discussed below, have indicated that cerulein induced acute pancreatitis results in chronic
pancreatitis as well as reduced tumor latency in Akt1/KRas mice compared to control wild type mice [7].

Through evaluating the variations in cytokine expression throughout the progression of acute pancreatitis and increasing damage to the pancreas, we expect to find expression patterns that could be used as parameters for detection of pancreatic distress. This strategy should allow doctors to identify patients with risk factors and prevent further damage or disease progression. The overall goal is to understand the changes within the pancreatic environment that lead to tumorigenesis in an effort to develop a therapy that inhibits overactive aberrant protein signaling that could otherwise circumvent conventional treatments.
BACKGROUND

Immune cell and cytokine populations play a large role in evaluating the progression of inflammatory diseases such as pancreatitis. Though pancreatitis and PDAC cannot be clearly linked to each other CP and PDAC do display similar infiltrating inflammatory cell populations [8]. The inflammatory process of acute pancreatitis is commonly initiated by environmental factors such as alcohol consumption, which damages the pancreas and can reoccur or progress to a chronic state which is hallmarked by irreversible fibrosis of the pancreas. Chronic pancreatitis may lead to the development of PDAC [9], through mechanisms associated with continued inflammation that induces cytokine production and alteration in the immune cell population which in turn sets off an elusive cascade resulting in immunosuppression and tumorigenesis.

**Inflammation: Extravasation and Diapedesis**

Inflammation, like that of acute pancreatitis, begins with recruitment and invasion of neutrophils and monocytes into pancreatic tissue. This process known as extravasation and/or diapedesis involves vasodilation and decreased rate of blood flow which allows for small separations in the vascular endothelium that increase vascular permeability to leukocytes and allows their eventual escape (diapedesis) from the bloodstream into the damaged tissue [10]. The diapedesis itself however requires the assistance of adhesion molecules expressed on the internal surface of the vascular endothelium in order for the immune cells to first adhere to the endothelial cells before they are able to traverse the blood vessel wall. The combination of the slowed blood flow and expression of adhesion molecules on the vascular endothelium allow for capture of
leukocytes onto the surface of the vessel and result in a rolling motion of the leukocytes as they pass from one adhesion molecule to the next atop the endothelium. Increased contact with the vascular endothelium in an area that has been activated in response to trauma brings the leukocytes into contact with pro-inflammatory cytokines that activate and signal the leukocytes to enter the tissue [10].

Akt is a survival factor that becomes activated in instances of trauma and is involved in signaling that modulates acute inflammation [11]. In the process of inflammation Akt regulates the level of permeability of the vascular endothelium, as a result Akt therefore also regulates the degree of leukocyte infiltration into damaged tissues [11]. Specifically in knockout Akt1 studies, vascular permeability is markedly decreased and acute inflammation is suppressed compared to normal Akt1 expressing genotypes. However Akt2, another Akt isotype, in the same studies did not impact vascular permeability [11].

Studies have shown that with cerulein injections, intracellular adhesion molecule 1 (ICAM-1) is upregulated thus facilitating immune cell invasion [12]. Furthermore after entering the pancreas, neutrophils themselves have the potential to activate trypsinogen, a digestive enzyme, and perpetuate the inflammatory process [8]. As further evidence for the connection between adhesion molecules, neutrophils, and acute pancreatitis, a study conducted by Frossard et al. demonstrated that drug or genetically induced deficiencies in both ICAM-1 and neutrophils decreased the severity of acute pancreatitis [13].

Interleukin 6 (IL-6) is a cytokine that plays a complex role in the onset of inflammation as well as the adaptations that occur within the tissue as an immune response occurs. Primarily, IL-6
functions to initially assist in recruiting neutrophils to the site of trauma via signaling that up
regulates expression of adhesion molecules such as ICAM-1 [14]. Neutrophils however have a
short lifespan compared to other immune cells and are therefore only active in acute inflammation
within the tissue for 24 to 48 hours after leaving circulation. Studies have shown that IL-6 has a
role in apoptotic signaling in neutrophils shortly after tissue infiltration. This leads to a necessity
for recruiting a new group of immune cells after the neutrophil population has been exhausted. At
this point IL-6 signaling is altered to instead up regulate expression of monocyte adhesion
molecules thus recruiting monocytes into the tissue [14].

**Monocytes: Maturation to Macrophage**

Once monocytes enter the tissue they differentiate into dendritic cells or macrophages and
become activated. In the presence of IL-6 the monocytes entering the tissue preferentially mature
into macrophages [14]. The level of macrophage activation within the pancreas thereafter may
assist in determining the degree of acute pancreatitis severity. Active macrophages secrete
cytokines including tumor necrosis factor (TNF) α along with monocyte chemotactic protein
(MCP) 1, and interleukins (IL) 1β, IL-6. MCP-1 has been shown to both attract monocytes to the
site of inflammation as well as activate them. In an effort to determine the role of MCP-1 in acute
pancreatitis, a study was conducted by Bhatia et al. that demonstrated MCP-1 synthesis blocker
(bindarit) administration in conjunction with cerulein injections markedly reduced the severity of
AP [15].

TNFα is a primary pro-inflammatory cytokine. Thus, it is expressed at instances of trauma
to elicit an immune cell response and inflammatory cascade of signaling. TNFα expression
influences the activation of Nuclear Factor (NF) κB [16]. NF-κB has been seen in studies of inflammatory diseases to enable production of cytokines that perpetuate the inflammatory response [16] by receiving environmental stimulus and responding by moving from the cell surface to the nucleus where it functions in initiation of transcription of cytokines. Interestingly Akt has been demonstrated to be a mediator in the activation of NF-κB via interaction of TNFα with Akt followed by Akt’s positive regulation of NF-κB [17]. In this way NF-κB and Akt can be considered regulators of cytokine production.

