ABSTRACT

Targeting Toll-Like Receptor 4 Related Inflammation Using Small Molecule, TAK-242, to Attenuate Inflammatory Damage in Pancreatitis and Islet Transplantation

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Inflammation is usually a carefully controlled process within the body consisting of the pro-inflammatory phase where damaged or infected tissue is removed followed by an anti-inflammatory stage when tissue repair and regeneration takes place. If the pro- or anti-inflammatory stages are not appropriately controlled, the pro-inflammatory process can lead to medical complications caused by excessive damage and the anti-inflammatory process can lead to complications relating to excessive fibrosis. Pancreatitis is a condition where excessive inflammation can result in pancreas and remote organ damage. Excessive inflammation also results in detrimental outcomes in pancreatic islet transplantation which can be utilized to replace beta cell mass lost during type 1 or type 3c diabetes.

Toll-like receptor 4, a pattern recognition receptor found on various immune, endothelial, and epithelial cells, can respond to both endogenous and exogenous ligands resulting in enhanced cytokine and chemokine production as well as immune infiltration and activation. In this study, I have used TAK-242, a small molecule inhibitor of TLR4 to analyze the beneficial effects of inhibition of TLR4 in pancreatitis and islet transplantation.

In my fist aim I demonstrate the therapeutic potential of inhibition of TLR4 signaling in the context of sterile inflammation using a mouse model of cerulein-induced pancreatitis. In this study I show that administration of TAK-242 prior to cerulein injections results in a less inflammatory environment within the pancreas of mice according to molecular, structural, and flow cytometric analysis.

In my next aim I show that targeting TLR4 with TAK-242 can result in enhanced islet transplant outcomes by attenuating innate inflammatory responses immediately after transplantation. In this study, I validate miR-375 and miR-200c as reliable biomarkers for measuring islet graft damage following total pancreatectomy with islet autologous transplantation. I then demonstrate a reduction in these biomarkers and decreases in immune cell activation through inhibition of TLR4.

Finally, I show not only does inhibition of TLR4 have effects by directly targeting TLR4 on immune cells, but it also modulates exosome production and cargo of islets in an inflammatory state. These exosomes by themselves have the ability to stimulate macrophage activation which can be subdued by inhibition of TLR4 signaling using TAK-242.

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by

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A Dissertation

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ATTRIBUTIONS

CHAPTER TWO

Jordan Mattke conducted literature searches, prepared original draft, created figures, and revised final manuscript. Carly M. Darden, Michael C. Lawrence, Jayachandra Kuncha, Yumna Ali Sha, Robert R. Kane reviewed and edited the original manuscript. Bashoo Naziruddin reviewed and edited the manuscript and supervised this study.

CHAPTER THREE

Jordan Mattke conceptualized the study, conducted experiments, and prepared the original manuscript. Carly M. Darden and Jayachandra Kuncha assisted with experimental procedures. Michael C. Lawrence helped with processing of RNA sequencing data. Bashoo Naziruddin conceptualized and supervised the study. All authors approved original manuscript.

CHAPTER FOUR

Jordan Mattke conceptualized the study, conducted experiments, and prepared the original draft and final manuscript. Carly M. Darden and Srividya Vasu assisted with carrying out experiments. Jeffery Kirkland catalogued and obtained clinical samples. Robert R. Kane provided the inactive TAK-242 analogue, MAP84, and reviewed and edited original manuscript. Bashoo Naziruddin conceptualized and supervised this study in addition to editing and approving the manuscript.

CHAPTER FIVE

Jordan Mattke and Srividya Vasu performed literature searches and prepared original manuscript. Jordan Mattke conceptualized and performed all experiments presenting new, original data. Carly M. Darden and Michael C. Lawrence reviewed and edited the original manuscript. Kenjiro Kumano created images used throughout this manuscript. Bashoo Naziruddin supervised the preparation of this manuscript.

DEDICATION

To my family, friends, and God who have always believed, supported, sacrificed, and loved me throughout this journey.

CHAPTER ONE

Introduction

The Pancreas and its Function

The pancreas is a glandular organ that plays key roles in aiding digestion as well as supporting the homeostasis of the body. The two types of glandular tissue within the pancreas are the exocrine acinar cells and the endocrine islets of Langerhans. The exocrine acinar cells serve to synthesize, store, and secrete digestive enzymes such as α-amylase, lipase, and proteases (Leung and Ip 2006). The endocrine pancreas is made up of the islets of Langerhans, which are estimated to make up only 1-2% of pancreatic tissue. Each islet is an organoid consisting of at least 5 major cell types. Glucagon secreting α cells make up approximately 35% of islet cells while insulin and amylin secreting β cells make up approximately 55% of the islet mass. Islet δ cells represent less than 10% of islet cells and primarily secrete somatostatin. F cells of islets secrete pancreatic polypeptide and account for less than 5% of islet cells. Finally, ε cells make up less than 1% of islet cells (Paniccia and Schulick 2017). The secretion of insulin and glucagon are essential to glucose homeostasis within the body. In the presence of elevated serum glucose concentrations β cells are stimulated to secrete insulin resulting in increased glucose uptake in adipose and muscle cells in order to achieve a euglycemic state (Norton et al. 2022). Glucagon is produced and released by α cells in response to low serum glucose levels and stimulates hepatic glucose output (Jiang and Zhang 2003).

It is widely accepted that normal blood glucose levels should be between 70 and 100 mg/dL following fasting. Dysregulation of blood glucose levels can have detrimental

effects on the body. Hyperglycemia (blood glucose levels above the normal range) can result in several health complications such as cardiovascular disease, central nervous system deficiencies, bone and joint degeneration, and retinopathy (Klein et al. 1994; Levitan et al. 2004; Sima et al. 2004; Y. Xie et al. 2021). Hypoglycemia (blood glucose levels below the normal range) can result in reduced cognitive function, tachycardia, arrythmias, endothelial dysfunction, and death (Amiel 2021). Therefore, the pancreas plays an integral role in the overall function of the human body.

However, the pancreas is not immune from complications resulting in damage and dysfunction of this organ. Inflammation is usually a carefully controlled process within the body consisting a of a pro-inflammatory phase to remove damaged or infected cells or tissue followed by an anti-inflammatory phase resulting in tissue repair and regeneration. When this process produces disproportionate pro-inflammatory or anti-inflammatory responses, significant health complications can occur. Within the pancreas, this can result in pancreatitis targeting exocrine acinar tissue or type 1 diabetes mellitus targeting endocrine tissue islet.

Pancreatitis

Acute pancreatitis has shown an increasing trend in incidence over time with current global incidence rate of about 34.8 cases per 100,000 (Li et al. 2021; Mederos et al. 2021; Iannuzzi et al. 2022). Acute pancreatitis most commonly presents with abdominal pain that often radiates to the back that is exacerbated by eating, drinking, or lying supine. Acute pancreatitis can also present with nausea, vomiting and low-grade fever. This disease can also be diagnosed by serum and/or amylase levels greater than 3 times the upper limit of normal. Finally, acute pancreatitis can be identified through CT or MRI findings of

diffuse or localized inflammatory enlargement of the pancreas in the case of interstitial edematous pancreatitis (85%) and necrosis of the pancreas parenchyma observed in necrotizing pancreatitis (15%) (Sandrasegaran et al. 2020; Mederos et al. 2021).

Acute pancreatitis can also be subdivided by disease severity as outlined by the revised Atlanta classification definitions. The most common presentation is mild acute pancreatitis, which is classified based on no local or remote organ complications. Patients with transient local and remote complications lasting less than 48 hours are classified as having moderately severe acute pancreatitis. Severe pancreatitis presents with persistent local and/or remote complications lasting longer than 48 hours (Mederos et al. 2021). In a worldwide study carried out by Matta et al. in 4 different regions, mild and moderately severe acute pancreatitis commonly showed mortality rates of less than 1% while severe pancreatitis showed mortality rates between 7.9-43.8% depending on the region (Matta et al. 2020). Following onset of the first episode of acute pancreatitis, patients are at risk for recurrence. Studies have noted a recurrence rate between 15-20% following the first episode of pancreatitis (Ahmed Ali et al. 2016; Li et al. 2023).

The most common etiologies associated with acute pancreatitis are gallstones, alcohol, and triglyceridemia. However, hypercalcemia, infection, hereditary factors, autoimmunity, certain medications, and structural abnormalities have also been identified as possible causes for this condition (Mederos et al. 2021).

Pancreatitis may also be characterized as chronic pancreatitis, which is defined as a repetitive inflammatory condition of the exocrine pancreas leading to irreversible pancreatic tissue damage (Beyer et al. 2020). Both acute and chronic pancreatitis can present with abdominal pain. However, acute pancreatitis and recurrent acute pancreatitis

show elevated lipase while chronic pancreatitis often presents within the normal limits (Cohen and Kent 2023). Chronic pancreatitis is often diagnosed through imaging studies which identify hyperechoic features with shadowing, main pancreatic duct calcifications, lobularity with honeycombing, pseudocysts, stranding, irregular main pancreatic duct, dilated main pancreatic duct, duct branch dilation, or hyperechoic main pancreatic duct wall (Beyer et al. 2020).

Acute pancreatitis and recurrent acute pancreatitis have been identified as risk factors for the development of chronic pancreatitis as a study by Sankaran et al. showed that 10% of patients developed chronic pancreatitis following their first episode of acute pancreatitis and 36% of patients developed chronic pancreatitis following recurrent acute pancreatitis (Sankaran et al. 2015). Drinking and smoking have also been identified as risk factors for developing chronic pancreatitis. Genetic factors such as mutations of SPINK1, CTFR, CTRC, CASR, and CLDN2 as well as ductal obstruction are other aspects which can contribute to increased risk of chronic pancreatitis (Beyer et al. 2020).

Complications associated with chronic pancreatitis include pseudocysts, bile-duct stricture, duodenal stricture, and splanchnic venous thromboses. This condition can also result in reduced islet mass causing glucose intolerance and eventually diabetes. Chronic pancreatitis can also result in total loss of exocrine function leading to steatorrhea, weight loss, sarcopenia, and deficiencies in fat-soluble vitamins.

While diagnosis of acute and chronic pancreatitis are well studied, targeted therapy for pancreatitis has remained elusive. Current treatment regimens for acute and chronic pancreatitis only aim to treat the symptoms of the disease. Current guidelines suggest oxygen, fluid resuscitation, and analgesia for the treatment of acute pancreatitis (Szatmary

et al. 2022). Surgical interventions can also be used in cases of acute pancreatitis when the underlying cause of pancreatitis has been identified (Heckler et al. 2021). Chronic pancreatitis is typically managed through a similar treatment regimen as acute pancreatitis where management of pain is the first goal. In pancreatitis caused by alcohol or smoking, pain can often be alleviated following cessation (Lew et al. 2017; Vege and Chari 2022). Chronic pancreatitis pain is also managed through the use of analgesics, antioxidants, and neuromodulators (Vege and Chari 2022). Pancreatic enzyme replacement therapy is important to mitigate the loss of exocrine function during chronic pancreatitis (Vege and Chari 2022). One surgical intervention option that has seen success for treating recurrent acute pancreatitis and chronic pancreatitis is pancreatectomy followed by autologous islet transplantation, which will be discussed in a later section.

Diabetes

Diabetes can broadly be described as an endocrine imbalance resulting in too little insulin being produced or reduced effects of insulin. Diabetes can be further divided into type 1, type 2, and type 3 diabetes depending on the etiology of the diabetic condition.

Type 1 diabetes mellitus is the result of autoimmune destruction of β cells leading to insulin insufficiency. Diabetes is diagnosed with fasting blood glucose higher than 126 mg/dL, any blood glucose level ≥ 200 mg/dL with symptoms of hyperglycemia, and a glycated hemoglobin of 6.5% or higher over a 3-month period. Type 1 diabetes mellitus can be further diagnosed with symptoms accompanied by the presence of autoantibodies targeting insulin, 65 kDa glutamic acid decarboxylase, insulinoma-associated protein 2, and zinc transporter 8 as well as genetic mutations associated with altered immune responses (Atkinson et al. 2014; Katsarou et al. 2017). Type 1 diabetes patients are also

sensitive and responsive to insulin (Lawrence et al. 2021). In type 2 diabetes mellitus, β cells are present and produce insulin. However, insulin responsiveness in insulin sensitive tissues and insulin secretion by β cells is altered leading to atypical blood glucose levels (Zheng et al. 2018). The most prevalent cause of type 2 diabetes mellitus is obesity along with lifestyle choices such as smoking and drinking. There are also genome wide association studies have implicated a number of possible genetic causes that may contribute to the development of type 2 diabetes (Zheng et al. 2018). Diabetes that does not fall into the categories of type 1 or type 2 is referred to as type 3 or type 3c diabetes. Type 3 diabetes has elements of both type 1 and type 2 diabetes as it is described as a metabolic syndrome linked to brain insulin resistance resulting in impairment of central insulin signaling processes, accumulation of neurotoxins, neuronal stress contributing to neurodegeneration. This condition is commonly linked to the progression of Alzheimer's disease (Nguyen et al. 2020). Type 3c diabetes mellitus most similar to type 1 diabetes in that this condition presents with impaired β-cell function and absence of insulin resistance in insulin sensitive tissues. However, type 3c diabetes mellitus also presents with exocrine insufficiency, consistent pancreatic abnormalities on imaging, and the absence of autoimmune markers of type 1 diabetes as type 3c diabetes presents secondary to destruction of exocrine pancreatic tissue in conditions such as chronic pancreatitis and pancreatic adenocarcinoma (Hart et al. 2016).

Although significant progress has been made in understanding diabetes mellitus in terms of risk factors and progression, this condition continues to be major concern globally evidenced by the increased incidence of diabetes over time (J. Liu et al. 2020). Diagnosis of type 2 diabetes has played an overwhelming role in this increase as incident cases of

type 2 diabetes mellitus more than doubled worldwide from 1990 to 2017 making up 98.3% of incident cases. Although type 1 diabetes mellitus accounted for only 1.8% of diabetes incident cases in 2017, this condition has also seen an increased trend in incidence from 1990 to 2017 (J. Liu et al. 2020).

Even though the different types of diabetes have similar presentation, the treatment and management of diabetes must be catered to the etiology of the condition. As 60% of type 2 diabetes mellitus patients present with obesity, lifestyle modifications such as controlled diet and increased physical activity resulting in weight loss have shown positive results in type 2 diabetes remission (DeFronzo et al. 2015; Chatterjee et al. 2017; Garber et al. 2020). Type 2 diabetes is also be managed through pharmacological intervention targeting glucose production in the liver, increasing insulin secretion, insulin sensitization, glucagon like peptide 1 modulation, glucose absorption, and addition of insulin (DeFronzo et al. 2015; Garber et al. 2020). Because type 1 and type 3c diabetes result from insulin insufficiency following loss of endocrine islet tissue, the management of these conditions is carried out through the use of exogenous insulin and continuous glucose monitoring. Significant progress has been made in recent years in regard to technologies for continuous glucose monitors as well as continuous subcutaneous insulin infusion pumps. There has also been progress in the manufacturing of insulin in different forms such as rapid-acting or basal insulin. Pharmacological drugs targeting the immune system can also be used to slow the progression of type 1 diabetes. However, the most exciting advances have come in the form of β-cell replacement using pancreatic progenitor cells as well as stem-cell derived β-cells (Quattrin et al. 2023). Another therapeutic option to replace endocrine

function in type 1 and type 3c diabetes is islet transplantation, which will be discussed in detail in the next section.

Islet Transplantation and Therapeutic Uses

Since dysregulation of blood glucose can result in many severe health complications associated with hyperglycemia and hypoglycemia, restoration of endocrine function following islet destruction in the progression of type 1 diabetes as well as following surgical resection of the pancreas performed for patients suffering from chronic and recurrent acute pancreatitis is essential. This can be achieved through the use of autologous islet transplantation following pancreatectomy for chronic and recurrent acute pancreatitis or allogenic islet transplantation utilized as a therapeutic option for type 1 diabetes. Islet transplantation presents as an appealing therapeutic option for restoration of endocrine function as it is considered a safe and minimally invasive transplant procedure (Shapiro et al. 2017).

In order to obtain islets for transplantation, they must first be isolated from pancreatic tissue. Even though technology and solution improvements have been made, the modern procedure of islet isolation still follows the same steps reported by Ricordi et al. published in 1989. This process consists of 1) procuring the pancreas with dissection of non-pancreatic tissue and decontamination, 2) perfusion and digestion of the pancreas through enzymatic and physical processes, and 3) enrichment of endocrine islets using density gradient purification of pancreatic digest (Ricordi et al. 1989).

Following isolation, the islets may be transplanted immediately by intraportal infusion or alternative sites, which is commonly done for patients following total pancreatectomy (Kirchner et al. 2017). They may also be cultured for a period of 24 to 72

h before being administered when transplantation is used for type 1 diabetes patients (Shapiro et al. 2017).

Restoration of endocrine function following total or near total pancreatectomy using islet autotransplantation did show promising results in a study carried out by Najarian et al. However, it was noted that a large percentage of patients were not able to achieve long-term insulin independence following the procedure (Najarian et al. 1980). Following this study, substantial progress has been made in islet isolation to improve islet purity and quality as well as therapeutic interventions targeting challenges covered in the next section resulting from a recent study of 409 patients carried out by Sutherland et al. reporting 66% of patients receiving islet transplant following pancreatectomy had at least partial islet function at least 3 years after the procedure performed for chronic pancreatitis (Sutherland et al. 2012). Another study analyzing responses from 564 autologous islet transplant recipients demonstrated significantly improved quality of life scores in patients with functioning islet grafts following pancreatectomy (Chinnakotla et al. 2022).

Allogenic islet transplantation also went through early challenges eventually culminating in the publication by Shapiro et al. reporting 7 consecutive patients achieving insulin independence following islet transplantation for the treatment of type 1 diabetes mellitus (Shapiro et al. 2000). However, long-term study in a larger cohort of patients revealed that median graft survival was only 5.9 years with graft failure eventually occurring in 36% of patients (Marfil-Garza et al. 2022). Although many patients do not achieve complete long-term insulin independence, the advancement in the field of islet transplantation is further motivated by studies demonstrating significantly improved

quality of life scores following islet transplantation for treatment of type 1 diabetes (Poggioli et al. 2006; Foster et al. 2018).

Challenges of Islet Transplantation

Although islet transplantation presents as a promising therapeutic option for restoration of endocrine function, there are still many challenges to overcome in this field. One of the first and most obvious challenges to overcome is obtaining a sufficient islet yield in order to restore endocrine function. In a study analyzing outcomes of 581 autologous islet transplant recipients, the most significant factor predicting islet function following transplant was islet yield per kilogram of body weight. In this study, it was noted that patients receiving < 2,000 IEQ/kg of body weight were 25 times more likely to experience graft failure than patients receiving ≥ 5,000 IEQ/kg of body weight (Chinnakotla et al. 2015). In allogenic islet transplantation for type 1 diabetes patients often required multiple islet infusions of islets in order to receive the necessary amount of islets to achieve sustained insulin production and glycemic control (Shapiro et al. 2000; Ryan et al. 2005; Gangemi et al. 2008; Barton et al. 2012; Marfil-Garza et al. 2022). However, studies have shown full islet function can be achieved using islets isolated from a single donor (Hering et al. 2005; Ryan et al. 2005)

One of the reasons for the high number of islets needed for endocrine function and glycemic control is the instant blood mediated inflammatory reaction (IBMIR) as islets are infused into the hepatic portal vein. This reaction is characterized by platelet consumption and activation of the coagulation and complement pathways (Bennet et al. 2000). Early studies suggested that 50-70% of islet mass is lost in the immediate post transplantation period (Korsgren et al. 2005). Therefore, several studies have focused on strategies to

mitigate this islet loss. An *in vitro* study carried out by Ramnath et al. demonstrated that culturing islets 24 or 48 hours can result in reduced islet damage following exposure to blood (Ramnath et al. 2015). Use of low molecular weight dextran sulfate as well as immobilization of heparin on islet surfaces has also shown promise in reducing the detrimental effects of IBMIR on islets exposed to blood (Johansson et al. 2006; Cabric et al. 2007).

If islets are able to avoid the obstacle of IBMIR, they still have many other challenges to overcome. Islets must also overcome hypoxia and innate immune responses following transplant. As islets are isolated and engrafted into the liver, they are subjected to hypoxic stress and damage, which can be addressed by the addition of exogenous antioxidants or increasing the expression of cellular antioxidants (Kanak, Takita, Kunnathodi, et al. 2014). Islets are also subject to responses of innate immune cells such as neutrophils, islet resident macrophages, and Kupffer cells further contributing to islet loss following transplantation. Administration of therapeutics such as etanercept and anakinra to block IL-1β and TNFα have shown positive islet transplant outcomes during the peritransplant period (Naziruddin et al. 2018). Islets are also capable of producing their own cytokines termed “isletokines” such as CXCL10, which further contribute to immune activation and infiltration leading to injury of grafted islets (Yoshimatsu et al. 2017).

Allogenic islet transplantation for type 1 diabetes also faces additional challenges not observed in autologous islet transplantation. First, transplant of allogenic islet tissue can lead to allorecognition leading to development of alloantibody production and development of alloreactive T cells, which leads to loss of transplanted islets. Transplant of islets for type 1 diabetes also presents the added hurdle of autoimmune responses as

most patients with type 1 diabetes also possess autoantibodies to antigens such as glutamic acid decarboxylase, insulinoma-associated protein 2 antigen, and zinc transporter type 8 antigen. Piemonti et al. demonstrate in their study that more than half of patients receiving islet transplants for type 1 diabetes developed increased donor-specific alloantibodies or autoantibodies following islet transplantation resulting in significantly reduced graft survival than compared to patients that did not develop alloantibodies or autoantibodies following transplant (Piemonti et al. 2013). These alloimmune and autoimmune responses often require induction immunosuppressive therapy such as inhibition of IL-2 mediated activation of T-cells or T-cell depletion prior to transplant and maintenance of immunosuppression following transplant (Shapiro et al. 2017). More recent approaches to achieving long-term transplant outcomes include encapsulation of islets to circumvent the need for immunosuppression. Development of drug or cytokine releasing scaffolds for localized immunomodulation is also an area seeing great promise (Smink et al. 2018). Co-transplantation of immunomodulatory cells such as mesenchymal stem cells or T_{reg} cells is another therapeutic strategy in the developmental stages for use in islet transplantation (Desai and Shea 2017).

Interestingly, it has been found that innate and adaptive immune responses do not function independently of one another as innate responses may affect adaptive responses and vice versa. One class of receptor implicated in the cross talk between the innate and adaptive immune responses leading to rejection and failure of transplanted organs and tissues is the class of receptors known as toll-like receptors (TLRs) (Kim et al. 2008).

Toll-Like Receptor 4

Toll-like receptor 4 (TLR4) is one of the most thoroughly studied pattern recognition receptors first recognized for its ability to stimulate NF-κB-mediated gene expression in response to lipopolysaccharide (LPS), a component of the outer membrane of gram negative bacteria (Chow et al. 1999). Therefore, TLR4 plays a crucial role in the recognition and removal of various infectious agents as demonstrated by several studies pointing out increased disease susceptibility and severity in many different diseases due to knockout or alterations of TLR4 (Miller et al. 2005).

TLR4 not only responds to exogenous ligands such as LPS. Later research demonstrated that TLR4 is also activated by endogenous ligands such as high mobility group box 1 protein (HMGB1), extracellular matrix glycoprotein tenascin-C, and various heat shock proteins (Gong et al. 2020). As all of these proteins are known to be upregulated following cell damage or necrosis, they are commonly referred to as damage-associated molecular patterns. Expression of HMGB1 has been associated with type 2 diabetes complicated by coronary artery disease (Yan et al. 2009). Increases serum HMGB1 have also been associated with severity and mortality of acute pancreatitis (Arriaga-Pizano et al. 2018). Release of HMGB1 from islets has also been correlated to the degree of damage of human islets. In a mouse model of islet transplantation, HMGB1 release also correlated with transplant outcomes (Itoh et al. 2012). Therefore, TLR4 and its signaling could have an important role in inflammatory damage of the pancreas and islet tissue. This is evidenced by a study that showed significantly increased TLR4 expression on monocytes of patients with type 1 diabetes (Devaraj et al. 2008). TLR4 as well as ligands HMGB1, heat shock protein 70, hyaluronan were significantly elevated and correlated with TLR4

mRNA levels in type 2 diabetes patients (Dasu et al. 2010). Increased TLR4 expression was also noted on monocytes of patients with systemic complications of acute pancreatitis (Gorsky et al. 2015). Although no human data is currently available for TLR4 expression in islet isolation and transplantation, many animal studies implicate TLR4 in islet graft failure and show promising results of islet isolation and transplant outcomes through inhibition or loss of TLR4 (Goldberg et al. 2007; Gao et al. 2010; Giovannoni et al. 2015; Chang, Murphy, et al. 2018; Chang, Akinbobuyi, et al. 2018).

Exosomes

Exosomes represent a classification of extracellular vesicle that contains a specific composition of protein, RNA, and DNA. While other types of extracellular vesicles are formed by the outward budding of the plasma membrane, exosomes are formed in a highly regulated process of endocytic vesicle formation, inward budding of the endosomal membrane with loading of DNA, RNA, or protein cargo, and exosome release following fusion of multivesicular bodies with the plasma membrane of the cell (Gurunathan et al. 2019). Exosomes range in size from 50-150 nm and express tetraspanin molecules such as CD63, CD9, and CD81. They can also possess molecules related to antigen presentation, adhesion, membrane transport and fusion, heat shock proteins, cytoskeletal proteins, raft associated proteins and glycolipids, and enzymes (Février and Raposo 2004). These particles play a large role in intercellular communication, carry biomarkers for various disease conditions, and are even used in a therapeutic capacity (Mattke et al. 2021).

One of the major cargoes carried in exosomes used as biomarkers in pancreatic diseases is miRNA. Saravanan et al. show in their studies differential expression of miRNAs such as miR-375, miR-200c-3p, miR-29b-3p, and miR-216a-5p in islet

exosomes, which correlated to islet stress and damage of islets following exposure to insult of cytokines and hypoxia (Saravanan et al. 2019). miRNA analysis of plasma exosomes collected for type 1 diabetes patients showed significantly altered miRNA expressions such as miR-16-5p, miR-302d-3p, and miR-574-5p (Garcia-Contreras et al. 2017). In patients undergoing total pancreatectomy with autologous islet transplantation (TPIAT), it has been demonstrated that elevated DAMP levels in exosomes released by islets prior to infusion resulted in increased insulin requirements and higher hemoglobin A1c levels following transplant (Saravanan et al. 2024 Feb 15). In a study focusing on allogenic islet transplantation, donor exosomes were able to be noninvasively monitored in recipients, which could be quantified and used as diagnostic biomarkers to monitor graft rejection (Vallabhajosyula et al. 2017). Exosomes have also been profiled and have been proven to be unique in terms of miRNA and protein cargo during acute pancreatitis (Jiménez-Alesanco et al. 2019).

Recent research has also demonstrated an immunomodulatory role for exosomes in pancreatic diseases. Exosomes from mesenchymal stem cells have generated promising experimental results in diabetes and islet transplantation (Wen et al. 2016; Nojehdehi et al. 2018; Chen et al. 2020; Keshtkar et al. 2020). However, exosomes have also been found to have detrimental effects in pancreatic diseases as evidenced in a study carried out by Marino et al. that demonstrated donor exosomes significantly contribute to islet allograft rejection (Marino et al. 2016).

Purpose

TLR4 has shown a diversity of roles in the progression of pancreatic diseases such as pancreatitis and diabetes. As TLR4 also has strong ties to innate inflammation, we aim

to investigate the immunomodulatory roles of TLR4 during sterile inflammation of acute pancreatitis and innate and adaptive immune responses during islet transplantation through inhibition of TLR4 using the small molecule TAK-242. Additionally, we have investigated the various roles for exosomes in islet transplantation and present my preliminary work demonstrating the immunomodulatory capacity of these islet exosomes, which can be affected by TLR4 signaling.

CHAPTER TWO

Toll-Like Receptor 4 in Pancreatic Damage and Immune Infiltration in Acute Pancreatitis

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Abstract

Acute pancreatitis is a complex inflammatory disease resulting in extreme pain and can result in significant morbidity and mortality. It can be caused by several factors ranging from genetics, alcohol use, gall stones, and ductal obstruction caused by calcification or neutrophil extracellular traps. Acute pancreatitis is also characterized by immune cell infiltration of neutrophils and M1 macrophages. Toll-like receptor 4 (TLR4) is a pattern recognition receptor that has been noted to respond to endogenous ligands such as high mobility group box 1 (HMGB1) protein and or exogenous ligands such as lipopolysaccharide both of which can be present during the progression of acute pancreatitis. This receptor can be found on a variety of cell types from endothelial cells to resident and infiltrating immune cells leading to production of pro-inflammatory cytokines as well as immune cell activation and maturation resulting in the furthering of pancreatic damage during acute pancreatitis. This review will address the various mechanisms mediated by TLR4 in the advancement of acute pancreatitis and how targeting this receptor could lead to improved outcomes for patients suffering from this condition.

Introduction

The pancreas is an organ comprised of exocrine acinar tissue and endocrine islet tissue. Inflammation targeting the endocrine islets of the pancreas results in type 1 diabetes mellitus, which can be managed through the use of exogenous insulin to replace insulin production lost following beta cell destruction. Acute pancreatitis is an inflammatory condition within the pancreas characterized by damage to the organ in the form of exocrine acinar cell death and local and systemic inflammation (Lee and Papachristou 2019). Acute pancreatitis may present in mild, moderate, severe forms. Mild pancreatitis presents with no local or systemic complications and no organ failure while moderate and severe pancreatitis both present with local and systemic complications and organ failure. Patients with acute pancreatitis present with epigastric pain radiating to the back, elevated serum amylase and lipase activity, and characteristics of pancreatitis based on ultrasonography, computed tomography, or magnetic resonance imaging (Cappell 2008; Walkowska et al. 2022). The most common causes of acute pancreatitis are gallstones and alcohol, but other causes include hypertriglyceridemia, medication toxicity, trauma, hypercalcemia, various infections, autoimmune, ischemia, and hereditary causes (Cappell 2008). One of the other hallmarks of acute pancreatitis is the infiltration of immune cells such as neutrophils and macrophages following initial insult of pancreatitis, which generates further pancreatic damage (Folch et al. 1998; Hu et al. 2020).

Although mortality due to pancreatitis has decreased over time, hospitalizations and cost of care have significantly increased for patients suffering from acute pancreatitis (Brindise et al. 2019). It is also estimated that ~18% of patients presenting with acute pancreatitis will have recurrence or develop chronic pancreatitis (Lee and Papachristou

2019). The current therapeutic strategies for managing acute pancreatitis include intravenous fluid resuscitation, nutritional support, and administration of analgesics (Lee and Papachristou 2019). However, there is currently no targeted therapy for the treatment of acute pancreatitis.

Many cell types are implicated in the progression of acute pancreatitis. It is believed that acute pancreatitis is initiated by one of many factors that results in dysregulation of acinar cells resulting in the production of proinflammatory cytokines and chemokines (Halangk and Lerch 2004). Following the induction of pancreatitis, neutrophils have been found to infiltrate the pancreas as soon as 1 hour after induction of pancreatitis (Folch et al. 1998). Proinflammatory M1 macrophages can be found infiltrating the pancreas during the early inflammatory stages of pancreatitis while M2 macrophages have been noted to be more prevalent during the resolution of phases of pancreatitis several days after pancreatitis induction (Wu et al. 2020). Infiltration of CD4+ T cells can be noted as soon as 6 hours following cerulein challenge in mice further contributing to pancreatic damage (Demols et al. 2000).

Toll-like receptors (TLRs) are the best characterized class of pattern recognition receptor. There are currently 11 recognized TLRs in humans while there are 13 TLRs in mice. These receptors can be found on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) as well as intracellularly localized to endosomes (TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13) (Kawai and Akira 2006).

TLR1 associates with TLR2 to recognize mycobacterial lipoprotein as well as triacylated lipopeptides (Takeuchi et al. 2002). TLR6 also forms a heterodimer with TLR2 recognizing macrophage-activating lipopeptide-2 and other diacylated lipopeptides

(Takeuchi et al. 2001). Although TLR1, TLR2, and TLR6 have been shown to be expressed on the surface, Motoi et al. have demonstrated that signaling of TLR1/TLR2 and TLR2/TLR6 actually happens in the endolysosomes (Motoi et al. 2014). TLR5 is known to recognize bacterial flagellin and is also able to form a physical complex with TLR4 (Yoon et al. 2012; Hussain et al. 2020). TLR10 is a unique toll-like receptor for several reasons. First, the ligands for TLR10 are currently unknown as it is a disrupted pseudogene in mice. TLR10 has been shown to be most homologous to TLR1 and TLR6 and binds to the TLR1/2 ligand, PAM_3CSK_4 (Guan et al. 2010). TLR10 is also the only toll-like receptor to demonstrate anti-inflammatory properties (Jiang et al. 2016). TLR4 is one of the most extensively studied TLRs, which responds to a variety of ligands and will be covered in more detail in the next section.

Bacterial translocation is a phenomenon which live bacteria that colonize the intestines or their products are able to cross the intestinal barrier into neighboring organs or the circulatory system resulting in remote organ inflammation and complications. This has been noted in both animal models and human trials during the progression of severe acute pancreatitis (Liu et al. 2019). Pancreatitis can also occur following viral infection with viral hepatitis, coxsackie and echoviruses, hemorrhagic fever viruses, CMV, and VZV being the most common (Simons-Linares et al. 2021). Because TLRs play a major role in bacterial and viral recognition, the activation of these receptors could play a protective role in the progression of pancreatitis following bacterial and viral infection.

Up to this point all of the discussed ligands for toll-like receptors are related to bacterial and viral infection and serve a role in inflammation in order to protect the body from infection. However, toll-like receptors are also able to respond to markers of sterile

inflammation and cell apoptosis. For example, it has been demonstrated that mRNA released from necrotic cells stimulates the activation of TLR3 (Karikó et al. 2004). High mobility group box 1 (HMGB1) released by innate immune cells in response to TNF or IL-1β is able to activate both TLR2 and TLR4 (Yu et al. 2006).

Heat shock proteins (HSP) are a class of protein originally found to be produced in response to a sudden increase in temperature. However, over time many different types of stresses have been associated with the upregulation of these proteins. These proteins fall into 3 main categories based on their size. HSP60 and HSP70 are both implicated in protein folding and unfolding as well as protein assembly while HSP90 has been shown to prevent steroid binding to DNA (Kaufmann 1990). Ethridge et al. demonstrated both HSP70 and HSP27 are significantly upregulated during the induction of pancreatitis in mice (Ethridge et al. 2000). Cao et al. also showed that HSP60 and HSP70 were significantly upregulated following the induction of pancreatitis in mice and the inhibition of p38 caused a decrease in expression of these heat shock proteins (Cao et al. 2015). In humans HSP60 serves to activate both TLR2 and TLR4 to promote proliferation of venous smooth muscle cells (de Graaf et al. 2006). HSP27 released after global ischemia is able to stimulate both TLR2 and TLR4 resulting in increased NF-KB signaling, which upregulates monocyte chemoattractant protein (MCP)-1 and intercellular adhesion molecule (ICAM)-1 as well as cytokine IL-6 (Jin et al. 2014).

Toll-Like Receptor 4 Signaling and Pathological Relevance in Acute Pancreatitis

Toll-like receptor 4 (TLR4) is a receptor expressed on a multitude of cell types recognizing bacterial lipopolysaccharides (LPS), viral RNA, saturated fatty acids, and damage associated molecular patterns (DAMPs) such as high mobility group box 1

(HMGB1) protein and heat shock proteins 60 and 70 (Figure 2.1) (Ohashi et al. 2000; de Graaf et al. 2006: 60; Lu et al. 2008; Laird et al. 2009; Rocha et al. 2016; Yang et al. 2020). HMGB1 has been found to be a key marker of many inflammatory conditions such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis in the nervous system (Fang et al. 2012). HMGB1 is also implicated in inflammatory heart disease as well as well as several vascular inflammatory diseases (de Souza et al. 2012; Bangert et al. 2016). HMGB1 has even been implicated in musculoskeletal diseases such as osteoarthritis (Shao et al. 2023). The production of HMGB1 and its signaling through TLR4 has been shown to be especially important in the progression of severe acute pancreatitis as administration of HMGB1 to mice resulted in increased pancreatic injury and NF-κB signaling that could be attenuated in TLR4-deficient mice (Li et al. 2016). Pancreatitis patients with higher HMGB1 levels have also corresponded to increased disease severity (Arriaga-Pizano et al. 2018). TLR4 can also be activated by pancreatic elastase, which has been shown to be elevated during acute pancreatitis (Antti Hietaranta et al. 2004).

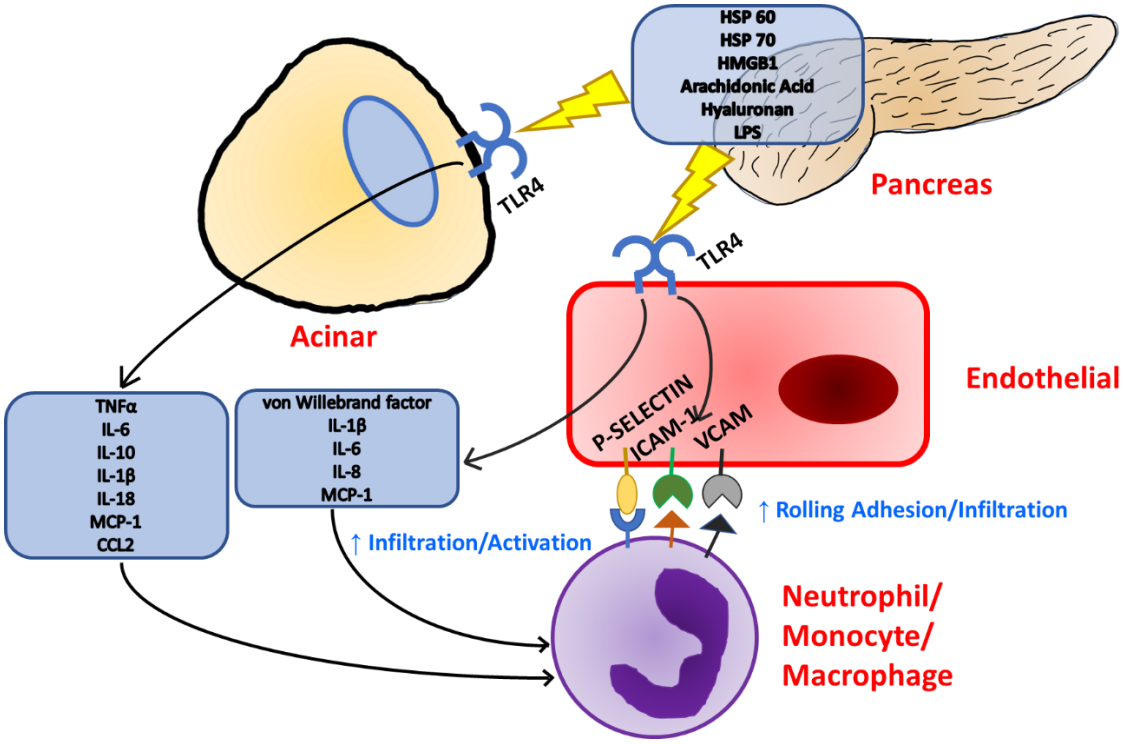

Figure 2.1. Inflammation in the pancreas can be initiated with TLR4 signaling as TLR4 has been known to respond to a multitude of ligands. Upon TLR4 stimulation, both acinar and endothelial cells release pro-inflammatory cytokines and chemokines which leads to immune cell recruitment and activation. Immune cell recruitment is further supported by TLR4 stimulation as endothelial cells upregulate the expression of surface adhesion molecules.