However, NF-κB also functions in terminating inflammation and thus limits the duration of the response. It has been discovered via NF-κB inhibition that the apoptosis of tissue infiltrating immune cells after trauma has been resolved is dependent on NF-κB signaling [18] similar to the way that IL-6 signals apoptosis of neutrophils.

**Macrophage Identification and Population Profiling**

Murine (mouse) macrophages express a set of antigens that make them uniquely identifiable from other immune cells, these antigens are F4/80, CD11b, and CD68 [19]. F4/80 specifically is expressed on the surface of macrophages and functions in adhesion, cellular binding, and recognition [20]. These qualities have made F4/80 the primary antibody for immunohistochemistry detailing macrophage populations, as well as a valuable marker for positively detecting macrophage infiltration.

The functions of macrophages are highly diverse. They can act as either pro- or anti-inflammatory cells as well as tumor promoting or tumor preventing. This versatility has generated two distinct macrophage classifications; M1 and M2. M1 macrophages are activated by interferon
(IFN) γ, lipopolysaccharides that are associated with the cell wall of bacteria, and tumor necrosis factors, as well as knockout of Akt1 [21]. They function in inflammation and tumor resistance. M2 macrophages are activated as a result of IL-4, IL-13, and IL-10 signaling, and act as immunosuppressors and tumor promoters [22]. Interestingly, knockout of Akt2 has been seen to also stimulate the differentiation of macrophages into the M2 state [21]. M1 is associated with high IL-12, IL-23, and TNFα secretion levels, while M2 secretes TGF β and IL-10.

Thus, M1 macrophages are commonly associated with inflammatory responses such as acute pancreatitis, and studies have even gone as far as to suggest that switching M1 to M2 could provide a new therapy for pancreatitis by suppressing the immune response and limiting fibrosis [8]. Fibrosis associated with the pancreas is the result of stellate cell activation, commonly caused by toxins like alcohol. However studies have shown that increased amounts of activated macrophages in the pancreas can also induce stellate cell activation and fibrogenesis through TGFβ signaling [23].

Previous cytokine based therapies that utilized IL-10 and anti-TNF have proved fruitless. In double-blind studies of IL-10’s effects, results have been inconclusive, one study found increased AP with IL-10 treatment and another found no discernable difference between treated and control. Other studies have attempted to switch macrophages between M1 and M2 using IL-4 and IL-13 but have failed in vivo. Anti-TNF studies have been halted by high risks of infection associated with treatment [8].
Animal Model

Utilization of transgenic KRas mouse models in conjunction with cerulein injections have allowed for the observation of AP and CP progression with an acceleration of tumor development, and has elucidated a potential connection between tissue damage due to pancreatitis and PDAC. This is the leading model for studying the immune response throughout the inflammatory processes of PDAC [8]. However, alterations of this transgenic KRas model in an effort to mimic human PDAC formation have turned to combination with knockouts of tumor suppressor genes such as PTEN. With certain tumor suppressor gene knockouts, like that of PTEN, deterioration of the mice is rapidly accelerated and thus there is decreased time to study initial or acute disease states [7]. Yet, with constitutively active Akt1, there is acceleration of tumor formation and increased longevity of the mice compared to other tumor suppressor gene manipulations, thus allowing for more longitudinal studies to be conducted [7]. This combination Akt1^{Myr}/KRas^{G12D} model allows the opportunity for a natural disease progression and aging of the mice which accurately reflects the human progression of pancreatic diseases, and lends itself to acute phase studies.

Cytokines and Regulators of Interest

The cytokines of particular interest at this stage of experimentation are those associated with initial acute immune responses. Beginning with the initial stages of the inflammatory response, extravasation and diapedesis which involves Akt1 vascular permeabilization and IL-6 dependent adhesion molecule expression, the overall perpetuation of a pro-inflammatory state via TNFα leads to an activation of NF-κB. This allows for a shift in immune cell population from
primarily neutrophils to macrophages and transcription of many pro-inflammatory signaling molecules, as well as positively identifying the presence of macrophages in the tissue via F4/80 that have been differentiated under the influence of IL-6 and secrete TNFα. Finally, further understanding the macrophage population via the presence or absence of Akt2 may be necessary in order to determine whether the tissue environment is pro or anti-inflammatory.
SPECIFIC AIMS

Pancreatic cancer is usually undiagnosed until the prognosis is inevitable death from the disease. Symptoms tend to be vague or all together nonexistent and testing methods are also problematic. A method for tracking disease progression from states of increased risk like that of pancreatitis is imperative in decreasing the instance of pancreatic cancer deaths across the globe.

Hypothesis

By quantifying inflammation signaling mRNA levels within inflamed pancreas, pathways of disease progression will be better understood and markers for early detection will be improved.

Specific Aim 1:

Determine if pancreatitis can be induced by injecting a group of transgenic KRas and Akt/KRas mice with a dose of cerulein (CCK) based on weight once hourly for 5 hours, and with Phosphate Buffered Saline (PBS) injections by the same regimen as a control in transgenic KRas and Akt/KRas mice.

Specific Aim 2:

Determine a method to extract RNA from pancreas and convert to cDNA in order to quantify levels of inflammatory cytokine expression utilizing SYBR Green RT-PCR and select specific primers for TNF-α, IL-6, F4/80, NF-κB, Akt1, and Akt2.
Specific Aim 3:

Compare cytokine levels across different genotypes and treatments thus elucidating cytokine expression patterns and their downstream effects on the pancreatic microenvironment.
PRELIMINARY RESULTS

**Inducing Inflammation: Cerulein Injections**

To determine the approximate time points of disease progression in the animal models, a related pilot study was referred to. In previous studies, KRas mice responded more severely to typical dosing of 50 µg/kg hourly for 6 hours twice a week than wild type mice [24], thus the scheme was paired down to achieve the desired level of inflammation in our model. In the pilot studies, the genotypes KRas, Ak1/KRas, and wild type were treated with 50 µg per 1 kg body mass Cerulein (CCK) once hourly for 5 hours from age 2.5 months to 3 months every other week for a total of 8 injections. Concomitantly a control group containing each genotype was treated using the same protocol with PBS. This injection scheme was designed uniquely for this project so as to induce pancreatitis in KRas and Akt1/KRas mice. Blood serum and tissue collections were done before and after the first set of injections and then after the fourth and eighth injections. Serum and histological stains at final collection were compared across genotypes for evidence of inflammation, fibrosis, and tumor formation.