Within the pancreas TLR4 is localized to pancreatic ductal epithelium, vascular endothelium, and islets while being absent in exocrine acinar cells of rats (Figure 2.2) (Li et al. 2005). However, TLR4 signaling has proven to play a significant role in acinar cell inflammation during acute pancreatitis, which will be discussed in a later section. TLR4 expression is increased on monocytes 24 hours after the onset of acute pancreatitis and reduces to normal levels after 7 days (Li et al. 2007). During severe acute pancreatitis, TLR4 is found to be elevated in liver, kidney, and intestinal tissue following induction of pancreatitis (Sawa et al. 2007).

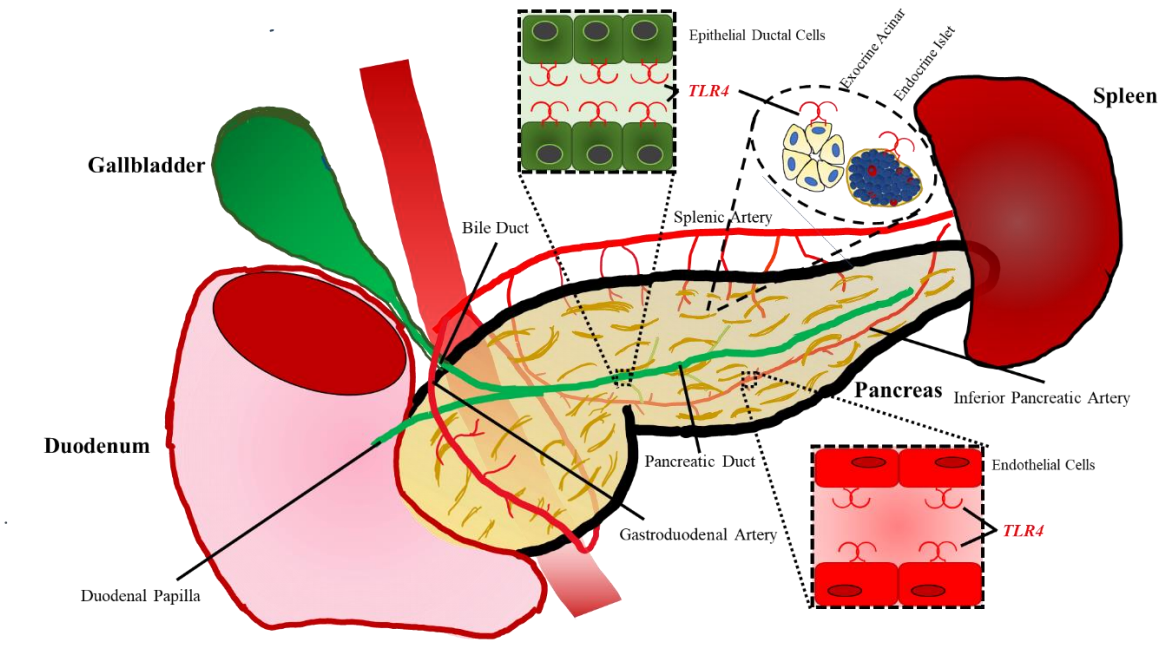

Figure 2.2. TLR4 is expressed on a variety of cell types within the pancreas. Epithelial cells lining the ductal tissue of the bile and main pancreatic duct are known to express TLR4. TLR4 is also expressed by exocrine acinar tissue as well as endocrine islets. The pancreas also has vasculature composed of endothelial cells that express TLR4.

The process of TLR4 signaling by LPS begins with LPS binding protein (LBP) binding and escorting LPS to CD14. CD14 then transfers LPS to the TLR4-MD2 complex (Ryu et al. 2017). Concentrations of LBP have been found to be significantly elevated in patients suffering from severe acute pancreatitis indicating the process of TLR4 signaling may have a role in systemic complications associated with severe acute pancreatitis (Erwin et al. 2000; Rau et al. 2003). Upon stimulation with LPS, TLR4-MD2 oligomerizes and is able to signal through the MyD88 dependent pathway to activate NF-κB and produce proinflammatory cytokines such as TNFα, IL-1β, IL-6, and IL12 (Lu et al. 2008). As markers such as IL-6 has proven to be significantly altered when looking at the severity of pancreatitis, TLR4 signaling could have a role in the progression of this disease (van den Berg et al. 2020). TLR4 signals through the MyD88 independent pathway through TRIF

activating NF-κB and IRF3 to produce type I interferons (Lu et al. 2008). Inhibiition of NF-κB signaling using Withaferin A has been shown to result in beneficial outcomes in cerulein induced pancreatitis as leukocyte infiltration was decreased as well as fibrosis (Kanak et al. 2017). Therefore, decreasing NF-κB signaling through inhibition of TLR4 could result in beneficial outcomes in the study of acute pancreatitis. An important downstream target of TLR4 signaling in pancreatitis is TRAF6, which is involved in both the TLR4 dependent and TLR4 independent pathways. This plays a significant role in the progression of pancreatitis as TLR4 deficient mice demonstrated much slower progression of pancreatic inflammation when compared to WT mice. TRAF6 was significantly higher in TLR4 deficient mice when compared to WT and TRAF6 was localized to pancreatic acinar cells (Zhou et al. 2010). TRAF6 has also demonstrated a protective role in the progression of acute pancreatitis in acinar cells as stimulation of TLR4 lead to increased SOCS1 and SOCS3 expression, which are responsible for the degradation of TRAF6 through polyubiquitination. Knockout of TLR4 prevented the progression of acute edematous pancreatitis to acute necrotizing pancreatitis with the administration of cerulein and LPS (Zhou et al. 2015).

Table 2.1 TLR4 responses to various ligands within pancreatic tissue

Ligand	Cell Type	TLR4 Response	References
LPS	Acinar	↑ ROS, apoptosis, TNFα, IL-6, IL-10, IL-1β, IL-18, MCP-1	(Kimura et al. 1998; Vaccaro et al. 2000; Gu et al. 2013; Pan et al. 2018)
	Endothelial	↑ P-Selectin, VCAM1, IL-1β, IL-6, IL-8	(Andonegui et al. 2002; Wang et al. 2011)
	Macrophages	M1 Polarization	(Colin et al. 2014)
	Neutrophil	↑ Survival	(Sabroe et al. 2003)
Arachidonic Acid	Acinar	↑ CCL2 and P-Selectin	(Mateu et al. 2015)
HMGB1	Macrophages	↑ IL-8, TNF	(Yu et al. 2006)
	Neutrophil	↑ NADPH, ROS	(Fan et al. 2007; Tadie et al. 2013)
Hyaluronan	Endothelial	↑ IL-8	(Taylor et al. 2004)
Heat Shock Protein 60	Vascular Smooth Muscle Endothelial Cells	↑ Migration, IL-8	(Zhao et al. 2015)
	Macrophages	↑ TNFα, NO	(Ohashi et al. 2000)
Heat Shock Protein 70	Macrophages	↑ TNFα	(Luong et al. 2012)
Heat Shock Protein 27	Endothelial	↑ MCP-1, ICAM-1	(Jin et al. 2014)
Fatty Acid	Endothelial	↑ IL-6, IL-8, CCL5, CXCL10	(Chen et al. 2018)

Another key outcome of TLR4 signaling via NF-κB is the activation of the NOD-leucine rich repeat family pyrin domain containing protein 3 (NLRP3) inflammasome. Upon activation, NLRP3 forms a complex with ASC and pro-caspase-1. This complex formation and activation results in the conversion of pro-caspase-1 to active caspase-1, which will then cleave pro-IL-1β and IL-18 to active IL-1β and IL-18 that will be released from the cell promoting further inflammation. This process also results in the cleavage of gasdermin D. The N-terminal fragment of this cleavage will then form a pore in the plasma membrane allowing for further release of IL-1β and IL-18. This inflammatory process mediated by the NLRP3 inflammasome has been termed pyroptosis (Figure 2.3)(Danielski et al. 2020). NLRP3, ASC, and caspase-1 are involved in inflammation associated with acute pancreatitis as loss of any of these signaling components has been shown to reduce the severity of acute pancreatitis (Hoque et al. 2011). In a study focusing on patients with acute pancreatitis, it was demonstrated that higher serum IL-18 levels corresponded to pancreatitis complicated by pancreatic necrosis and remote organ failure showing that pyroptosis was increased in the organs of these patients (Rau, Baumgart, et al. 2001). Targeting of the TLR4/NLRP3 axis with emodin, rhein, baicalin, and chrysin significantly reduced acinar cell necrosis and decreased nitric oxide production in macrophages demonstrating more beneficial outcomes in mice by targeting this pathway (Wen et al. 2020). NLRP3 is also a promising target in severe acute pancreatitis in that Sendler et al. propose that activation of this pathway in macrophages initiates innate inflammatory responses such as neutrophil recruitment and maturation as well as acts as a Th2-cell mediator for the adaptive immune system (Sendler et al. 2020). It has also been shown that inhibition of caspase-1 leads to reduced acinar cell death by necrosis in severe acute

pancreatitis (Rau, Paszkowski, et al. 2001). Therefore, targeting of TLR4 upstream of caspase-1 could at least partially inhibit cleaving and activation of caspase-1, which would result in more beneficial outcomes to those suffering from pancreatitis. Taken further, TLR4 on platelets can also signal through the NLRP3-ASC inflammasome leading to activation of caspase-1 promoting increased neutrophil extracellular trap (NET) formation leading to increased production of TLR4 agonist, S100A8/A9, which creates a positive feedback loop of inflammation during pyroptosis (Su et al. 2022).

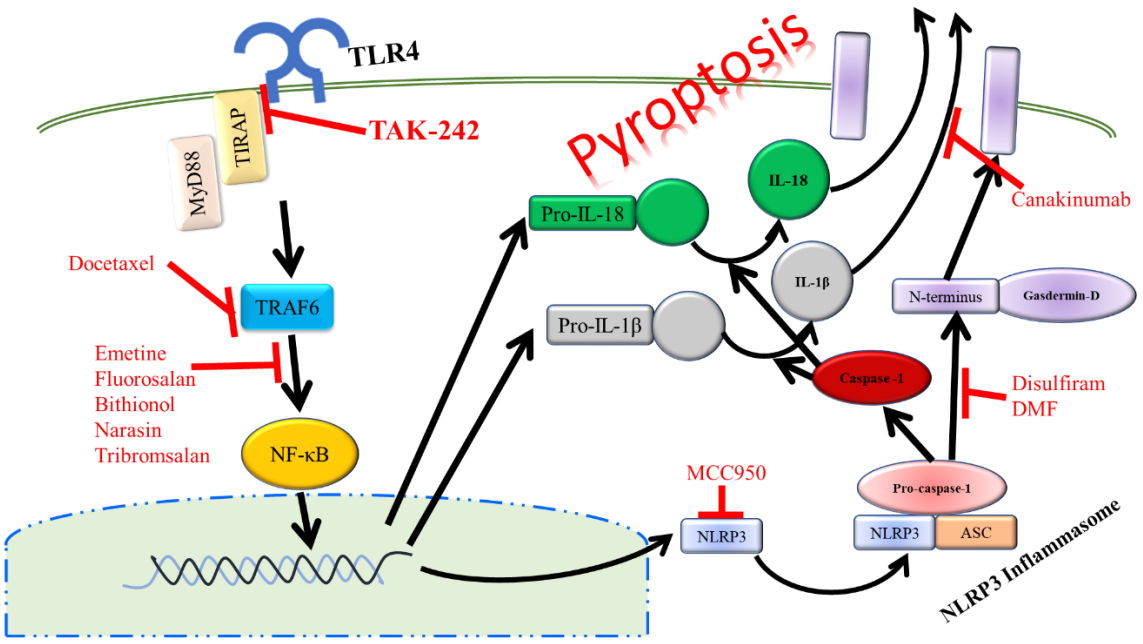

Figure 2.3. Activation of TLR4 can lead to the inflammatory process of pyropotosis. Many therapeutic drugs have been developed to target the different components of pyroptosis pathway.

Another outcome of TLR4 signaling is the production of macrophage migration inhibitory factor (MIF), which has been shown to be a significant indicator of the severity of pancreatitis in both humans and mice (Roger et al. 2001; Sakai et al. 2003; Zhu et al. 2020). Inhibition of MIF has led to alleviated damage in pancreatic and renal tissues in a

severe acute pancreatitis model through the attenuation of the NLRP3 pathway (Liu et al. 2021). MIF plays a key role in the NLRP3 inflammasome assembly leading the production and release of IL-1β in macrophages (Lang et al. 2018).

Activation of TLR4 can also lead to the activation of RIP3 and the induction of necroptosis (Su et al. 2019). While apoptosis is usually associated with an orderly process of cell disassembly with little or no DAMP release, necroptosis is associated with massive release of DAMP molecules resulting in enhanced inflammation as a result of cell death (Pasparakis and Vandenabeele 2015). Inhibition of TLR4 signaling using small molecule inhibitor of, TAK-242, has led to attenuated necroptosis in an acute pancreatitis model as evidenced by a reduced expression of RIP3 within the pancreas of treated mice (Hong et al. 2020).

TLR4 in Acinar Cells

TLR4 plays a significant role in inflammation within acinar cells of the pancreas. Treatment with LPS has been shown to significantly increase the amount of apoptotic acinar cells in a cerulein induced pancreatitis (Kimura et al. 1998). Upon stimulation with LPS, primary pancreatic acinar cells were found to have a significant increase in intracellular reactive oxygen species (ROS) as viability was decreased (Pan et al. 2018). LPS also induces apoptosis and expression of TNF-α, IL-1β, and IL-18 mRNA in AR4-2J cells (derived from azaserine-induced malignant nodules in rat pancreas) (Vaccaro et al. 2000). Another study found that TLR4-positive acinar cells respond to LPS by activating the inflammasome and producing TNF-α, IL-6, IL-10, IL-1β, IL-18, and MCP-1 during acute pancreatitis, and these effects could be exacerbated by alcohol (Fig. 1) (Gu et al. 2013). Vona-Davis et al. investigated the effects of LPS and TNFα treatment on AR4-2J

cells and noted that there was an activation of both STAT3 and SOCS3 in response to this treatment with significant increases in SOCS3 expression with treatment of LPS and IL-6 while TNFα enhanced expression of STAT3, which also stimulated SOCS3 expression (Vona-Davis et al. 2005). This highlights the importance of SOCS3 in the resolution of inflammation and a possible role of TLR4 in not only the initiation of inflammation, but the resolution of inflammation within acinar cells.

Isolated pancreatic acini are able to respond to arachidonic acid via TLR4 resulting in the upregulation of monocyte chemoattractant protein 1 (CCL2) and P-selectin, which would cause increased immune recruitment and adhesion in the pancreas (Mateu et al. 2015). In a study carried out by Sztefko and Panek it was noted that elevated levels of arachidonic acid could be involved in the development of complications in acute pancreatitis (Sztefko and Panek 2001). In vivo $TLR4^{-/-}$ and $CD14^{-/-}$ mice showed reduced acinar atrophy in a severe acute pancreatitis model (R Sharif et al. 2009). Small molecule inhibitor of TLR4, TAK-242, increased the viability of pancreatic acinar cells, decreased lactate dehydrogenase, and reduced apoptotic cell death following exposure to taurocholate. This was accompanied by decreased release of cytochrome c into the cytoplasm, reduced mitochondrial swelling, and decreased mitochondrial Ca^{2+} buffering capacity following exposure to taurocholate (Pan et al. 2016). Administration of natural product, biochanin A, resulted in reduced pancreas damage through decreased TLR4 and NLRP3 signaling (Pan et al. 2023). This is further evidenced in a study that showed knockout of NLRP3 or gasdermin D in acinar tissue of cerulein treated mice resulted in reduced pyroptosis (Gao et al. 2021). In TLR4 deficient mice there is significantly less acinar cell necrosis (Awla et al. 2011).

TLR4 in Endothelial Cells

TLR4 plays a key role in the inflammation of endothelial cells, which could contribute to the infiltration of immune cells during the progression of acute pancreatitis. First, stimulation of TLR4 in endothelial cells leads to significant increases in surface adhesion proteins as well as increases in P-selectin and von Willebrand factor expression (Figure 2.1) (Beckman et al. 2021). P-selectin plays a significant role in the adhesion and infiltration of neutrophils as inhibition of P-selectin has resulted in reduced damage and neutrophil infiltration in experimental pancreatitis (Hackert et al. 2009; Hartman et al. 2012). Elevated von Willebrand factor has also proven to be a useful biomarker for severe acute necrotizing pancreatitis (Reuken et al. 2022). TLR4 signaling in endothelial cells can lead to Weibel-Palade body degranulation, NF-κB activation, and vaso-occlusion (Belcher et al. 2014). It has been further demonstrated that TLR4 participates in HLA class I signaling to upregulate P-selectin and von Willebrand factor expression leading to increased rolling adhesion and infiltration by monocytes (Jin et al. 2022a). Vein endothelial cells have been shown to respond to LPS via TLR4 signaling to produce cytokines such as IL-1β, IL-6, and IL-8 further contributing to immune infiltration and activation (Wang et al. 2011). Increased IL-8 concentrations in serum are correlated to more complicated pancreatitis as well as neutrophil elastase (Gross et al. 1992). These results implicate serum IL-8 as a biomarker for neutrophil activation leading to pancreatitis complications. This also shows that reduction in production of IL-8 through the inhibition of TLR4 signaling could result in improved pancreatitis outcomes.