*Figure 1: Cerulein Injection Scheme*
Results from Hematoxylin and Eosin (H&E), Trichrome to detect collagen deposition, and general histological stains form macrophages (F4/80) as well as neutrophils (Myeloperoxidase; MPO) suggested that inflammation, fibrosis, and tumor formation were all occurring throughout the time points of the injections with increasing severity except in wild type mice. Most notably, mice with KRas and especially Akt and KRas showed that infiltration of immune cells and collagen deposition were exacerbated compared to control wildtype mice of the same time point and treatment. This was indicated by a noticeable inflammatory response, antibody binding to expected proteins and cell types, in tissue collected 48 hours post injection.

![Hematoxylin & Eosin and Trichrome images]

*Figure 2: Cerulein Injected Tissue Injury at 24 Hours*
Based on this evidence it was concluded that the presence of transgenic oncogenes, Akt and KRas, altered the sensitivity of these models to experimentally induced inflammation and fibrosis. These findings support the need for investigation into cytokine expression throughout the course of the disease starting with treatment to induce acute pancreatitis in order to further delineate potential mechanisms of response.
Attempt at Analyzing Cytokine Expression: mRNA Quantification

In order to establish a benchmark for methods of evaluating cytokine expression levels, pancreatitis was induced in 25 mice via intraperitoneal CCK injections following the cerulein injection scheme from the above study. The mice genotypes included KRas, Akt/KRas, and wild type which were divided into 2 groups which received either CCK or PBS. Tissue was collected according to IACUC protocol 24 hours post 1 round of injections and frozen at -80° C.

Tissue was then homogenized in liquid nitrogen using a mortar and pestle prior to RNA extraction. Once RNA extraction was complete cDNA was synthesized. This cDNA was used to analyze mRNA expression of housekeeping genes via RT-PCR. RNA quality however was not ideal which lead to a lack of viable quantification.
METHODS

Optimization

RNA Preservation: RNase Inhibitors

Due to the fact that RNA is easily degraded, and was not adequately preserved in previous studies, use of RNase inhibitors at the time of pancreas collection became a main interest. The pancreas as previously mentioned is the center for production of many exocrine digestive enzymes and is therefore full of tissue destroying enzymes, in fact a mouse’s pancreas can hold up to 75 mg of ribonucleases [25]. As such, coupled with the instability of RNA, the pancreas is a difficult organ to harvest RNA from. In order to prevent leakage of digestive enzymes into the pancreas and destruction of RNA at harvest, a different method of collection was needed.

Based on Azevedo-Pouly et al. stress on the animals and potential for pancreatic autolysis [25] was limited by eliminating submandibular bleeding from the collection protocol. Next, a more gentle IACUC protocol for sacrifice was considered, cervical dislocation under isoflurane gas followed by diaphragm puncture which eliminated trauma to the body and potential for release of destructive enzymes into the tissue. Finally better methods for storing of the tissue were evaluated considering the immediate placement of the harvested pancreas into RNase inhibiting chemical solutions. TRIzol (ThermoFisher, Waltham, MA) and RNALater (ThermoFisher, Waltham, MA) were tested side by side with both whole and cut tissue.

For this side by side comparison 2 ml microcentrifuge tubes were labeled as follows; “whole tissue RNALater”, “cut tissue RNALater”, “whole tissue TRIzol”, “cut tissue TRIzol” and then filled with 1ml of the corresponding RNase inhibitor and placed on ice. Mice (animal numbers
5533, 5539, 5543, and 5544) were then sacrificed according to the variant IACUC protocol previously outlined. For “whole tissue” samples the pancreas was removed and directly placed into the corresponding tube using sterile tweezers. For “cut tissue” samples the pancreas was removed and placed into a sterile petri dish containing 1ml of the corresponding RNase Inhibitor and cut using a sterile scalpel into approximately 0.5 cm by 0.5 cm sections before moving them to their labeled tube via a disposable sterile pipette. After collection was complete all tubes were incubated in 4\(^{\circ}\) C overnight to allow the RNase inhibitors to fully infiltrate and protect the tissue before moving to -80\(^{\circ}\) C for long term storage.

**Homogenization with RNase Inhibitors**

Collected tissue was then homogenized in liquid nitrogen using a mortar and pestle in order to prepare for RNA extraction. Because cytokine expression is measured by mRNA expression, only RNA was desired from the tissue. Working under a chemical hood and using proper protective equipment to avoid cryogenic hazards, samples moved from -80\(^{\circ}\) C storage to ice to thaw before further processing. The mortar and pestle was prepared by cleaning with soap and water followed by a rinsing with nuclease free water. Once the TRIzol treated samples were thawed on ice and the entire contents of the tube was dumped into the mortar followed by the immediate poring of approximately 5 ml of liquid nitrogen atop the sample. The samples were crushed with a clean pestle in the presence of liquid nitrogen. It was extremely important to work quickly at this stage in order to prevent additional exposure of the tissue to RNases. If the tissue was not finely ground enough by the time the liquid nitrogen evaporated more liquid nitrogen was added and the crushing repeated.
Once all liquid nitrogen had evaporated and tissue was crushed, 5 ml of TRIzol was transferred immediately into mortar with powdered pancreas. Again, this step was performed quickly to prevent exposure to RNases as TRIzol is an RNase inhibitor. TRIzol was pipetted up and down to pick up all crushed pancreas and transferred to an RNase free 15 ml conical tube labeled with the corresponding animal identification number. The tube was vortexed for approximately 30 second and then incubated at room temperature vortexing intermittently for 5 to 10 minutes or until all tissue was dissolved in TRIzol and no lumps were apparent.

The RNAlater treated whole pancreas was thawed on ice and then the tissue was transferred to the mortar using sterile tweezers followed immediately by the poring of liquid nitrogen over the tissue and crushing just as previously described. The RNAlater treated cut tissue was thawed on ice and then the entire contents, RNAlater included was poured into the mortar and homogenized in the same way as all previous samples. A phase separation of the RNAlater and TRIzol was noted, the TRIzol layer being less dense than the RNAlater. All samples were aliquoted into 1ml volumes and stored at -80⁰ C until RNA extraction.

**RNA Extraction**

RNA was then extracted from the homogenized pancreas samples. 0.2 ml chloroform was added to the homogenized sample per each ml of TRIzol and vortexed (thus 200 µl per 1 ml homogenized aliquot), then incubated at room temperature for 2-3 minutes. Then all samples were centrifuged at 4⁰ C for 15 minutes at 10,000xg to allow phase separation. The aqueous phase was carefully transferred to a new RNase free tube immediately after centrifugation, taking care to avoid the opaque interphase. For final extraction, PureLink RNA Mini Kit spin columns (Ambion,
Waltham, MA) were used. This system utilizes silica beads that bind RNA specifically and allow all other substances to elute freely. One volume of RNase free 70% ethanol was added to the aqueous phase previously separated, next the tube was vortexed.

Up to 700 µl at a time of sample was transferred to a spin cartridge and spun at 12,000xg for 15 seconds. Flow through was discarded and these steps were repeated until all of the sample was processed through the column no greater than 700 µl at a time, for this study this step was repeated on average 3 times for each sample. Next the columns were washed with a series of proprietary buffers, Wash Buffer 1 and 2 provided in the Ambion kit. Finally the column was dried with a 1.5 minute 12,000xg spin. Elution of the purified RNA was performed by moving the dried column into a collection tube provided within the Ambion kit and adding 100 µl of RNase free water to the membrane. The RNA and water was incubated for 1 minute at room temperature and finally the column within the collection tube was spun for 2 minutes at 12,000xg after which the membrane was removed and the viscous RNA containing liquid was saved within the collection tube.

Immediately following extraction the RNA was run on an EtBr agarose gel to test quality. The gel was prepared by combination of 1.2 g agarose powder with 60 ml TBE buffer in a flask and warming until the agarose powder was dissolved. Then 4 µl EtBr was pipetted into the flask under a chemical hood and swirled until combined. The mixture was then poured into a gel mold and allowed to cool. After cooling the gel was placed into its docking station and the compartment was filled with 1X TBE buffer. The agarose gel was loaded as follows; 1st lane 100 bp ladder, and the rest with a loading mix made up of 1 µl sample, 4 µl TE buffer, and 1 µl loading dye, 2nd lane TRIzol whole tissue, 3rd lane TRIzol cut tissue, 4th lane RINAlater whole tissue, and 5th lane
RNA later cut tissue. The agarose gel was run at 120 V for 45 minutes and then imaged using a Kodak Imaging machine and Carestream MI software. The results of the visualization indicated degradation of all RNA samples except for the whole tissue preserved in RNAlater which clearly possessed the identifying 28S and 18S rRNA bands.

![Image: RNAlater Preserved Whole Tissue Agarose Gel with 28S and 18S rRNA Bands]

**Figure 5: RNAlater Preserved Whole Tissue Agarose Gel with 28S and 18S rRNA Bands**

**cDNA Synthesis**

For cDNA synthesis iScript cDNA Synthesis Kit (BioRad, Hercules, CA) was used. This kits parameters allow for a maximum of 1 µg of RNA to be converted to cDNA per reaction, thus RNA was nanodropped. In the process of nanodropping nuclease free water was used to clean the
pedestals followed by the loading of 2 µl TE buffer onto each pedestal for blanking. Finally, 2 µl of each sample was loaded in duplicate and measured, cleaning in-between each set of samples with nuclease free water. Once the concentrations in ng/µl of RNA was known via nanodrop samples were and diluted to 0.5 µg/µl.

A positive control RNA sample was obtained from another lab to run in comparison with the whole tissue RNAlater RNA sample. A Reverse Transcriptase master mix was prepared with 1 µl iScript Reverse Transcriptase and 4 µl 5X iScript Reaction Mix per reaction. For these purposes only one reaction was performed for each sample, thus a total of 2 reactions. Then into each labeled PCR tubes 5 µl master mix was pipetted. Based on nanodrop concentrations, the volume of positive control RNA pipetted into the master mix was 7 µl, leaving 8 µl of the 20 µl total reaction volume to be made up by nuclease free water. The RNAlater whole tissue RNA sample was diluted to 0.5 µl thus 2 µl were pipetted into the master mix followed by 13 µl of nuclease free water. Thermocycling was performed in an Eppendorf Mastercycler proS with the following parameters; Priming at 25° C for 5 minutes, Reverse Transcription at 42° C for 30 minutes, RT Inactivation at 85° C for 5 minutes and finally a hold at 4° C. Finally, the tubes now containing cDNA were transferred to -80° C storage until the analysis stage.