Within the pancreas, intraperitoneal administration of LPS lead to significant increases in P-selectin and vascular cell adhesion molecule-1 (VCAM-1) expression within

the pancreas (Andonegui et al. 2002). As VCAM-1 and P-selectin are both associated with increased immune cell adhesion and infiltration, a reduction of these markers through inhibition of TLR4 signaling could serve as a therapeutic strategy to attenuate damage due to immune cell infiltration and activation. TLR4 of endothelial cells is also able to sense components of the extracellular matrix such as hyaluronan to trigger inflammation and the initial stages of would defense and repair (Taylor et al. 2004). As hyaluronan has been shown to be accumulated in the edematous interstitium during acute pancreatitis, this could lead to the activation of TLR4 in endothelial cells further contributing to inflammation and immune cell recruitment to the pancreas(Johnsson et al. 2000).

TLR4 in Macrophages

TLR4 and the associated pathways are essential to the maturation and polarization of macrophages (Figure 2.4). Macrophages can be divided into different categories based on stimulus. M0 macrophages are non-activated macrophages, which can differentiate into proinflammatory M1 macrophages or anti-inflammatory M2 macrophages. M1 macrophages develop following exposure to inflammatory signals such as IFNγ, LPS, or TNFα, which activates STAT1, NF-κB, p65/p50, and IRF5 resulting in the production of IL-6, TNFα, IL-23, and iNOS. M2 macrophages can be further subdivided into M2a, M2b, M2c, and M2d subtypes all of which play a role in resolution of inflammation and wound healing and form in response to different stimuli (Colin et al. 2014). As mentioned previously, the activation of TLR4 not only signals through TRAF6 to promote inflammation and survival signals, but it also signals through SOCS1 and SOCS3 to provide negative feedback for this process. SOCS1 has proven to be especially important in dampening immune infiltration and inflammation responses as mice deficient in SOCS1

showed increases in inducible nitric oxide synthase expression in the pancreases of these mice, which appeared to preferentially damage exocrine over endocrine tissue (Chen et al. 2004). Qin et al. also show that deficiency of SOCS3 in macrophages resulted in higher levels of M1 macrophage genes (Qin et al. 2012). However, this contrasts to a study carried out by Gordon et al. who found that silencing of SOCS3 promoted a shift of activated M1 macrophage markers to increased expression of M2 macrophage markers. This same study also noted that silencing of SOCS3 resulted in increased phagocytic capacity of these macrophages (Gordon et al. 2016). In a study conducted by Arnold et al. focusing on renal and peritoneal inflammation, SOCS3 expression correlated strongly with disease severity and renal injury. SOCS3 was also shown to colocalize with markers of M1 macrophage polarization (Arnold et al. 2014). The role of SOCS3 in the activation of macrophages in pancreatitis is further supported by a study showing that macrophage-specific deletion of SOCS3 developed less severe pancreatitis and produced less TNFα in response to cerulein injections. This same study also highlighted the importance of CCL2 in the migration of macrophages from the bone marrow to the pancreas during the induction of pancreatitis (Saeki et al. 2012). This further emphasizes the importance of TLR4 response in acinar cells as CCL2 has been found to be upregulated by TLR4 in response to arachidonic acid. NLRP3 signaling also plays a large role in the activation of macrophages and associated pro-inflammatory as well as the compensatory anti-inflammatory responses. In a study where bone marrow-derived macrophages were incubated with pancreatic acini, macrophages were found to secrete increased IL-1β and IL-18 showing an upregulation of the NLRP3 pathway. In a study conducted by Sendler et al. NLRP3 activation was shown to have a significant role in both the hyperinflammation responses as macrophages are

activated promoting enhanced recruitment and activation of other immune cells. Inhibition of the NLRP3 inflammasome using MCC950 resulted in significantly reduced neutrophil infiltration, T cell activation, and disease severity in mice. They also showed that NLRP3 signaling has a role in the anti-inflammatory response syndrome in which Th2 and Tregs are activated by IL-18 in the absence of IL-12, which results in enhanced pancreatic fibrosis and permits bacterial translocation into pancreatic necrosis or severe sepsis (Sendler et al. 2020). Since TLR4 is known to activate the NLRP3 inflammasome, inhibition of TLR4 could also lead to some of these beneficial effects.

Macrophages play an important role in the progression of pancreatitis as a delicate balance between M1 and M2 macrophages is needed in order for pathogen clearing and wound healing to occur. During acute pancreatitis, infiltrating macrophages are mainly activated and differentiated into an M1 phenotype (Hu et al. 2020). This is further confirmed by Sendler et al. who showed M2 macrophages were found in non-necrotic areas of pancreatic tissue while M1 macrophages were found in necrotic fields during induction of pancreatitis. Co-culture of bone marrow derived macrophages with acinar cells showed significant increases in IL-6 and TNFα as well as anti-inflammatory IL-10 (Sendler et al. 2020). TLR4 signaling plays a large role in the differentiation of M1 macrophages. In macrophages advanced glycation end products have been found to stimulate TLR4 signaling resulting in an M1 phenotype, which could be inhibited by treatment with TAK-242 (Z. Liu et al. 2020). The fungal protein paracoccin has also been found to stimulate TLR4 resulting in a pro-inflammatory M1 phenotype (Freitas et al. 2016). Treatment of macrophages with berberine results in a reduction in the production of inflammatory factors and reduced polarization of macrophages to an M1 phenotype by inhibiting the

binding of MyD88 to TLR4 (Gong et al. 2019). Blocking of TLR4 and TNFR1 promotes of a shift of macrophages to an M2 phenotype (Sawoo et al. 2021). Therefore, inhibition of TLR4 could serve as a therapeutic strategy to dampen inflammation induced by macrophages by promoting a shift from inflammatory to anti-inflammatory macrophages.

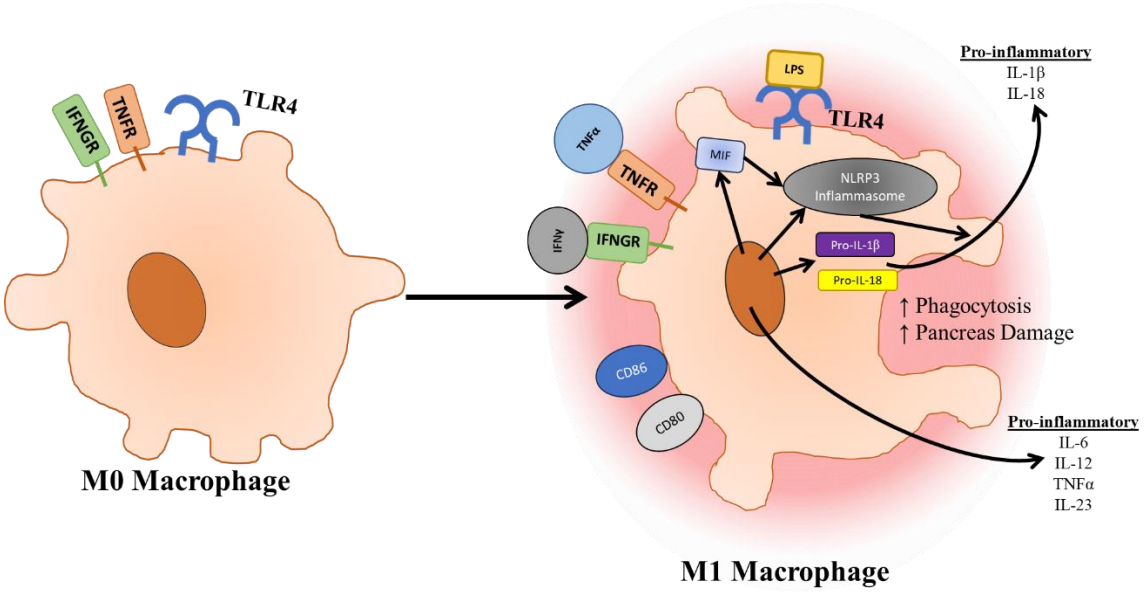

Figure 2.4. Once macrophages have infiltrated the pancreas, they can be activated and polarized to an M1 state through TLR4 signaling. Activation of macrophages is further supported by factors released by pancreatic tissue. Upon activation, polarized macrophages increase their phagocytic capacity as well as release pro-inflammatory cytokines which further immune infiltration and destruction.

Administration of pancreatic elastase results in increased inflammatory responses in THP-1 cells mediated by TLR4 (Antti Hietaranta et al. 2004; A. Hietaranta et al. 2004). High fat diet in acute pancreatitis rats aggravated infiltration of activated, inflammatory macrophages by signaling of TLR4 which could be inhibited by the administration of TAK-242 (Hong et al. 2020). Carbon monoxide releasing molecule has been found to inhibit TLR4 signaling in macrophages leading to reduced production of TNFα and proinflammatory cytokines in cerulein-induced pancreatitis (Xue and Habtezion 2014).

TLR4 in Neutrophils

TLR4 signaling plays a significant role in the activation and lifespan of neutrophils. Stimulation of TLR4 using purified LPS almost completely prevents neutrophil apoptosis at early timepoints by signaling through NF-κB (Sabroe et al. 2003). TLR4 also signals through MEK kinase leading to the inhibition of G-protein-coupled receptor kinases 2 and 5, which are responsible for the internalization or surface chemokine receptor CXCR2, resulting in increased neutrophil activation and migration (Fan and Malik 2003). TLR4 signaling is also unique in neutrophils in that neutrophils only utilize the MyD88-dependent signaling pathway when stimulated with ligands such as LPS (Tamassia et al. 2007). HMGB1 during hemorrhagic shock/resuscitation results in increased TLR4 signaling resulting in increased NADPH oxidase activity in neutrophils leading to an increased production of reactive oxygen species (Fan et al. 2007). The activity of NADPH oxidase and production of reactive oxygen species by neutrophils results in increased TLR2 and ICAM-1 expression on endothelial cells, which creates a positive feedback loop of inflammation and cellular adhesion (Fan et al. 2003). High fat diet in acute pancreatitis induced rats results in increased activation and infiltration of neutrophils into the pancreas by signaling through TLR4 (Hong et al. 2020). $TLR4^{-/-}$ mice have demonstrated significantly less neutrophil infiltration in an acute pancreatitis model (Akbarshahi et al. 2011; Awla et al. 2011).

One unique feature of neutrophils is their ability to produce web-like structures composed of decondensed chromatin fragments wrapped in histones, proteases, granules, and cytoplasmic proteins referred to as neutrophil extracellular traps (NETs) (Fig. 4) (Li et al. 2022). NETs have been found to play a significant role in the progression of a

taurocholate induced acute pancreatitis model as neutrophil depletion and administration of DNase I lead to attenuated pancreas damage (Merza et al. 2015). In patients with acute pancreatitis increases in platelet microparticles were observed. When these platelet microparticles were mixed with healthy neutrophils, there was an increase in myeloperoxidase, neutrophil elastase, and histone H3 production as well as the release of NETs. This indicates that the production of NETs also plays a role in the progression of pancreatitis in humans (Qi et al. 2020). TLR4 on platelets induces platelet binding to neutrophils resulting in neutrophil activation and NET formation (Clark et al. 2007). The presence of free radicals has been shown to play a role in the progression of pancreatitis and the production of NETs (Guice et al. 1986). These free radicals such as superoxide can lead to an increased production of NETs at the site of sterile inflammation through signaling of TLR4 (Al-Khafaji et al. 2016). Treatment of neutrophils with HMGB1 (elevated during severe acute pancreatitis) results in increased production of NETs through increased signaling of TLR4 (Tadie et al. 2013). The production of NETs is also supported by the activation of the NLRP3 inflammasome under sterile conditions as NLRP3 was found to support both nuclear envelope and plasma membrane rupture during the release of NETs (Münzer et al. 2021). Not only are NETs produced directly through TLR4 signaling, but NET release is also supported by cytokines produced in other tissues during the progression of acute pancreatitis. IL-8 (elevated following TLR4 signaling in endothelial cells) promotes the production of NETs through its interaction with CXCR2, which is upregulated on neutrophils in response to TLR4 signaling (An et al. 2019). IL-1β (upregulated in response to TLR4 signaling in acinar and endothelial cells in response to

TLR4 signaling) has also shown the ability to stimulate the production of NETs in neutrophils as well (Meher et al. 2018).

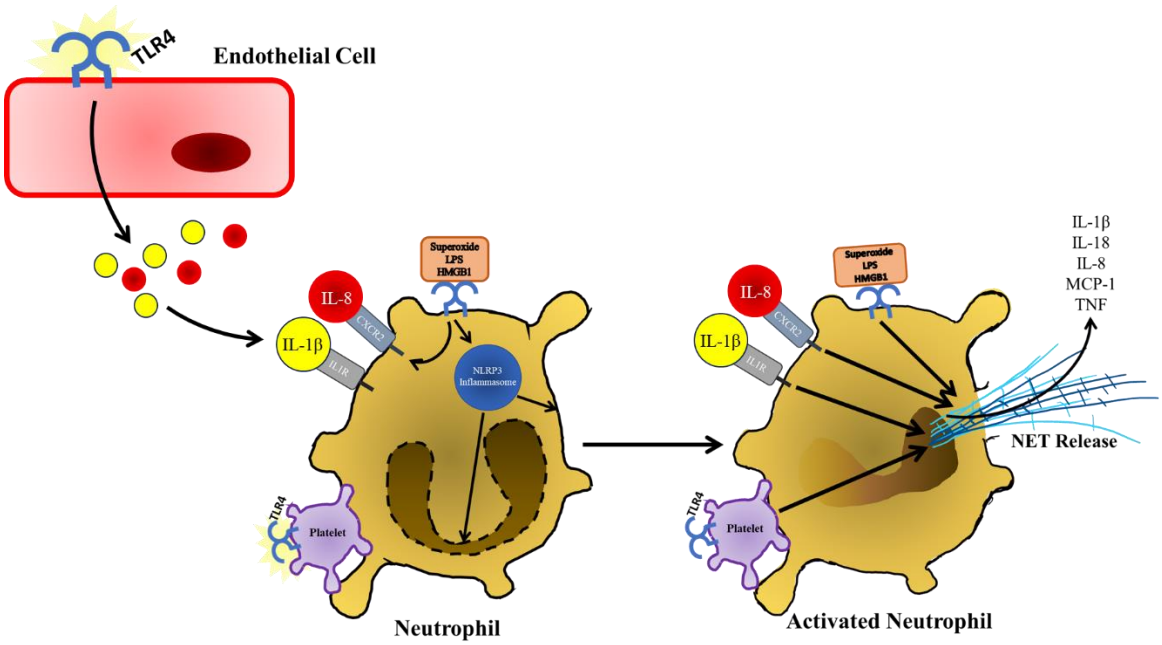

Figure 2.5. Following TLR4 stimulation and other damage associated signals present in the pancreas, neutrophils respond by secreting NETs, which further contribute to pancreatic damage and immune infiltration during the progression of acute pancreatitis. NET formation is also supported by TLR4 activation and binding of platelets. TLR4 stimulation also supports the production of NETs by upregulating CXCL2, which is stimulated by IL-8 that presents at increased concentrations during the progression of acute pancreatitis. NETs are also released in response to increased IL-1β, which is also increased in response to TLR4 stimulation in various cell types in response to TLR4 signaling.

TLR4 in Remote Organ Complications During Severe Acute Pancreatitis

TLR4 has a demonstrated role in pancreatic inflammation and in the progression of acute pancreatitis. In addition, previous studies have also demonstrated a role for TLR4 in remote organ complications as well. It has long been recognized that severe acute pancreatitis can lead to lung damage, which in turn leads to increased mortality of patients with pancreatitis (INTERIANO et al. 1972). During the induction of acute pancreatitis mice utilizing LPS, an upregulation of both TLR4 and MIF were observed in the lungs of treated mice. The inhibition or loss of MIF resulted in lower TLR4 expression in the lungs

of mice following the induction of pancreatitis as well as increased survival outcomes further implicating TLR4 in respiratory distress following the onset of pancreatitis (Matsuda et al. 2006). $TLR4^{-/-}$ mice also showed significantly less lung myeloperoxidase activity following the cerulein induction of pancreatitis (R Sharif et al. 2009). Induction of pancreatitis in rats using L-arginine also resulted in increased serum creatinine and BUN indicating increased renal injury during this induction of pancreatitis. This same study also showed increased liver biomarkers aspartate transaminase (AST) and alanine transaminase (ALT) during the induction of pancreatitis. Down regulation of HMGB1/TLR4/NF-KB signaling using protocatechuic acid resulted in significant reduction in levels of pancreatic amylase and lipase as well as reduced AST, ALT, creatinine, and BUN (Abdelmageed et al. 2021). The trends of reduced renal and hepatic damage were also noted in TLR4 deficient mice as well as significantly lower serum levels of interleukin-1 and tumor necrosis factor (Sawa et al. 2007). Taken together, not only could inhibition of TLR4 affect immune infiltration and damage within the pancreas during the onset of acute pancreatitis, but inhibition of TLR4 could also alleviate remote organ complications during severe acute pancreatitis such as adult respiratory distress syndrome.

Table 2.2. TLR4 related molecules used as diagnostic biomarkers

Marker	Source	Findings	References
IL-1β	Mouse Pancreas	Neutrophils and macrophages are major producers of IL-1β	(Fink and Norman 1996)
	Mouse Pancreas	IL-1β correlates with the severity of pancreatitis	(Fink and Norman 1997)
	Human Serum	Associated with severity of disease	(Sternby et al. 2021)
IL-18	Human Serum	Elevated with increased severity of pancreatitis	(Wereszczynska-Siemiatkowska et al. 2002)
	Human Serum	Elevated with renal and respiratory failure during acute pancreatitis	(Malmstrøm et al. 2012)
	Human Serum	Significantly elevated in patients with complicated by pancreatic necrosis and remote organ failure	(Rau, Baumgart, et al. 2001)
IL-6	Human Serum	Elevated in mild and severe acute pancreatitis	(Pooran et al. 2003)
	Human Serum	Associated with severe disease	(Sternby et al. 2021)
	Human Serum	Increased in severe acute pancreatitis starting 5 hours after onset	(Inagaki et al. 1997)
	Human Serum	Elevated in patients with acute pancreatitis complications	(Fisic et al. 2013)
	Human Serum	Elevated in patients that did not survive	(Arriaga-Pizano et al. 2018)
IL-8	Human Serum	Elevated in severe acute pancreatitis	(Pooran et al. 2003)
	Human Serum	Elevated in patients that did not survive	(Arriaga-Pizano et al. 2018)
TNF	Human Serum	Elevated in severe acute pancreatitis	(Pooran et al. 2003)
HMGB1	Human Serum	Elevated in patients with severe acute pancreatitis	(Yang et al. 2017)
	Rat Pancreas and Serum	Elevated in pancreatic tissue 12 hours after induction of acute necrotizing pancreatitis. Significantly elevated in serum 12 hours after pancreatitis induction	(Yu et al. 2016)
	Human Serum	Elevated in patients that did not survive	(Arriaga-Pizano et al. 2018)

Summary

TLR4 is a pattern recognition receptor expressed on a diverse population of cells and plays a multitude of roles in the progression in acute pancreatitis. The initial insult of pancreatitis results in the production of key ligands for TLR4 such as HMGB1 and heat shock proteins associated with sterile inflammation. These ligands can then stimulate localized inflammation in acinar and endothelial tissue through TLR4 to promote the production of many inflammatory factors which increase infiltration and activation of innate immune cells (Figure 2.1). Once these innate immune cells enter the pancreas, TLR4 signaling stimulates the polarization of macrophages to an inflammatory M1 phenotype, which will lead to further destruction of exocrine tissue as well as continued infiltration of immune cells (Figure 2.4). However, further study is warranted in the area of TLR4 signaling and the polarization of macrophages as overproduction of M2 macrophages can result in chronic pancreatitis (Xue et al. 2015). Clarification of the role of SOCS3 and TLR4 in macrophage polarization is also of interest in the progression of pancreatitis as deletion of SOCS3 resulted in more beneficial outcomes following induction of pancreatitis. SOCS3 also colocalized with M1 macrophage markers. However, conflicting information states that SOCS3 deficiency results in increased M1 gene expression. TLR4 also has a significant role in the infiltration and activation of neutrophils as TLR4 deficient mice show reduced neutrophil infiltration and pancreas damage. TLR4 has a role in the production of NETs, which are shown to be increased in the progression of pancreatitis and play a role in destruction of exocrine tissue (Figure 2.5). I would also like to acknowledge that very little research has been carried out on TLR4 signaling in ductal epithelial cells. TLR4 has been identified in cells of the ductal epithelium of the pancreas (Li et al. 2005).

As TLR4 has been shown to be upregulated in epithelial cells of the bowel during inflammatory conditions such as Crohn's disease and ulcerative colitis, it would also be worth investigating TLR4 expression and signaling of the pancreatic ductal epithelial cells during the progression of acute pancreatitis as this could also contribute to immune cell infiltration and activation (Cario Elke and Podolsky Daniel K. 2000). Investigation of TLR4 inhibition has been shown to be clinically feasible as small molecule inhibitor of TLR4, TAK-242, has been used in a clinical trial for use in the treatment of sepsis and has proven to be safe for use in humans (Rice et al. 2010). Because of the diversity of roles TLR4 plays in the progression of pancreatitis and pancreas damage, the inhibition of TLR4 could be worth investigating for the treatment of acute pancreatitis in humans.

CHAPTER THREE

TLR4 Inhibition Attenuates Damage and Immune Infiltration in Cerulein-Induced Pancreatitis

This chapter submitted to *Journal of Gastroenterology* as: Jordan Mattke, Carly M. Darden, Jayachandra Kuncha, Michael C. Lawrence, Bashoo Naziruddin. TLR4 Inhibition Attenuates Damage and Immune Infiltration in Cerulein-Induced Pancreatitis.

Abstract

Background: As toll-like receptor 4 (TLR4) plays multiple roles in different cell types in response to inflammation and immune infiltration, we aimed to investigate whether targeting TLR4 using a specific small molecule inhibitor, TAK-242, could lead to improved outcomes in cerulein-induced pancreatitis in mice.

Methods: C57BL/6 mice were treated with control saline and repeated injections of 50 μg/kg cerulein to induce pancreatitis. TLR4 inhibition by 3 mg/kg or 10 mg/kg TAK-242 intervention was performed prior to pancreatitis induction. RNA isolation and bulk RNA sequencing was carried out on mice from each group followed by RT-qPCR analysis. Pancreases were also sectioned for histological analysis. Pancreases were processed into single cells, which were examined by flow cytometry to monitor immune infiltration. Finally, serum HMGB1 levels were determined using ELISA assay.

Results: RNA analysis revealed significantly increased inflammation pathways in response to cerulein injections, which could be significantly attenuated by TAK-242. Histological analysis showed improved scores for mice treated with TAK-242 when compared to cerulein. Flow cytometric analysis of pancreatic single cells obtained from mice subjected to acute pancreatitis or recurrent acute pancreatitis revealed significantly

reduced monocyte, macrophage, and neutrophil populations when 3 mg/kg TAK-242 was administered. Finally, serum HMGB1 exhibited a dose dependent decrease reaching significance when 10 mg/kg TAK-242 was administered during pancreatitis induction during recurrent acute pancreatitis.

Conclusions: These results validate TLR4 as a therapeutic target to attenuate immune infiltration as well as pancreatic and remote organ damage in the progression of acute and recurrent acute pancreatitis.

Keywords: Toll-like receptor 4; TAK-242; Macrophage; Pancreatitis

Introduction

Pancreatitis can have symptoms ranging from mild and localized to severe and necrotizing, and it can be fatal in some scenarios (Awla et al. 2011). Pancreatitis can present as an acute condition with a variety of causes such as obstruction of the pancreatic duct secondary to gallstones, alcohol, endoscopic retrograde cholangiopancreatography, and various drugs causing organelle dysfunction. All of these causes result in acinar cell death as well as localized and systemic inflammation (Lee and Papachristou 2019). Chronic pancreatitis is characterized by inflammation of the pancreas that results in acinar cell loss or atrophy with or without replacement with fibrotic tissue ending in recurrent or constant abdominal pain, diabetes, and maldigestion (Kleeff et al. 2017).

Toll-like receptor 4 (TLR4), a receptor expressed by a variety of cell types, is a major contributor to inflammation and local immune responses in conditions such as sepsis (Kuzmich et al. 2017). TLR4 is a pattern recognition receptor that can be activated by both pathogen-associated molecular patterns such as lipopolysaccharide (LPS) and danger-associated molecular patterns such as oxidized low-density lipoprotein and oxidized phospholipids (Rocha et al. 2016). Upon stimulation with LPS or saturated fatty acids with the assistance of myeloid differentiation factor 2 and cluster of differentiation 14 (CD14), TLR4 undergoes dimerization and signals through the myeloid differentiation primary response 88 pathway ending in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), stimulating the transcription of inflammatory cytokines. TLR4 can also signal independently of the myeloid differentiation primary response 88 pathway, leading to the production of interferon (Rocha et al. 2016). Another important result of TLR4 signaling is the transcription and activation of the NOD-like receptor family

pyrin domain containing 3 (NLRP3) inflammasome. It has been shown that activation of the NLRP3 inflammasome and its components is required for full pancreatic injury during acute pancreatitis (Ferrero-Andrés et al. 2020).

In a sodium taurocholate model of pancreatitis, it was noted that knockout of TLR4 resulted in reduced tissue damage, pancreatic and lung myeloperoxidase activity, serum and pancreatic levels of chemokine (C-X-C motif) ligand 2 (CXCL2), and blood amylase (Awla et al. 2011). TLR4 was also shown to be upregulated in the early stages of cerulein-induced pancreatitis localized around the pancreatic ductal epithelium, vascular endothelium, and islets (Li et al. 2005). High mobility group box 1 (HMGB1), a key ligand for TLR4, has been shown to be significantly elevated in the circulation of patients suffering from severe acute pancreatitis (Yasuda et al. 2006). These results were validated in a rat model of pancreatitis that showed elevated levels of HMGB1 starting 12 hours after beginning the experiment (Z.W. Zhang et al. 2010). Administration of recombinant human HMGB1 in a mouse model led to exacerbated pathogenesis of experimental pancreatitis that could be alleviated with the knockout of TLR4 (Li et al. 2016). It has also been proposed that TLR4-positive acinar cells are able to respond to LPS by activating the inflammasome and producing pro- and anti-inflammatory molecules during mild subclinical acute pancreatitis (Gu et al. 2013). LPS also enhances transforming growth factor beta-1 production and signaling in pancreatic stellate cells, leading to increased type I collagen and α-smooth muscle actin production and Smad 2 and 3 phosphorylation in an alcoholic chronic pancreatitis model in rats (Sun et al. 2018). A recent study has also shown that there is cross-talk between TLR4 and HLA class I mediating P-selectin expression on endothelial cells (Jin et al. 2022b). This is significant in that it has shown that P-selectin

expression mediates neutrophil recruitment in different models of pancreatitis (Hartman et al. 2012).

One of the main hallmarks of pancreatitis is immune cell infiltration. TLR4 plays a large role in the activation and maturation of these immune cells. Macrophages play a key role in the progression of pancreatitis. Acute pancreatitis is characterized by infiltration of the inflammatory M1 macrophage while macrophages in chronic pancreatitis tend to exhibit an M2 phenotype, which promotes pancreatic fibrosis (Hu et al. 2020). Activation of TLR4 has been shown to polarize macrophages to an M1 phenotype, and stimulation of TLR4 can switch M2 macrophages to M1 macrophages (Wang et al. 2014). Another important cell type that infiltrates immune cells is neutrophils. Administration of cerulein induces a significant increase in the expression of CXCL2, which is a neutrophil attractant (Wan et al. 2021). An important aspect of neutrophil infiltration is the production of neutrophil extracellular traps (NETs) made of extracellular chromatin that are at least partially responsible for occlusion of the pancreatic ducts (Leppkes et al. 2016). It has been shown that platelet TLR4 detects TLR4 ligands in the blood and induces binding to adherent neutrophils, leading to the production of NETs (Clark et al. 2007). Treatment of neutrophils with the TLR4 ligand LPS resulted in increased production of NETs (Tadie et al. 2013).

Because of the multitude of roles TLR4 has in the progression of pancreatitis, we hypothesized that administering the TLR4 inhibitor TAK-242 would lead to attenuated damage and immune cell infiltration during cerulein-induced pancreatitis.

Methods

Mice

Male and female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) were used in this study. Mice were housed in a climate-controlled, pathogen-free environment with access to standard laboratory chow and water as needed. All experimental protocols were approved by the North Texas Veterans' Affairs Institutional Animal Care and Use Committee in compliance with published guidelines for animal care.

Induction of pancreatitis

For the acute model of pancreatitis, mice were administered 0.5 mL of saline, 3 mg/kg of TAK-242, or 10 mg/kg of TAK-242 (Tocris Bioscience, Bristol, UK) intraperitoneally. This injection was followed by 7 hourly injections of 0.1 mL of saline or 50 μg/kg cerulein (Sigma Aldrich, St. Louis, MO, USA) intraperitoneally. To establish a recurrent acute model, this process was carried out twice weekly for 4 weeks. Twelve hours after the last cerulein injection, mice were sacrificed and the pancreas was harvested. For histology and immunohistochemistry, the pancreas was placed in 10% formalin (Sigma Aldrich, St. Louis, MO, USA) and stored at 4°C. Pancreases were also harvested and divided into 2 parts for immune cell infiltration analysis and for RNA extraction.

RNA isolation and sequencing

Sections of pancreas were stored in Qiazol (Qiagen, Hilden, Germany). Samples were then homogenized with a handheld dounce homogenizer for 30 seconds. mRNA was extracted using chloroform, precipitated with isopropanol, and washed with ethanol. RNA was then quantified with a Cytation 5 (BioTek, Winooski, VT, USA). RNA samples were

then sequenced on a NovaSeq 6000 instrument using single end reverse strand sequencing. Usegalaxy.org was used to process each fastq file. Each file quality was assessed using the FastQC tool. The Trimmotatic tool was used to trim the ends of each read, and HISAT2 was used to align and quantify reads. Reads were then annotated using the annotateMyIDs tool before building a feature count matrix using the featureCounts tool. Finally, differential expression of each RNA was analyzed using the limma tool.

RNA sequencing analysis

After the completion of RNA sequencing, RNAs were organized by false discovery rate *P* value, and all $P > 0.05$ were excluded from the analysis. The remaining RNAs were then organized by fold change. In the comparison of cerulein vs. control RNA, fold changes above 2.00 were collected and entered into Enrichr, which was used to perform KEGG analysis of the highest upregulated RNAs in the cerulein samples (Z. Xie et al. 2021). Appyter was then used to generate volcano plots and find the most upregulated pathways based on the lowest *P* values. This same process was repeated to compare TAK- vs. cerulein-treated mice to find the lowest fold changes (< -2.00) when comparing TAK-treated mice to cerulein-treated mice.

Real-time quantitative polymerase chain reaction

RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. Primers for glyceraldehyde 3-phosphate dehydrogenase, Cxcl2, Ccl4, Il1b, Cxcl10, Tnfaip3, Il6, and Cd14 were purchased from Qiagen. Quantitative polymerase chain reaction (PCR) was carried out on a QuantStudio 7 Flex system utilizing

RT^2 SYBR Green Rox Mastermix (Qiagen, Hilden, Germany). Data were analyzed using the ΔΔCT method followed by fold change calculation.

Histology and immunofluorescence

Each pancreas used for histology and immunohistochemistry was harvested and immediately placed in 10% formalin and stored for at least 48 hours at 4°C before being processed. Each pancreas was then embedded in paraffin and cut into 5 μM sections. Sections were stained with hematoxylin and eosin and scored according to the Schmidt Scoring Criteria (SCHMIDT et al. 1992). Sections used for immunohistochemistry were deparaffinized in xylene and rehydrated with decreasing concentrations of ethanol. Slides were then submerged in citrate buffer of pH 6 at 97°C for 20 minutes. Following antigen retrieval, slides were washed 3 times with Tris-buffered saline containing 0.025% Triton X-100. Slides were then blocked with Tris-buffered saline containing 1% bovine serum albumin for 1 hour to block nonspecific binding. Slides were incubated overnight with rabbit anti-mouse F4/80 (Cell Signaling Technology, Danvers, MA, USA) and insulin polyclonal antibody (Invitrogen, Waltham, MA, USA). Slides were then incubated for 1 hour with secondary antibody before administering ProLong™ Gold antifade reagent with DAPI (Invitrogen, Waltham, MA, USA) and being imaged on a Zeiss LSM 880 Airyscan.

Flow cytometry

Sections of the pancreas were suspended in Hanks balanced salt solution containing 2 mg/mL collagenase. The tissue suspensions were incubated at 37°C for 15 minutes to digest the pancreas and obtain a single cell suspension. The resulting suspensions were then washed with Roswell Park Memorial Institute medium with 10% fetal bovine serum and passed through a 70 μM filter. The resulting single cells (5×10^5) were labeled with

anti-CD45 Brilliant Violet 786, anti-CD14 Brilliant Violet 605, anti-F4/80 Brilliant Violet 510, anti–cluster of differentiation 86 (CD86) APC, anti–lymphocyte antigen 6 complex locus G6D (Ly6G) Alexa Fluor 488, anti-CD206 Brilliant Violet 711, and anti-CD163 Brilliant Violet 421 (Biolegend, San Diego, CA, USA). Cells were then detected on a BD Fortessa. The resulting data were processed with FlowJo.

HMGB1 enzyme-linked immunosorbent assay

Blood samples were collected immediately following the last injection of cerulein by tail vein puncture. Samples were then transferred to microcentrifuge tubes and spun at 2,000 g for 10 minutes. The top serum layer was collected and stored at -80°C. Samples were thawed and HMGB1 content in serum was then measured by the Mouse HMGB1/HMG1 ELISA Kit (Colorimetric; Novus Biologicals, Littleton, CO, USA) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was carried out in GraphPad Prism9 (GraphPad Software, La Jolla, CA, USA). Comparison of more than two groups was carried out using one-way analysis of variance and Tukey's multiple comparison test. Statistical analysis of two groups was determined by an unpaired one-way Student's *t* test. Differences were considered significant when P values were < 0.05.

Results

TAK-242 reduces RNA markers of inflammation within the pancreas

To begin this experiment, I first performed sequence analysis of isolated RNA from the pancreases of all mice. Following the elimination of insignificant P values ($P > 0.05$), we collected a list of the most upregulated genes (fold change > 2.00) in cerulein samples

compared to control samples. I then repeated this process to find the most downregulated genes (fold change < -2.00) when comparing TAK-242–treated mice to cerulein-treated mice. Examples of common genes from these analyses can be found in Figure 1d. We then entered the most downregulated genes from the TAK-242 and cerulein comparison into Enrichr to perform a KEGG pathway analysis to determine the pathways most significantly affected by the administration of TAK-242. This process was repeated with the control and cerulein RNA sequencing results to generate a list of the most significantly upregulated pathways in response to cerulein administration. The most downregulated pathways in the TAK-242 vs. cerulein were compared to the most upregulated pathways in the cerulein vs. control analysis to reveal several common pathways in this study. Many of the common pathways such as TNF signaling, chemokine signaling, NF-κB signaling, and mitogen-activated protein kinase signaling pathways related to inflammatory pathways. A more comprehensive list of pathways can be found in Figure 1b. To confirm the results of the RNA sequencing and KEGG pathway analysis, I evaluated the mRNA expression of several markers using RT-qPCR. We noted that chemokine (C-C motif) ligands 4 (CCL4) had a noticeable increase in response to cerulein, which was decreased by TAK-242 treatment (cerulein = 4.842 ± 2.319 FC, TAK-242 = 1.240 ± 0.518 FC, $P = 0.134$). This same trend was observed in CD14 (cerulein = 4.416 ± 2.240 FC, TAK-242 = 0.821 ± 0.214 FC, $P = 0.126$) and CXCL2 (cerulein = 19.153 ± 6.914 FC, TAK-242 = 3.879 ± 0.7455 FC, $P = 0.080$). Significant changes were observed when comparing the mRNA expression of C-X-C motif chemokine ligand 10 (CXCL10) (cerulein = 2.453 ± 0.466 FC, TAK-242 = 0.713 ± 0.117 FC, $P = 0.034$), tumor necrosis factor alpha-induced protein 3 (TNFAIP3) (cerulein = 5.512 ± 0.237 FC, TAK-242 = 3.319 ± 0.166 FC, $P = 0.011$), interleukin (IL)-

1β (cerulein = 9.004 ± 1.900 FC, TAK-242 = 1.428 ± 0.178 FC, $P = 0.044$), and IL-6 (cerulein = 7.044 ± 0.871 FC, TAK-242 = 1.900 ± 0.836 FC, $P = 0.030$) (Figure 3.1).