Primer Selection and Design

Glutaminyl-tRNA synthetase (QARS), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were selected as potential housekeeping genes for this study based on their previous uses as reference genes. Of these housekeeping gene primers GAPDH was already designed and available, however the QARS primer was designed specifically for this study. QARS was of
interest because studies have demonstrated that expression remains relatively constant within normal and cancerous pancreatic tissue [26]. This allows the potential for continued use of the QARS housekeeping gene in studies profiling cytokines with this animal model in more detrimental pancreatic inflammatory states than AP and even potentially cancerous states. The sequences decided upon were:

Fw: 5’ ATCACTCCATATACCATGGATCTG 3’

Rv: 5’ TGTAAGGTGTATAACCCAGCCA 3’

The primers were received in dry powder form and reconstituted in nuclease free water based on nmoles, and then a 1:10 diluted working stock was aliquoted.

**Housekeeping Gene Amplification**

PCR was performed on an Eppendorf Mastercycler proS. The thermocycler was programmed according to the Tm of GAPDH (58° C) with the parameters 95° C for 5 minutes followed by 35 cycles of 95° C for 1 minute, 53° C for 1 minute, and 72° C for 1 minute, then 72° C for 10 minutes and a 15° C hold.

The cDNA synthesized from the positive control RNA was amplified for both QARS and GAPDH, and the RNAlater whole tissue synthesized cDNA was amplified for both as well using the following recipe per reaction; 10 µl GoTaq 2X master mix, 0.5 µl forward primer (QARS or GAPDH), 0.5 µl reverse primer (QARS or GAPDH), 2.5 µl cDNA diluted below 250 ng (0.5 µl cDNA added to 2.5 µl nuclease free water per reaction), and 6.5 µl nuclease free water. Afterwards an agarose gel was made and loaded with a 100 bp ladder in the 1st lane, QARS whole tissue
RNA later sample in the 2\textsuperscript{nd} lane, GAPDH whole tissue RNA later sample in the 3\textsuperscript{rd} lane, QARS positive control in the 4\textsuperscript{th}, and GAPDH positive control in the 5\textsuperscript{th} lane. The agarose gel was run at 120 V for 45 minutes and then imaged. Analysis of the gel indicated 237 bp bands in both QARS lanes and 151 bp bands in both GAPDH lanes meaning that amplification was successful.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Housekeeping Gene Amplification Agarose Gel (QARS and GAPDH)}
\end{figure}

The faintness of the GAPDH bands lead to consideration of another potential housekeeping gene, $\beta$2 Microglobulin ($\beta$2M). This gene is considered one of the best for reference in gastric tissues including the pancreas [27].

Thus another round of housekeeping gene amplification was performed following the same parameters as the former and adding in $\beta$2M for both the positive control and RNA later whole
tissue samples. An agarose gel was made and loaded accordingly; 100 bp ladder, QARS RNAlater whole tissue, GAPDH whole tissue, β2M whole tissue, QARS positive control, GAPDH positive control, and β2M positive control. The agarose gel was run at 120 V for 40 minutes and visualized. Upon analysis of this gel the bands for QARS and GAPDH were once again identifiable and distinct bands were also present for β2M. Both GAPDH bands appeared much fainter in comparison to QARS and also β2M

![Figure 7: Housekeeping Gene Amplification Agarose Gel (QARS, GAPDH and B2M)](image)

**RT-PCR: QARS, β2M, and GAPDH**

Utilizing a 7900HT Fast Real-Time PCR System with SYBR green as the detector expression levels were analyzed for three potential housekeeping genes, QARS, β2M, and GAPDH. To begin this process a housekeeping gene optimization plate was designed to evaluate
the mRNA expression of the RNAlater whole tissue cDNA sample. For optimization purposes the sample was tested in duplicate with each of the three housekeeping genes QARS, GAPDH, and β2M at varying cDNA sample concentrations within the well. Concentrations were varied by adding 0 µl of sample cDNA as a negative control and increasing volumes of 0.5 µl, 1 µl, and 2 µl of sample to other wells as outlined in Table 1.

Table 1: 96-Well Plate Organization for RT-PCR Optimization of Housekeeping Genes (QARS, GAPDH and B2M)

<table>
<thead>
<tr>
<th>QARS</th>
<th>QARS</th>
<th>B2M</th>
<th>B2M</th>
<th>GAPDH</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0µl</td>
<td>0µl</td>
<td>0µl</td>
<td>0µl</td>
<td>0µl</td>
<td>0µl</td>
</tr>
<tr>
<td>0.5µl</td>
<td>0.5µl</td>
<td>0.5µl</td>
<td>0.5µl</td>
<td>0.5µl</td>
<td>0.5µl</td>
</tr>
<tr>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>2µl</td>
<td>2µl</td>
<td>2µl</td>
<td>2µl</td>
<td>2µl</td>
<td>2µl</td>
</tr>
</tbody>
</table>

After designing the plate layout a master mix was made for each gene and multiplied by 9 reactions in order to fill 8 wells with each. In a labeled 1.5 ml microcentrifuge tube on ice 10 µl PowerUp SYBR green (Applied Biosystems, Foster City, CA), 1 µl forward primer (QARS, GAPDH, or β2M), 1 µl reverse primer (QARS, GAPDH, or β2M), and 6 µl nuclease free water per reaction were combined. The total reaction volume per well was 20 µl, 18 of which was made up of gene specific master mix leaving 2 µl of volume for cDNA. Master mix was pipetted into
each well of a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA) according to gene followed by 2 µl of the corresponding concentration of cDNA. The wells that were to contain 2 µl of sample were pipetted directly from the cDNA sample and the wells that were to contain 0 µl of sample were filled with 2 µl nuclease free water. For the wells that were to contain between 0 and 2 µl of cDNA a diluted aliquot was made. Using the RNA later whole tissue cDNA sample a 0.5 µl diluted aliquot was made combining 0.5 µl cDNA with 1.5 µl nuclease free water times 7 reactions, a 1 µl diluted aliquot was made combining 1 µl cDNA and 1 µl nuclease free water times 7 reactions, then 2 µl of the diluted aliquots were pipetted into their corresponding wells.

Once all wells were filled the plate was sealed with an Optical Adhesive Cover (Applied Biosystems, Foster City, CA). The plate was then spun at 200xg for 1 minute before loading into the 7900HT Fast RT-PCR System. The cycling parameters were set according to Applied Biosystems PowerUp SYBR green protocol; UDG Activation at 50⁰ C for 2 minutes, DNA Polymerase Activation at 95⁰ C for 2 minutes followed by 40 cycles of Denaturing at 95⁰ C for 1 second and Annealing at 62⁰ C for 30 seconds, and finally a Dissociation step. All 24 wells filled with sample and master mix were ensured to have the reporter and detector selected as SYBR and then the cycle was run.

After the run was complete analysis of Ct values was conducted and it was determined that QARS at a maximum sample volume of 2µl had an average Ct value of 23.5, and GAPDH 21. The lowest Ct value average at maximum sample volume was that of β2M which was 18. Based on these findings β2M was decided upon as the housekeeping gene for this experiment going forward.
**Successful Completion of Methods**

Based on the successes of these optimization trials many methods were set going forward. The animal sacrifice and collection methods were modified to remove additional stress and autolysis of the tissue. Final method being cervical dislocation under isoflurane followed by diaphragm puncture. Tissue collection and immediate storage was altered to placing whole intact pancreas into 1ml of RNAlater and incubating at 4°C overnight before long term storage at -80°C. Tissue homogenization was set to use of mortar and pestle with thawed tissue to be placed into the mortar with sterile tweezers. The RNA extraction protocol was decided to combine the methods of chloroform extraction and spin column extraction. Finally the cDNA synthesis system of choice was BioRad iScript system and its protocol.

As a final result of successful cDNA synthesis and several rounds of housekeeping optimization β2M was decided upon as the ideal reference gene for this study. All things considered at this point the study was ready to be started from injections.

**Experiment**

**Animal Protocol**

12 mice, animal numbers: 5550, 5554, 5556-5558, 5563, 5565, 5566, 5568, 5571, 5575, 5578, were intraperitoneally injected once hourly for 5 hours, 6 mice with genotypes KRas and Akt/KRas were used as control and as such were treated with PBS. Another group of 6 mice genotypes KRas and Akt/KRas were treated with Cerulein. Dosing of PBS and Cerulein were based on mouse body weight and followed a standard of 50 µg per 1 kg body mass, as defined by the preliminary studies and optimization.
24 hours following injections all 12 mice were sacrifice according to IACUC protocol cervical dislocation under isoflurane followed by diaphragm puncture. Pancreas was collected whole and placed directly into labeled 2μl microcentrifuge tubes containing 1ml RNAlater using sterile tweezers. All tubes were incubated at 4° C overnight before moving to -80° C long term storage.

Table 2: Organization of Mice into Injection Groups

<table>
<thead>
<tr>
<th>Animal I.D.</th>
<th>Genotype</th>
<th>Sex</th>
<th>BG</th>
<th>Weight (g)</th>
<th>Injections 2.25.16</th>
</tr>
</thead>
<tbody>
<tr>
<td>5550</td>
<td>Akt/Ras</td>
<td>Female</td>
<td>198</td>
<td>32</td>
<td>CCK ip. (hourly 5x)</td>
</tr>
<tr>
<td>5556</td>
<td>Akt/Ras</td>
<td>Male</td>
<td>156</td>
<td>32</td>
<td>CCK ip. (hourly 5x)</td>
</tr>
<tr>
<td>5575</td>
<td>Akt/Ras</td>
<td>Female</td>
<td>540</td>
<td>24</td>
<td>CCK ip. (hourly 5x)</td>
</tr>
<tr>
<td>5554</td>
<td>Ras</td>
<td>Male</td>
<td>29</td>
<td>24</td>
<td>CCK ip. (hourly 5x)</td>
</tr>
<tr>
<td>5571</td>
<td>Ras</td>
<td>Female</td>
<td>32</td>
<td>24</td>
<td>CCK ip. (hourly 5x)</td>
</tr>
<tr>
<td>5563</td>
<td>Ras</td>
<td>Male</td>
<td>33</td>
<td>24</td>
<td>CCK ip. (hourly 5x)</td>
</tr>
<tr>
<td>5565</td>
<td>Akt/Ras</td>
<td>Female</td>
<td>136</td>
<td>27</td>
<td>PBS ip. (hourly 5x)</td>
</tr>
<tr>
<td>5568</td>
<td>Akt/Ras</td>
<td>Male</td>
<td>191</td>
<td>37</td>
<td>PBS ip. (hourly 5x)</td>
</tr>
<tr>
<td>5557</td>
<td>Akt/Ras</td>
<td>Female</td>
<td>137</td>
<td>24</td>
<td>PBS ip. (hourly 5x)</td>
</tr>
<tr>
<td>5566</td>
<td>Ras</td>
<td>Female</td>
<td>22</td>
<td>24</td>
<td>PBS ip. (hourly 5x)</td>
</tr>
<tr>
<td>5558</td>
<td>Ras</td>
<td>Female</td>
<td>26</td>
<td>24</td>
<td>PBS ip. (hourly 5x)</td>
</tr>
<tr>
<td>5578</td>
<td>Ras</td>
<td>Male</td>
<td>26</td>
<td>24</td>
<td>PBS ip. (hourly 5x)</td>
</tr>
</tbody>
</table>

Tissue Homogenization

Whole tissue stored at -80° C and preserved in RNAlater was thawed on ice. All equipment was prepared and used in the same manner as outlined in the optimization run. All tools were washed with soap and water followed by a simple rinse with nuclease free water in order to prevent direct contact of the thawed exposed tissue with chemicals and RNases residing on the surface of the tools. The thawed whole pancreas on ice was then transferred via sterile tweezers to the clean mortar and covered with liquid nitrogen. The tissue was pulverized with the clean pestle until
mostly powdered. However, given that the tissue was thawed prior to homogenization powder was not easily formed. Thus, the tissue was crushed as much as possible in liquid nitrogen and then 5 ml TRIzol was added and grinding was continued until a viscous pink paste formed which was then transferred to a labeled sterile 15 ml conical tube. The tubes were vortexed until all tissue clumps were dissolved. At this point all samples were aliquotted into 1ml volumes and placed in 4°C for approximately 1 hour before RNA extraction was performed.