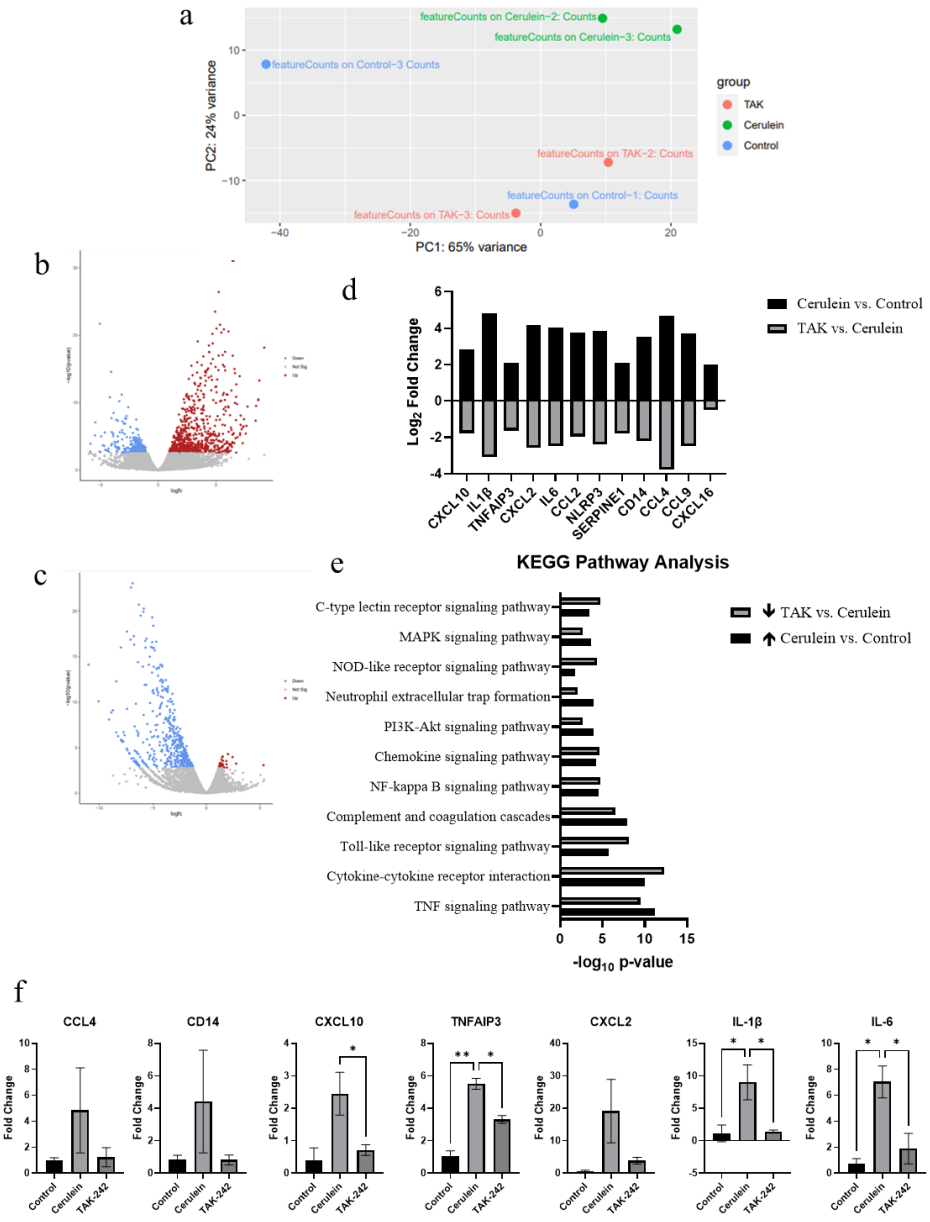

Figure 3.1. (a) Principal component analysis plot of RNA sequencing results. Volcano plots show significantly altered RNAs in (b) control vs. cerulein-treated mice and (c) TAK-242 vs. cerulein-treated mice. RNA sequencing analysis showed (d) significant upregulation of inflammatory RNAs in response to cerulein injection that were significantly downregulated by TAK-242. Significantly altered RNAs were input into (e) KEGG pathway analysis to reveal pathways that were increased by cerulein and decreased by TAK-242. (f) Expression of CCL4, CD14, CXCL10, TNFAIP3, CXCL2, IL-1β, and IL-6 were used to confirm and evaluate the expression of RNAs within the significant pathways from the KEGG analysis. (* $P < 0.05$. ** $P < 0.005$)

TAK-242 prevents morphological changes induced by cerulein

Since the RNA analysis revealed significant changes in inflammatory mRNAs and inflammatory pathways, I next investigated whether there were significant changes in the pancreas structure as well as altered immune cell infiltration. Following fixation in formalin and staining with hematoxylin and eosin, pancreases were scored according to Schmidt's Scoring Criteria, which take into account interstitial edema, leukocyte infiltration, acinar cell necrosis, and hemorrhage. The cerulein-treated animals had significantly higher scores than control animals, and although TAK-242 did not completely ameliorate pancreatic damage, it did result in significantly lower scores than those in cerulein-treated animals (cerulein = 10.33 ± 0.94, TAK-242 = 6.33 ± 0.47, $P = 0.003$) (Figure 3.2).

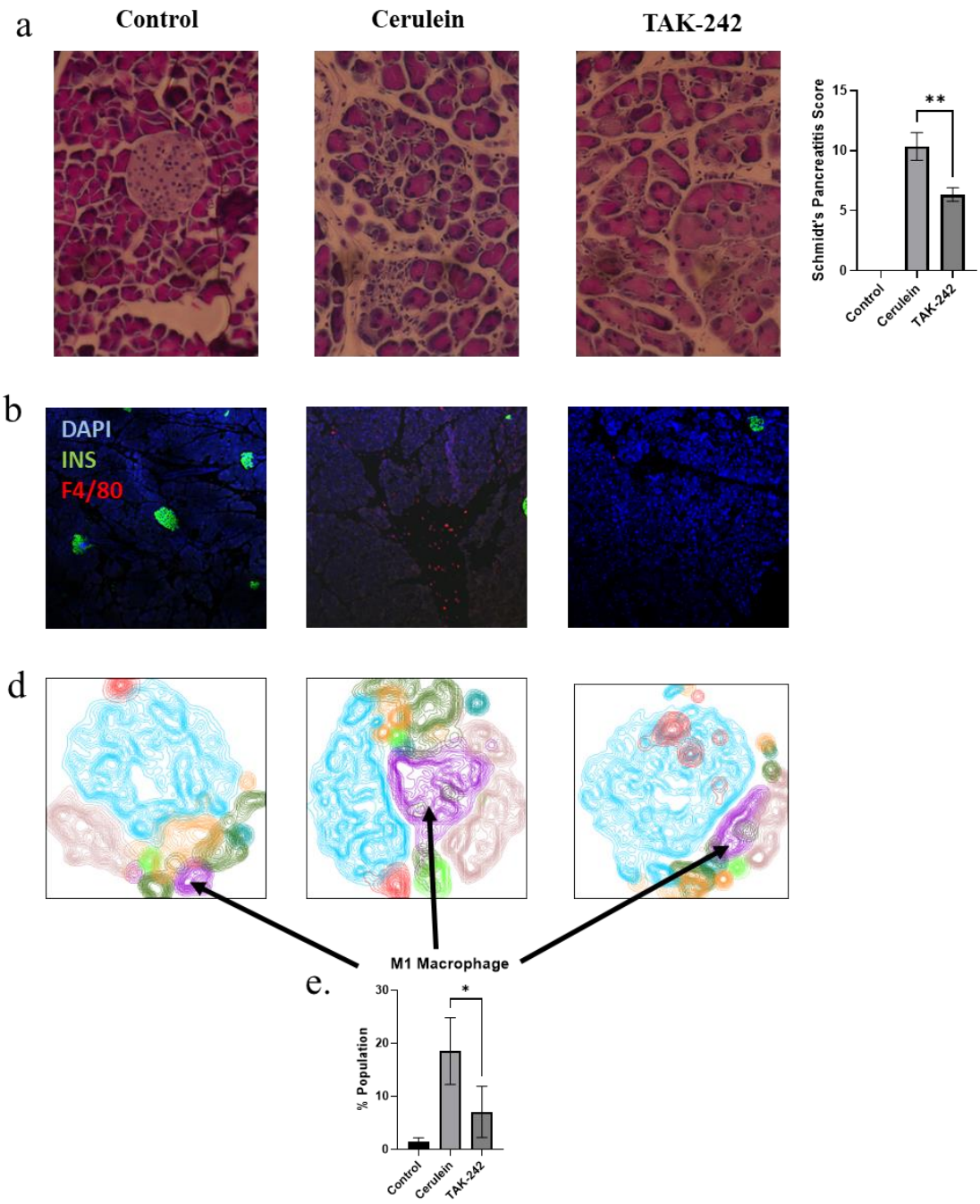

Figure 3.2 (a) Hematoxylin and eosin–stained sections from control, cerulein, and TAK-242–treated mice were used to generate (b) Schmidt's pancreatitis scores. (c) Immunofluorescent staining revealed an increased presence of F4/80+ cells within the pancreas of cerulein-treated mice, which was reduced by TAK-242. (d) Flow cytometric analysis using flowSOM population mapping of pancreatic single cells showed an increased population of (e) M1 macrophages within the pancreas of cerulein-treated mice that was reduced by TAK-242. (* $P < 0.05$, ** $P < 0.005$)

TAK-242 reduces immune cell infiltration in cerulein-induced pancreatitis

As improved scores were observed in TAK-242–treated pancreases when compared to cerulein-treated pancreases, I evaluated macrophage infiltration by monitoring the expression of the macrophage marker F4/80. Observation of pancreatic sections revealed a clear increase in macrophage infiltration that was reduced by treatment with TAK-242 (Figure 3.2). I next processed each pancreas into a single cell suspension to analyze cells by flow cytometry. Using flowSOM population mapping, we noted a significant increase in the M1 macrophage population ($CD45^+/F4/80^+/CD86^+$) (control = 1.125% ± 1.128%, cerulein = 18.533% ± 6.307%, TAK-242 = 7.070% ± 4.833%, P = 0.033) (Figure 3.2).

To confirm the results of our population mapping, we analyzed each immune cell population individually to find significant differences between cerulein- and TAK-242–treated mice. This analysis revealed a significant decrease in total $CD45^+/CD14^+$ cells (cerulein = 27.87% ± 3.51%, TAK-242 = 2.52% ± 0.50%, P = 0.0003) within the pancreases of TAK-242–treated mice as well as total $CD45^+/F4/80^+$ cells (cerulein = 32.3% ± 6.19%, TAK-242 = 7.16 % ± 2.70%, P = 0.003) (Figure 3.3).

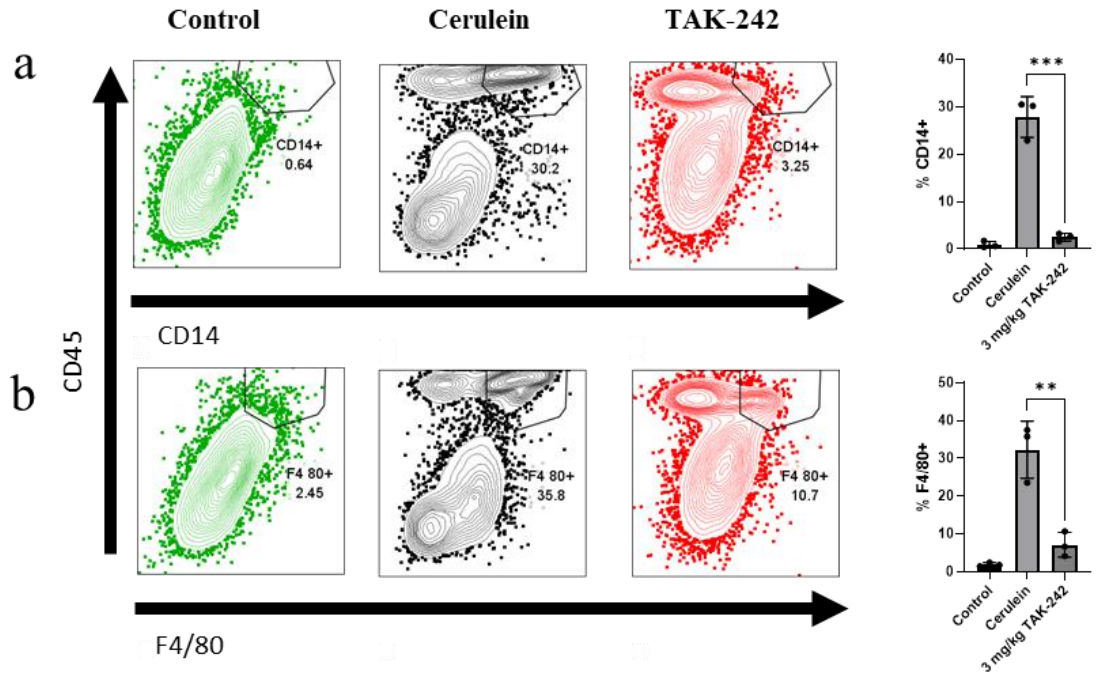

Figure 3.3. Flow cytometric analysis of (a) monocytes and (b) macrophages within the pancreas of control, cerulein, and TAK-242–treated mice following induction of acute pancreatitis. (** $P < 0.005$, *** $P < 0.0005$)

TAK-242 protects the pancreas from immune cell infiltration following the recurrence of pancreatitis

It has been estimated that 21% of patients will experience recurrence of pancreatitis after their first episode (Li et al. 2023). Therefore, I sought to investigate whether inhibition of TLR4 with TAK-242 could protect the pancreas from immune infiltration following repeated insult with cerulein. Following 4 weeks of treatment with cerulein twice per week, the pancreases were procured from mice, and a single cell digest was prepared and analyzed by flow cytometry. This analysis showed a noticeable reduction in $CD45^+/CD14^+$ cells (cerulein = 12.73% ± 1.64%, TAK-242 = 9.91% ± 0.62 %, $P = 0.107$) and significant differences in $CD45^+/F4/80^+$ cells (cerulein = 12.48% ± 0.92%, TAK-242 = 9.12% ± 0.08%, $P = 0.015$) as well as $CD45^+/Ly6G^+$ cells (cerulein = 12.83% ± 1.40%, TAK-242 = 9.07% ± 0.34%, $P = 0.046$). This experiment also demonstrated an elevated

CD45+/F4/80+/CD86+ population in cerulein that was significantly reduced by treatment with TAK-242 (cerulein = 7.96% ± 1.63%, TAK-242 = 5.42% ± 0.07%, $P = 0.047$) (Figure 3.4).

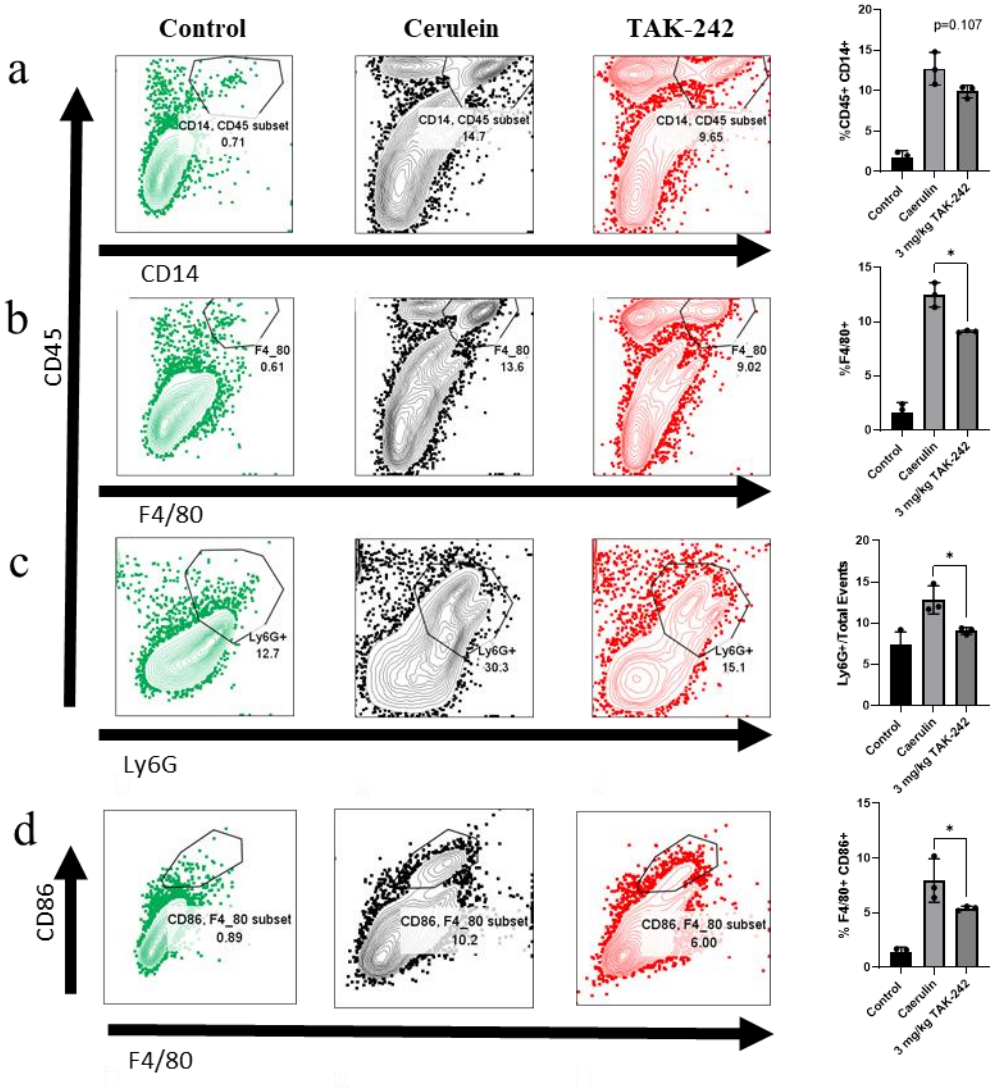

Figure 3.4. Flow cytometric analysis of (a) monocytes, (b) macrophages, (c) neutrophils, and (d) M1 macrophages following 4 weeks of cerulein injections. (* $P < 0.05$)

TAK-242 inhibits inflammatory HMGB1 in cerulein-induced pancreatitis

As pancreatitis can result in remote organ damage, I investigated whether biomarkers of the severity of pancreatitis could be affected by the presence of TAK-242. Interestingly, we found that HMGB1, a known ligand for TLR4, was increased in the serum of mice treated with cerulein and decreased in a dose-dependent manner until a significant decrease was noted at the 10 mg/kg dose in our recurrent model of pancreatitis (cerulein = 2.768 ± 1.124 ng/mL, 10 mg/kg TAK-242 = 0.595 ± 0.128 ng/mL, $P = 0.023$) (Figure 2.5).

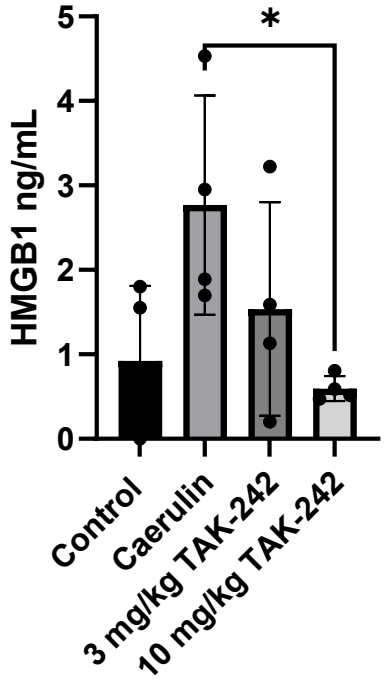

Figure 3.5. HMGB1 expression in the serum of cerulein- and TAK-242–treated mice. (* $P < 0.05$)

Discussion

In this study, I began by showing that injections of cerulein result in significant differences in the transcriptome of mice within the pancreas. RNA sequencing analysis revealed many significantly upregulated RNA expressions when cerulein was administered

to mice. When these RNAs were put into Enrichr for KEGG pathway analysis, the most significantly upregulated pathways were related to inflammation. When repeating this process looking at the most significantly downregulated genes in TAK-242 and cerulein samples, it was noted that many genes upregulated by cerulein were downregulated by TAK-242. KEGG pathway analysis of these RNAs showed that the most significantly altered pathways were again related to inflammation (Figure 3.1).