RNA Extraction

This protocol was followed exactly as outlined in the optimization run up to the elution step. Due to the expected high concentration of RNA being obtained the columns were eluted twice, each time with 100 μl nuclease free water at 12,000xg for 2 minutes. This alteration created a total final volume of approximately 200 μl viscous RNA solution, 200 μl more of nuclease free water was added to each sample in order to dilute for cDNA synthesis. All samples were stored at -80°C until cDNA synthesis.

cDNA Synthesis

In order to meet the RNA concentration parameters for cDNA synthesis reactions all samples were nanodropped. The approximate RNA concentrations for each sample were around 2 μg/μl. In order to reduce the concentration by slightly more than half, 500 μl nuclease free water was added to each 400 μl sample, yielding a final concentration of slightly less than 1 μg/μl.

The BioRad iScript cDNA Synthesis Kit was used as in the optimization protocol. However an additional genomic DNA elimination step was incorporated. For this step a DNase master mix was made combining 0.5 μl iScript DNase and 1.5 μl iScript DNase Buffer per reaction times 25
reactions. Each of the 12 samples was converted in duplicate thus requiring reagents for 24 reactions.

In order to produce a reaction volume of 20 µl the 1 µg/µl RNA had to be diluted up to a volume of 14 µl. For each sample an RNA dilution was made combining 2 µl RNA with 26 µl nuclease free water. Next 14 µl of this diluted RNA was pipetted into PCR tubes followed by 2 µl of the DNase master mix. This mix of RNA and DNase was incubated in an Eppendorf thermocycler with the following parameters; DNA Digestion at 25º C for 5 minutes then DNase Inactivation at 75º C for 5 minutes. Immediately following the genomic DNA elimination step 4 µl of iScript RT Supermix was added to each reaction and placed back into the thermal cycler under the following parameters; Priming at 25º C for 5 minutes, RT at 46º C for 20 minutes, RT Inactivation at 95º C for 1 minute finally ending in a 4º C hold.

Following the completion of cDNA synthesis a final EtBr agarose gel was made and loaded. 1st lane 100 bp ladder, 2nd lane 5565, 3rd 5558, 4th 5557, 5th 5575, 6th 5571, 7th 5563, 8th 5556, 9th 5554, 10th 5550, 11th 5578, 12th 5568, 13th 5566. The gel was run at 120 V for 45 minutes and imaged. Results pictured in Figure 8 indicated intact cDNA for all samples and confirmed the effectiveness of the changes made to methods allowing logical proceeding to cytokine expression analysis via RT-PCR.
Real-Time PCR: Cytokines

Using the single batch of cDNA produced a full 96-Well plate was designed to test in duplicate all samples at 2 μl volume with the cytokines IL-6, F4/80, TNFα, and the housekeeping gene β2M. All samples were loaded into their corresponding wells with 18 μl of the SYBR green master mix made of 10 μl SYBR, 1 μl forward primer, 1 μl reverse primer and 6μl nuclease free water per reaction. Once loaded the plate was sealed and spun at 200xg for 1 minute before loading into the RT-PCR machine. Thermocycling parameters were set to 50⁰ C for 2 minutes, 95⁰ C for 2 minutes, 40 cycles of 95⁰ C for 1 second and 62⁰ C for 30 seconds, ending with a dissociation step. The reporter and detector were selected as SYBR and the program was run.
Following this plate’s analysis, another plate was designed to test in duplicate at a volume of 2 µl sample the genes NF-κB, Akt1, and Akt2. The plate was prepared and run in the exact same manner as previously outlined and the results were compiled and compared to the β2M values produced on the first plate.
RESULTS

The results of the RT-PCR for the first plate containing β2M, IL-6, F4/80, and TNFα were analyzed for fold gene expression. This was accomplished by averaging the duplicate β2M Ct values for each gene then normalizing each gene’s Ct values against the samples’ β2M expression. Each duplicate sample’s Ct value for any of the respective genes was divided by the samples’ corresponding β2M average to yield a normalized expression value. Next all of these housekeeping gene normalized values for duplicates of the same sample and gene were averaged. Finally data was grouped together by gene expression being tested, IL-6, F4/80, or TNFα, as well as genotype of the animals, Akt/KRas or KRas, and finally by treatment, PBS or CCK.

Utilizing Prism Graphpad software all data was compiled and statistically analyzed with ANOVA multiple comparison.
Figure 9: IL-6 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression

Figure 10: F4/80 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression
After analysis of the first plate, IL-6, F4/80, and TNFα data, the next round of amplification was decided to include Akt1, Akt2, and NF-κB based on studies conducted by Xu et al. [28]. All data from this second plate was analyzed by the same method as previously described for the first plate and graphed.
Figure 12: Akt1 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression

Figure 13: Akt2 Fold Expression Values for CCK and PBS Treated Groups of Akt/KRas and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression
Figure 14: NF-κB Fold Expression Values for CCK and PBS Treated Groups of Akt/Ras and KRas Mice Referenced by B2M Expression and Normalized Against Average KRas PBS Injected Expression Indicating ** Significant Variance
DISCUSSION