To confirm the results of this analysis, I examined several mRNA expressions by RT-qPCR. One of the significantly altered RNAs of interest was CXCL10. CXCL10 has shown increased expression in patients with chronic pancreatitis and is largely localized to the cytoplasm of pancreatic acinar cells (Singh et al. 2007). It has been demonstrated that CXCL10 plays a significant role in the polarization of macrophages to an M1 phenotype during the induction of pancreatitis (Peng et al. 2023). Another altered RNA of interest was TNFAIP3. Wang et al showed in their study that TNFAIP3 was elevated in acute pancreatitis pancreatic tissues as well as in a cell model of acute pancreatitis and that overexpression of TNFAIP3 resulted in phosphorylation and deubiquitination of RIP, which activates the NLRP3 inflammasome (Wang et al. 2021). These results also confirmed an increased expression of CXCL2 within the pancreas of cerulein-treated mice, which was significantly decreased by TAK-242. These results confirmed previous work showing an increase in CXCL2 levels in the serum and pancreas of mice treated with taurocholate. Another study showed that the damaging effects of taurocholate were largely reduced in TLR4-deficient mice (Awla et al. 2011). The results of our experiment further validated those results, as we targeted TLR4 in our experiment with TAK-242. IL-1β also showed significant changes in response to cerulein and TAK-242 treatment. As IL-1β

activation is closely tied to NF-κB and NLRP3 activity, which were both significantly affected by cerulein and TAK-242 treatment, it should come as no surprise that IL-1β expression was significantly altered (Cullen et al. 2015). IL-1β has also been shown to be increased during the progression of pancreatitis in the pancreas and serum (Fink and Norman 1997). I also observed a significant increase in IL-6 in response to cerulein administration that was attenuated by TAK-242. IL-6 has proven to be a useful marker of the severity of pancreatitis (J Mayer et al. 2000; Pooran et al. 2003; Sternby et al. 2021). Taken together, these RNA results support that TAK-242 reduces inflammation within the pancreas that could lead to lessened damage following the onset of pancreatitis.

As I observed significant changes in inflammation according to RNA signatures, I next showed that TAK-242 administration could result in beneficial outcomes in regard to pancreas structure and immune cell infiltration. Although TAK-242 administration did not completely alleviate pancreas structural changes and immune infiltration in response to cerulein treatment, hematoxylin and eosin staining and Schmidt's pancreatitis scoring showed significantly reduced scores when compared to cerulein. This is to be expected, as many receptors contribute to inflammation and damage within the pancreas during pancreatitis. In a study of a rat model, it was shown that TLR4 is localized to pancreatic ductal cells, endothelial cells, and islets while being absent from exocrine acinar cells in rats during cerulein induction of pancreatitis (Li et al. 2005). However, a contradicting study utilizing isolated pancreatic acinar cells showed that TLR4 plays a role in the upregulation of chemokine (C-C motif) ligand 2 and P-selectin within acini (Mateu et al. 2015). Another study demonstrated that the administration of LPS to pancreatic acinar cells resulted in increased reactive oxygen species and decreased viability (Pan et al. 2018). As

both of the previously mentioned studies used isolated pancreatic acini for their experiments, it would be useful to develop a mouse model that could be used for *in vivo* studies of TLR4 within acinar cells.

In my next experiment, I showed by immunofluorescence a dramatic increase in $F4/80^+$ within the pancreases of cerulein-treated mice, which was decreased by the administration of TAK-242. Previous work demonstrated that intraperitoneal administration of LPS leads to significant increases of P-selectin and VCAM-1 within the pancreas, which shows that TLR4 could play a role in monocyte rolling adhesion and infiltration following cerulein insult (Andonegui et al. 2002).

I next used flow cytometry and flowSOM population mapping to confirm the increased presence of macrophages within the pancreas. Our analysis of a pancreatic single cell suspension showed a significantly increased population of $CD45^+$, $F4/80^+$, $CD86^+$ cells, which corresponds to M1 macrophages. This is in agreement with previous work stating that during acute pancreatitis, infiltrating macrophages are differentiated into an M1 phenotype (Hu et al. 2020). However, our study showed a significant reduction in this population of macrophages in response to TAK-242 administration, validating results from our previous experiment showing a less inflammatory environment in the pancreas and reduced F4/80 immunofluorescence staining in the TAK-242–treated mice. We confirmed the flowSOM population mapping results by looking at each cell phenotype individually. Results of this study indeed showed reduced monocyte presence as well as macrophage presence.

In a recent study, it was noted that 21% of patients with acute pancreatitis will have a recurrence. I also demonstrated that treatment with TAK-242 leads to beneficial

outcomes in a recurrent acute pancreatitis model, where mice were administered cerulein twice per week for 4 weeks. My results showed a noticeable reduction in monocyte infiltration into the pancreas as well as significant decreases in macrophage and neutrophil infiltration into the pancreas of TAK-242–treated mice. As previous work has demonstrated that neutrophils and neutrophil extracellular traps play a significant role in the progression of pancreatitis, I demonstrated another beneficial outcome with the administration of TAK-242 (Wan et al. 2021). My results also showed a significant decrease in the presence of M1 macrophages, as was noted in the acute pancreatitis model. Finally, we demonstrated that inhibition of TLR4 using TAK-242 could lead to reduced expression of HMGB1 in the serum of treated animals. This is significant, as it has been demonstrated that HMGB1 has an important role in multiple organ dysfunction and systemic inflammation during severe acute pancreatitis (Yang et al. 2017). HMGB1 is also a noted ligand for TLR4, which can further contribute to immune activation and inflammation within remote organs (Yu et al. 2006).

This study does have some limitations. First, in the analysis of the RT-qPCR data, several markers showed noticeable change but did not achieve statistical significance. Increasing the number of test subjects in future studies could lead to smaller standard deviations, resulting in statistically significant changes. Next, the focus of this study was specifically inflammation and immune infiltration. Although I was able to demonstrate altered expressions of cytokines and chemokines in RNA, I did not perform any proteomic analysis from tissue or cell samples. A goal of future studies should be to investigate different cell populations during the progression of pancreatitis and the role that TLR4 plays in each stage of this disease. As I did note a significant increase in serum HMGB1

that was inhibited by TAK-242, it would also be worth investigating the protective effects of TAK-242 on remote organs outside the pancreas.

Conclusion

The results of this study demonstrate that the inhibition of TLR4 using TAK-242 leads to less inflammatory markers within the pancreas. This reduction of inflammation is further supported by reduced immune infiltration to the pancreas following insult with cerulein. Taken together, these results support the utility of TAK-242 for use in attenuation of pancreatic injury and damage following the onset of acute pancreatitis.

CHAPTER FOUR

Inhibition of Toll-Like Receptor 4 Using Small Molecule, TAK-242, Protects Islets from Innate Immune Responses

This chapter published as: Jordan Mattke, Carly M. Darden, Srividya Vasu, Michael C. Lawrence, Jeffery Kirkland, Robert R. Kane, Bashoo Naziruddin. Inhibition of Toll-like Receptor 4 Using Small Molecule, TAK-242, Protects Islets from Innate Immune Responses. 2024. Cells. 13(5):416.

Abstract

Islet transplantation is a therapeutic option to replace β cell mass lost during type 1 or type 3c diabetes. Innate immune responses, particularly the instant blood-mediated inflammatory reaction and activation of monocytes, play a major role in the loss of transplanted islet tissue. In this study we aimed to investigate the inhibition of toll-like receptor 4 (TLR4) on innate inflammatory responses. We first demonstrate significant loss of graft function shortly after transplant through the assessment of miR-375 and miR-200c in plasma as biomarkers. In vitro models we used to investigate how targeting TLR4 mitigates islet damage and immune cell activation during the peritransplant period. The results of this study support the application of TAK-242 as a therapeutic agent to reduce inflammatory and innate immune responses to islets immediately following transplantation into the hepatic portal vein. Therefore, TLR4 may serve as a target to improve islet transplant outcomes in the future.

1. Introduction

Type 1 diabetes mellitus is a chronic disease characterized by insufficient insulin production following autoimmune destruction of pancreatic β-cells leading to

hyperglycemia (Katsarou et al. 2017). Although this condition is usually managed through the administration of exogenous insulin, one therapeutic option for patients suffering from type 1 diabetes is allogenic islet transplantation to replace the β-cell mass lost to autoimmune destruction (Shapiro et al. 2017). Islet transplantation can also be performed to restore glycemic function following total pancreatectomy (Sutherland et al. 2012b).

Previous reports have demonstrated glycemic control after islet transplantation to treat type 1 diabetes and prevention of diabetes following pancreatectomy with improved quality of life. However, many patients require large quantities of islets to achieve complete insulin independence (Shapiro et al. 2000; Toso et al. 2007; Sutherland et al. 2012b; Foster et al. 2018). One of the major hurdles still to overcome in the field of autologous and allogenic islet transplantation is the challenge of innate immune responses immediately following transplantation. It has been estimated that 50% to 70% of islet mass is lost due to this innate immune reaction to islets as they are infused into the portal vein (Delaune et al. 2017). One of the earliest challenges for transplanted tissue is the instant blood mediated inflammatory reaction (IBMIR) characterized by platelet consumption and activation of the coagulation and complement pathways resulting in clot formation around islets as well as immune infiltration immediately following infusion into the portal vein (Bennet et al. 2000). Effective control of the innate immune response will minimize islet damage and improve islet transplant function.

Toll-like receptor 4 (TLR4) is an innate immune receptor expressed on a variety of cell types within the body. Most notably, TLR4 is known to be stimulated in the presence of bacterial lipopolysaccharide (LPS) as well as damage associated molecular patterns (DAMPs). Upon stimulation, TLR4 oligomerizes and can signal through the MyD88

dependent and MyD88 independent pathways resulting in the production of proinflammatory cytokines and recruitment of leukocytes (Lu et al. 2008).

In monocytes stimulation of TLR4 using LPS produces a more inflammatory M1 macrophage phenotype (Wang et al. 2014). This is significant in that it has been noted that M1 macrophages are one of the first leukocytes infiltrating into transplanted tissue following islet transplant in a mouse model (Mok et al. 2019). A study has also shown that inhibition of TLR4 using siRNA leads to lowered chemotactic and phagocytic activity as well as reduced production of IL-1β and IL-6 in recipients (Wu et al. 2009). Inhibition of TLR4 using peptide results in reduced IL-1β, TNF-α, iNOS, and IL-6 in Raw264.7 macrophages exposed to LPS and results in reduced macrophage infiltration in islet grafts (Dong et al. 2016). Stimulation of TLR4 within dendritic cells leads to dendritic cell maturation and increased T cell priming (Shen et al. 2008). TLR4 also has a significant role in the process of cross presentation within dendritic cells as TLR4 stimulation engages Rab34-dependent reorganization of lysosomes and delays antigen degradation as well as increasing Rab14 activity leading to increased anterograde transport resulting in altered antigen presentation and activation of T cells (Alloatti et al. 2015; Weimershaus et al. 2018).

Previous studies in mice have shown that TLR4 deficient allogenic islets display improved survival when transplanted into BALB/c recipients (Goldberg et al. 2007). In vitro blockade of TLR4 using TLR4 antibody resulted in reduced β-cell apoptosis and T-cell activation and proliferation against allogenic islets as well as indefinite allogenic islet graft survival, although tolerance was not achieved in this study (Giovannoni et al. 2015). These results were further confirmed by another study that showed that deficiency of the

TLR4 in donor islets or blockade of the TLR4 ligand HMGB1 resulted in prolonged graft survival (Krüger et al. 2010).

TAK-242 is a small molecule inhibitor shown to be highly specific for TLR4 with little off target effects and has been used in clinical trials for the treatment of severe sepsis (Rice et al. 2010; Naoko Matsunaga et al. 2011). Since previous work has demonstrated TLR4 having a substantial role in islet transplantation leading to early graft failure, our laboratory sought to investigate whether inhibition of TLR4 signaling using TAK-242 could lead to more favorable transplant outcomes (Gao et al. 2010). Results of the study by Chang et. al. revealed that islets transplanted under the kidney capsule with soluble TAK-242 or islets surface-modified with a NHS-PEG linker connected to a releasable TAK-242 showed improved islet recovery when compared to mice treated with control islets (Chang, Akinbobuyi, et al. 2018).

This study shows that inhibition of TLR4 using TAK-242 reduces innate immune responses such as IBMIR and leukocyte activation leading to attenuation of islet stress and damage. The study uses miR-375 and miR-200c as biomarkers to assess islet damage in both clinical samples and cell culture experiments. We also report that TAK-242 inhibits activation of inhibition of innate immune cells, which then reduced the proliferation and activation of CD8+ T cells. Overall, our results reveal a broader role for TLR4 in the protection of islet transplants from innate immune responses.

2. Materials and Methods

2.1. Patients and Sample Collection

All patients included in this study underwent total pancreatectomy with islet autologous transplantation (TPIAT) at Baylor University Medical Center in Dallas, TX. To

validate miR-375 and miR-200c as reliable biomarkers to assess islet damage, we analyzed plasma samples collected from TPIAT patients during the time of islet infusion. Previous published work showed that optimal outcomes following TPIAT occur when patients receive >5000 IEQ/kg (Chinnakotla et al. 2015). We therefore narrowed our focus to patients receiving >5000 IEQ/kg during TPIAT. Blood samples were collected prior to islet infusion and 1 hour after the completion of infusion. Three-month follow-up data were collected for each patient in this study. Table 4.1 presents patient and pancreas characteristics.

Table 4.1 Patient and pancreas characteristics at the time of TPIAT as well as islet isolation outcomes.

Variable	***Value (n = 10)***
Gender (female : male)	8 : 2
Age (years)	40 ± 11.7
Height (cm)	165 ± 9.88
Weight (kg)	70.2 ± 11.82
Body mass index (kg/m^2)	25.75 ± 3.71
Disease duration (years)	9.4 ± 5.9
Fasting blood glucose (mg/dL)	91.1 ± 12.3
Stimulated blood glucose (mg/dL)	153.5 ± 54.5
Basal C-peptide (ng/mL)	1.91 ± 1.39
Stimulated C-peptide (ng/mL)	7.39 ± 4.48
Δ C-peptide (ng/mL)	5.48 ± 3.47
Initial trimmed pancreas weight (g)	134.6 ± 25.3
Pancreas weight processed (g)	88.0 ± 23.3
Total islet yield (IEQ)	560,973 ± 125,830
Islet particle number (IN)	335,700 ± 96,743
Islet yield (IEQ/g pancreas)	5,986 ± 2012
Dose (IEQ/kg patient)	8,147 ± 2040

2.2. Islet Isolation

Islet isolations were carried out at the Baylor Scott and White Research Institute from deceased donor and chronic pancreatitis pancreases. Islet isolations were performed according to previously established practices for clinical transplantation (Kin 2010). Pancreases were decontaminated in antibiotic before being perfused with collagenase enzyme (Vitacyte, IN, USA) solution followed by mechanical digestion in a Ricordi chamber and purification in density gradient solution (Kin 2010). Viability of islets was confirmed using fluorescein diacetate and propidium iodide staining. Islets were allowed to recover overnight in PIM(S) media supplemented with PIM(ABS) Human AB Serum Supplement and PIM(G) Glutamine and glutathione supplement (Prodo Laboratories Inc, CA, USA).

2.3. Surface Modification of Islets

Islet surfaces were modified with TAK-242 using copper free click chemistry previously carried out by Chang, et. al (Chang, Akinbobuyi, et al. 2018). Islets were suspended in Kreb's Ringer bicarbonate buffer containing 5.6 mM glucose and 20 μM NHS-Linker-TAK-242 dissolved in DMSO for 30 minutes. Control islets were incubated in Kreb's Ringer bicarbonate buffer containing an equivalent amount of DMSO as NHS-Linker-TAK. Islets were then washed and suspended in RPMI 1640 with 10% fetal bovine serum (FBS) and Antibiotic-Antimicotic (ThermoFisher) prior to beginning experiments.

2.4. In vitro IBMIR

After 24 hours of culture, 500 IEQ of islets were administered into heparinized tubes. Control samples were mixed with 500 μL of RPMI with 10% FBS. For IBMIR simulation, islets were mixed with 500 μL of whole allogenic blood. Tubes were then

incubated for 3 hours at 37˚C with agitation. For samples treated with soluble TAK-242, TAK-242 was administered to a final concentration of 10 μM. The concentration of 10 μM was determined in order to keep consistency between this experiment and all other assays which showed significant changes in immune responses starting at 10 μM. All other samples contained an equivalent amount of DMSO as TAK-242-treated samples. At the beginning of the experiment and after 3 hours of incubation with agitation, all samples were centrifuged and plasma was collected for miRNA analysis.

2.5. Clotting Assay

50 IEQ of islets were suspended in 100 μL of platelet poor plasma in the presence and absence of 10 μM TAK-242 in a 96 well plate. Plasma without islets was added to wells to serve as a negative control. 25 mM $CaCl_2$ was then added to each well of the plate before the plate was transferred to a Cytation 5 (BioTek, Winooski, VT, USA) to measure the absorbance every minute at 405 nm.

2.6. One Way Mixed Lymphocyte Reaction

Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque gradient centrifugation. PBMCs were labelled with CFSE cell proliferation dye (ThermoFisher Scientific, Waltham, MA, USA). A total of 1 x 10^5 PBMCs were mixed with 1 x 10^5 mitomycin C treated human splenocytes labeled with CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific) in a U bottom 96 well plate with 55 μM IL-2 and 5 μg/mL LPS.

2.7. CD8+ T Cell Activation Assay

Following isolation of PBMCs, T cells were enriched with EasySep™ Human T Cell Enrichment Kit (STEMCELL Technologies, Vancouver, BC, Canada). CD8+ T cells were then selected using EasySep™ Human CD4 Positive Selection Kit II (STEMCELL Technologies, Vancouver, BC, Canada). Cells were then labelled with CFSE or CellTrace™ Violet Proliferation Violet Proliferation Kit. The CD8+ T cells were then mixed with Dynbeads™ Human T -Activator CD3/CD28 (ThermoFisher Scientific, Waltham, MA, USA) at a 1:1 ratio in a U-bottom 96 well plate. Cells were monitored for activation and proliferation by flow cytometry.

2.8. In Vitro Co-Culture of Allogenic PBMC and Islets

PBMCs were isolated by Ficoll Paque gradient centrifugation. PBMCs were labelled with CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific,Waltham, MA, USA). A total of 1 x 10^5 PBMCs were then mixed with ~15 IEQ of hand-picked islets in RPMI medium supplemented with 10% FBS, Anti/Anti (Gibco), and 55 μM IL-2 in a U-bottom 96 well plate.

2.9. Cytokine Quantification

After 24 hours of co-culture supernatant from each well was removed. Supernatant was then analyzed for the quantities of IL-1β, IL-6, IL-8, IP10, MCP-1, and TNFα by Milliplex Human Cytokine/Chemokine/Growth Factor Panel A (MilliporeSigma, Burlington, MA, USA) according to the manufacturer's protocol.

2.10. RT-qPCR for Secreted Stress and Damage miRNA

Following collection of plasma and cell culture supernatant, miRNA was isolated using miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany). UniSp6

synthetic spike in was added prior to column isolation. miRNA was reverse transcribed using miRCURY LNA RT Kit (Qiagen, Hilden, Germany). qPCR was performed using miRCURY LNA SYBR Green PCR Kit (Qiagen, Hilden, Germany) using primers for miR-375 and miR-200c (Qiagen, Hilden, Germany).

2.11 Flow Cytometry

Islets were removed from each co-culture by hand-picking. Cells were then administered into 5 mL polystyrene tubes through 70 μM filters. T cells were stained with anti-human CD3 Alexa Fluor 700, anti-human CD4 APC, anti-human CD8 APC-Cy7, anti-human CD69 PE, and anti-human CD25 BV786 (Biolegend, San Diego, CA, USA). Dendritic cells were stained with anti-human CD14 Brilliant Violet 605, anti-human CD11c PE-Cy7, anti-human CD80 BUV737, and anti-human CD83 BV785 (Biolegend, CA, USA). Cells were then analyzed on a BD LSRFortessa^{TM} Cell Analyzer.