Given that all fold expression values for the first plate, IL-6, F4/80, and TNFα demonstrated very minute variability across both gene and genotype no significant differences in expression could be noted. P values were much larger than 0.05; 0.5895 for IL-6, 0.2182 for F4/80, and 0.1843 for TNFα. At this point the experiment turned to literature review to explain the lack of variable data. An article inducing AP in rats in order to evaluate Akt expression was utilized for reference, Xu et al., and brought to light an explanation for the relatively unchanged expression levels. Xu et al. utilized two tissue collection time points that were far closer to the instances of AP induction than 24 post injection. The first time point being 3 hours post AP induction and the second 6 hours post. Testing for IL-6 and TNFα, as in this study it was noted that interestingly both of these cytokines were highly expressed at the 3 hour post time point, TNFα at approximately 90 pg/ml and IL-6 at approximately 350 pg/ml. However, by the 6 hour time point expression had already greatly decreased, TNFα to less than 50 pg/ml and IL-6 to less than 150 pg/ml [28]. This data suggests that at our collection time point of 24 hours post AP, induction is too far out to accurately depict the AP cytokine response that occurs within the tissue.

Xu et al. also evaluated expression of NF-κB and Akt by the same parameters as detailed for IL-6 and TNFα. However, NF-κB and phospho-Akt did not follow this decreasing pattern and instead increased expression over the 2 time points from 12 pg/ml at 3 hours to 17 pg/ml at 6 hours for NF-κB, and 0.7 pg/ml at 3 hours to 0.8 pg/ml at 6 hours for phospho-Akt [28]. Though both of
these genes displayed only a small increase in expression this data provided validity for proceeding with the RT-PCR of NF-κB and Akt isotypes.

Unfortunately, the fold expression in both Akt1 and Akt2 were near identical to that of IL-6, F4/80, and TNFα, in that there was no significant variation between any of the groups, with P values of 0.1808 and 0.4840 respectively. However, the graphed expression levels of NF-κB displayed a ** significant pattern of greater expression in CCK treated over PBS treated in both genotypes with a P value of 0.0005. This increased expression suggests that CCK is in fact causing an inflammatory response as indicated in the preliminary studies.

NF-κB’s role in inflammation of clearing immune cells from the tissue post response serves as a possible explanation as to why it’s mRNA levels remained elevated at the 24 hour post injection collection time point while all others did not. Being the regulator of cytokine transcription that it is, NF-κB may also still be upregulated this far out based on continued signaling within the microenvironment that simply does not involve the cytokines we targeted in this study. Some studies utilizing cerulein to induce pancreatitis have discovered that this method of inflammation induction directly stimulates NF-κB activation by degrading NF-κB associated inhibitors [29]. As evidence for the theory that NF-κB assists in eliminating inflammation and even protecting tissue from further damage, cerulein induced pancreatitis in NF-κB knockouts shows a notable increase in severity compared to normal functioning NF-κB genotypes [29].

As a result of uniformity in expression across nearly all treatments, genotypes and genes, with the exception of NF-κB, as well as the evidence presented by Xu et al. it was concluded that the collection time point of 24 hours was too far out from induction of AP to analyze peak mRNA
expression levels of these cytokines and cytokine regulators. However, for clinical diagnostic purposes it is valuable to note the longevity of elevated NF-κB mRNA levels and its potential for use as a marker of pancreatitis. It is of interest to for these purposes to continue characterizing NF-κB mRNA levels as well as its upstream, downstream and regulatory counterparts at other time points post inflammation induction.

The peak of pro-inflammatory cytokine mRNA levels occurs in a narrow margin of (1.5 to 4) hours after trauma as evidenced by studies of IL-6 and TNFα [30]. Thus, in the interest of characterizing pro-inflammatory cytokine mRNA levels and elucidating the specifics of the initial communication that occurs between cells at the onset of pancreatitis the collection time point should be moved up considerably. For future studies, multiple time points will be evaluated and compared, including 3 hours, 24 hours, and perhaps 48 hours. Also, baseline levels of cytokine expression will be collected for each sample through serum and a true negative control wild type group will be added.
CONCLUSION

Inflammation itself is part of the human body’s wound healing machinery and is meant to help us survive. However, when left unchecked by mutations in genetics or environmental stresses like diet, alcohol consumption, and smoking it can do more harm than good. To such an end, pancreatitis among other inflammatory diseases has been correlated with increased risk for cancer development. Pancreatic cancer has become the fourth leading cause of cancer related deaths in the U.S., yet it is still relatively untreatable given the fragility of the pancreas and the late stages of detection.

By investigating mRNA expression levels associated with inflammatory pathways, hallmarks may begin to be identified that will indicate increased risk for severe disease progression. Furthermore, in delineating the pathways by which the inflammatory cascade occurs, potential points of inhibition will come to light and be evaluated for drug development. By inhibiting an unchecked inflammatory response, tissue damage could be prevented thereby reducing the risk of tumor formation.

This study focused on profiling cytokines and cytokine regulators related to the acute stage of pancreatitis. By inducing AP with cerulein, inflammatory related mRNA expression was also induced. Thus mRNA expression levels of IL-6, F4/80, TNFα, Akt1, Akt2, and NF-κB were quantified via RT-PCR and evaluated for significant variance between groups of genotypes and treatments. Results showed significant variance across treatments for NF-κB mRNA expression levels. The elevation of NF-κB in cerulein treated Akt/KRas compared to Akt/KRas PBS treated
as well as cerulein treated KRas compared to PBS treated KRas shows promise of NF-κB as a potential marker for pancreatic distress. This study also provided successful methods for isolating intact RNA from the RNase inundated pancreas which will be used in future studies.

Given the longer duration of NF-κB elevation, it is a particularly valuable gene for clinical application due to the unlikelihood that patients will seek out medical care within the approximate 3 hour window of the initial pro-inflammatory response. Thus, in combination with evaluation of upstream, downstream, and regulatory genes of NF-κB, it is hopeful that through future studies a panel of markers can be developed to indicate pancreatic distress and disease progression.
REFERENCES