2.12. Statistical Analysis

Different groups were compared by unpaired two tailed Students t-test. Flow cytometry data were analyzed using FlowJo 10.8.1. All data were analyzed using GraphPad Prism 9.1.2 (GraphPad Software, CA, USA). Clinical data sample size was confirmed appropriate using power calculation which used 0.8 for the correlation coefficient, 0.05 for the alpha value, and 0.8 for the power value.

3. Results

3.1. Elevated miR-375 and miR-200c Corresponds to Poor Islet Function Following TPIAT

First, we aimed to identify biomarkers to assess islet damage. Previously, it was shown that intraportal infusion of islets into patients immediately results in islet damage as evidenced by elevated microRNA levels in the plasma (Saravanan et al. 2019). We analyzed plasma samples from TPIAT patients who received an islet dose of >5000 IEQ/kg to reliably assess islet damage and observed a several-fold increase in both miR-375 and miR-200c 1 hour after islet infusion when compared to pretransplant samples. When comparing the fold change of miR-375 and miR-200c to transplant outcomes 3 months after surgery, several trends were observed. First, we demonstrated that both miR-375 and miR-200c correlate strongly with HbA1c levels at 3 months following TPIAT (Figure 1). miR-375 and miR-200c also had a positive correlation to insulin usage 3 months following TPIAT ($p < 0.05$) (Figure 4.1) indicating that miR-375 and miR-200c can be used as biomarkers to assess islet damage and also to predict islet function following transplantation.

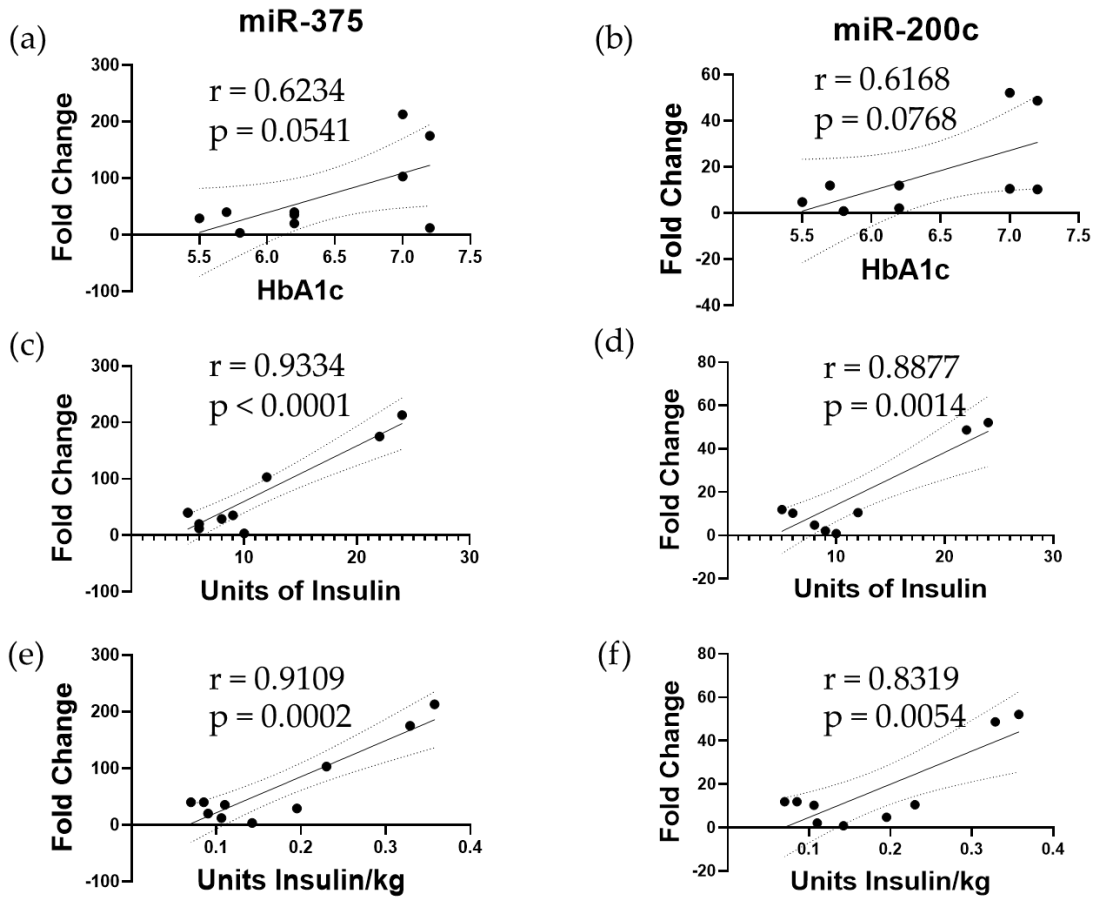

Figure 4.1. Correlation analysis of miRNA expression 1 h post-infusion with 3-month transplant outcomes. The fold change in (a) miR-375 and (b) miR-200c showed a positive trend when com-pared to 3-month hemoglobin A1c values. (c) miR-375 and (d) miR-200c fold change also showed a significant positive trend when compared to insulin use at 3 months post-TPIAT. This same trend was observed when (e) miR-375 and (f) miR-200c were compared to the insulin dose 3 months after TPIAT.

3.2 TAK-242 Reduces Damage to Islets Exposed to IBMIR In Vitro

To evaluate the protective effects of TAK-242 on islet damage, we created an in vitro model of IBMIR and measured miR-375 and miR-200c release. When islets were exposed to whole blood, there was a significant increase of miR-375 (control islet = 6.017 ± 0.177 FC vs. control IBMIR = 12.294 ± 1.448 FC, $p<0.001$) and miR-200c (control islet=6.327±0.761 FC vs. control IBMIR=33.273±0.666 FC, $p < 0.001$) in response to the presence of blood that was not noted in samples that did not contain a mixture of blood and

islets (Figure 4.2). However, when TAK-242 was present in soluble form or released locally from the surface of islets, islets secreted less miR-375 (control IBMIR = 12.294 ± 1.448 FC, MAP84 IBMIR = 12.154 ± 0.411 FC, TAK-242 IBMIR = 8.149 ± 0.375 FC, modified IBMIR= 8.157 ± 1.102 FC, $p < 0.05$) and miR-200c (control IBMIR = 33.273 ± 0.666 FC, MAP84 IBMIR = 31.674±1.990 FC, TAK-242 IBMIR = 17.665 ± 0.35 FC, modified IBMIR = 19.761 ± 3.26 FC, $p < 0.05$) when compared to untreated islets or islets treated with the inactive TAK-242 analog, MAP84 (Figure 4.2). The decrease in these miRNAs can be taken as a sign that islets are undergoing less stress and damage due to the IBMIR reaction in the presence of TAK-242 (Saravanan et al. 2019). In order to assess whether this reduction in damage was due to reduced immune cell activation or reduced complement and coagulation cascades, we performed a clotting assay using islets and platelet poor plasma to evaluate whether TAK-242 affects the clotting process. In this experiment we did not note a significant reduction in clotting times in mixtures with TAK-242 compared to clotting times with no treatment. Therefore, the remainder of our study focused on inflammation and damage due to immune cell infiltration and activation.

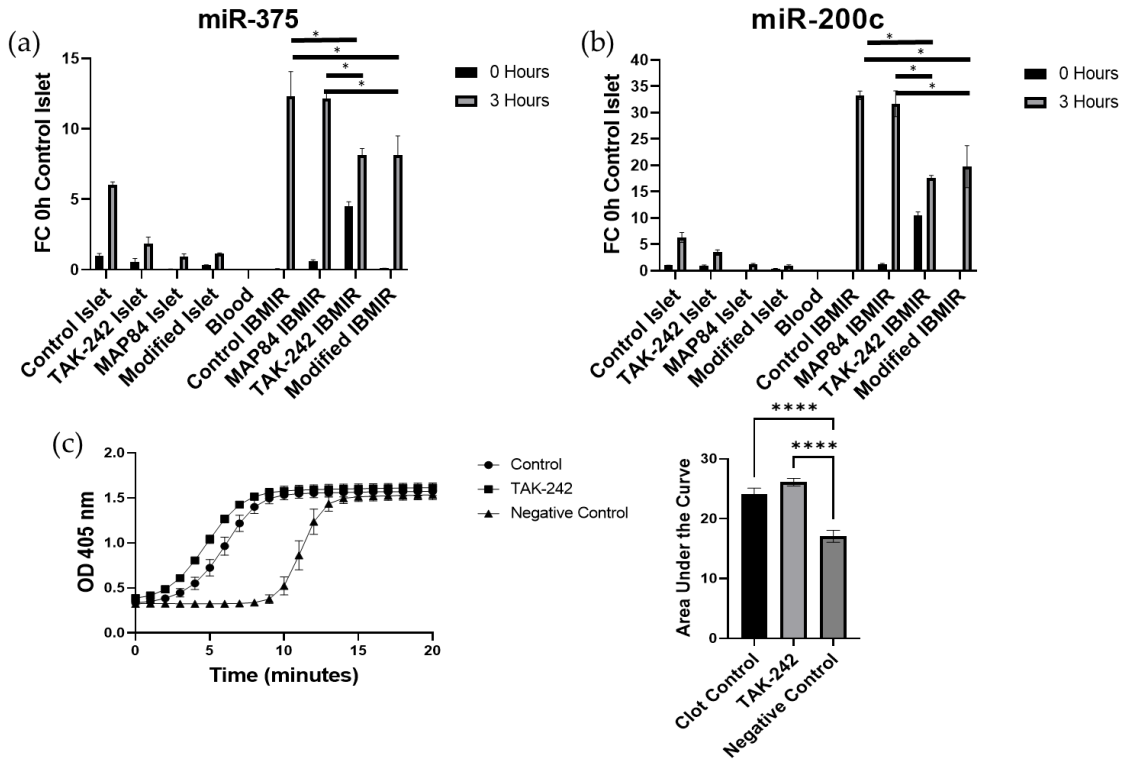

Figure 4.2. Inhibition of islet damage by TAK-242 as measured by the expression of (a) miR-375 and (b) miR-200c during an in vitro IBMIR reaction. Aliquots of islets and blood were mixed in vitro in the presence of TAK-242 or structural analog, MAP84, and the supernatant was analyzed for miR-375 and miR-200c using RT-PCR; (c) clotting assay results for islets exposed to platelet-poor plasma. * $p < 0.05$, **** $p < 0.0001$.

3.3 TAK-242 Inhibits T Cell Activation and Proliferation in a One-Way Mixed Lymphocyte Reaction with Splenocytes

Because β cells of islets are able to secrete their own cytokines, termed "isletokines,", we assessed whether TAK-242 was affecting isletokine secretion from islets or whether TAK-242 was directly inhibiting innate immune responses (Yoshimatsu et al. 2017). To do this, we conducted a one-way mixed lymphocyte reaction using human allogenic PBMCs and mitomycin C-treated splenocytes in the presence of supplemental IL-2 to assist with T cell proliferation and LPS to assist with the activation of dendritic cells responsible for presenting antigen and priming T cells. To begin, we first observed the activation markers CD83 and CD80 on dendritic cells. Following an overnight

incubation of PBMCs and splenocytes, LPS treated samples showed a remarkable increase in CD83 (control = 2,947 ± 256, LPS control = 4,672±64, LPS MAP84 = 5,679±331, $p < 0.005$) and CD80 expression (control = 2,011±364, LPS control = 15,691 ± 463, LPS MAP84 = 12,054 ± 631, $p < 0.05$) in the LPS control samples and LPS MAP84 samples (Figure 4.3). However, this increase in CD83 (LPS TAK-242 = 2,864±99, $p < 0.0005$) and CD80 (LPS TAK-242 = 653 ± 106, $p < 0.001$) was not noted in the LPS TAK-242-treated samples indicating a reduction in the activation of dendritic cells in these samples.

We then analyzed the supernatant from this mixed lymphocyte reaction using a Luminex assay. In this assay administration of TAK-242 ligand, LPS, resulted in significantly increased expression of IL-1β (control = 12.98 ± 16.83 pg/mL, LPS control = 4,072 ± 625 pg/mL, LPS TAK-242 = 0 ± 0 pg/mL, $p<0.0001$) , IL-6 (control=114±107 pg/mL, LPS control = 11,506 ± 1,409 pg/mL, LPS TAK-242 = 13.1 ± 1.6 pg/mL, $p < 0.0001$), IL-8 (control = 7,866 ± 2,071 pg/mL, LPS control = 53,984 ± 3,944 pg/mL, LPS TAK-242 = 5,297 ± 1,144 pg/mL, $p < 0.0001$), and TNFα (control = 446 ± 277 pg/mL, LPS control = 8,330 ± 2,214 pg/mL, LPS TAK-242 = 111 ± 36 pg/mL, $p < 0.005$). The expression of these cytokines in the supernatant was significantly inhibited by the presence of TAK-242 (Figure 3). Although MCP-1 (control=1,513±665 pg/mL, LPS control=1,380±171 pg/mL, LPS TAK-242=182±29 pg/mL, $p < 0.0005$) and IP-10 (control=451±60 pg/mL, LPS control=460±50 pg/mL, LPS TAK-242=338±13 pg/mL, $p < 0.05$) were not increased by LPS, the addition of TAK-242 significantly reduced the expression of each of these cytokines (Figure 4.3).

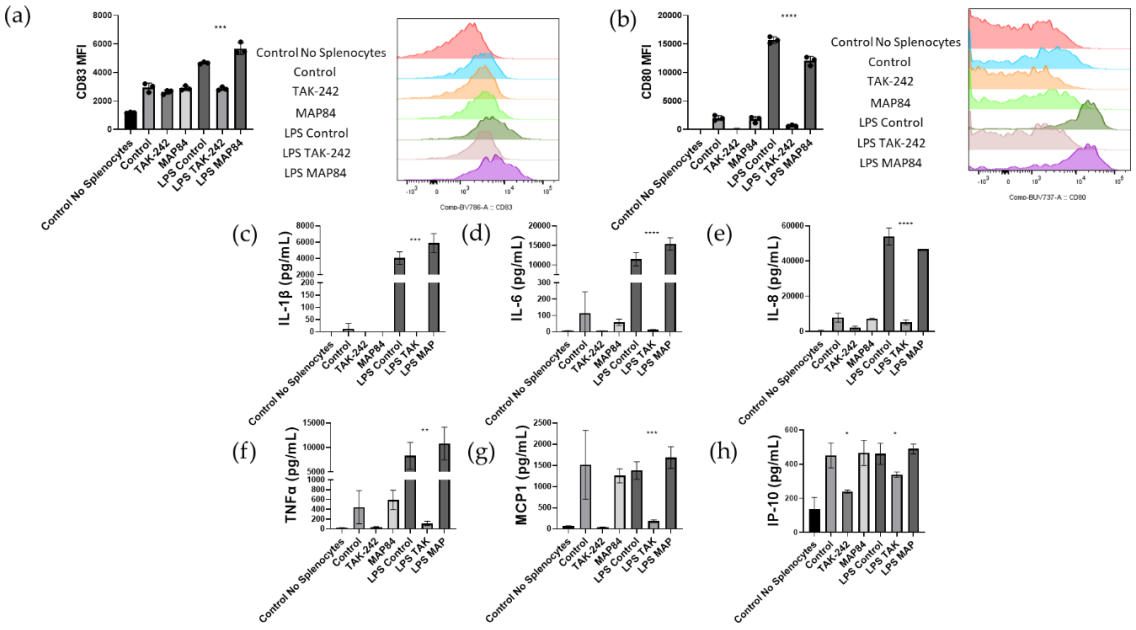

Figure 4.3. (a) CD83 and (b) CD80 expression was measured on dendritic cells 24 h after PBMCs were mixed with allogenic splenocytes. Cytokines (c) IL-1β, (d) IL-6, (e) IL-8, (f) TNFα, (g) MCP1, and (h) IP-10 were quantified in the supernatant of this one-way mixed lymphocyte reaction. (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$).

3.4 TAK-242 Directly Inhibits the Activation of CD8+ T Cells

We next investigated whether administration of TAK-242 resulted in decreased priming and activation of T cells in the mixed lymphocyte reaction. Initial results showed that TAK-242 decreased the proliferation of CD8+ T cells in a dose dependent manner (Figure 4.4). These observations indicated that combining allogenic splenocytes with LPS as an adjuvant resulted in a synergistic effect leading to an increased number of CD8+ CD69+ CD25+ T cells. As CD8+ T cells are known to express TLR4 and react to markers of sterile inflammation such as IL-12, We next investigated whether TAK-242 directly inhibits CD8+ T cell activation (Wienhöfer et al. 2021). Upon stimulation with CD3/CD28 antibody coated beads, we noted a dose dependent decrease in CD8+ T cell activation as well as proliferation in the presence of TAK-242 that was not affected by the inactive

analog, MAP84 (Figure 4.5). We also confirmed the presence of TLR4 on these cells and noted increased TLR4 expression as these cells became activated (Figure 4.5).

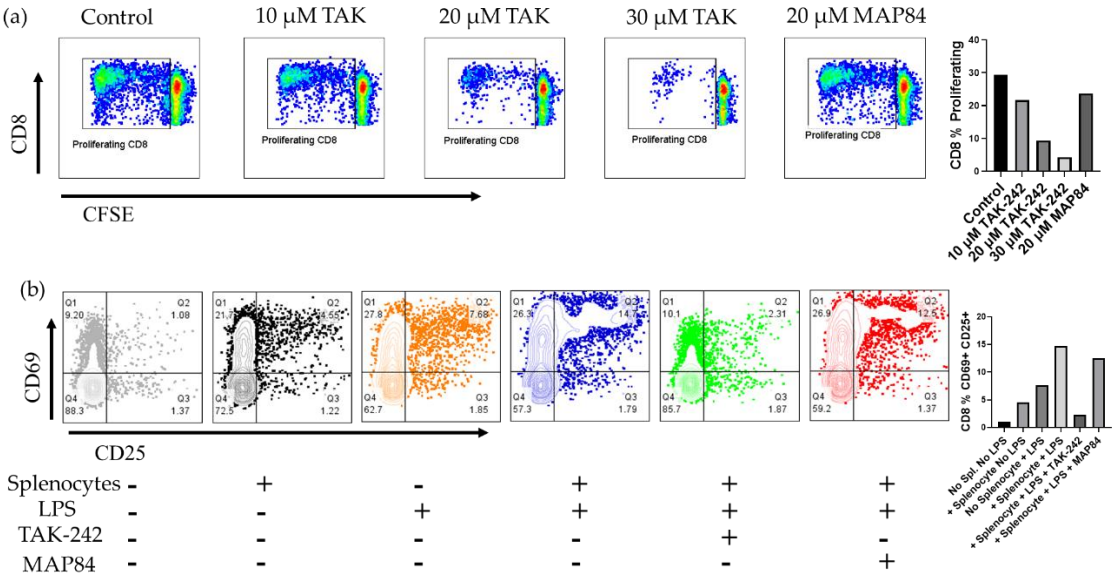

Figure 4.4. (a) Proliferation of CD8+ T cells in a one-way mixed lymphocyte reaction of allogenic PBMCs and mitomycin C-treated splenocytes. (b) CD69 and CD25 expression was assessed on CD8+ T cells in this one-way mixed lymphocyte reaction, which also utilized LPS as an adjuvant to enhance immune cell responses.

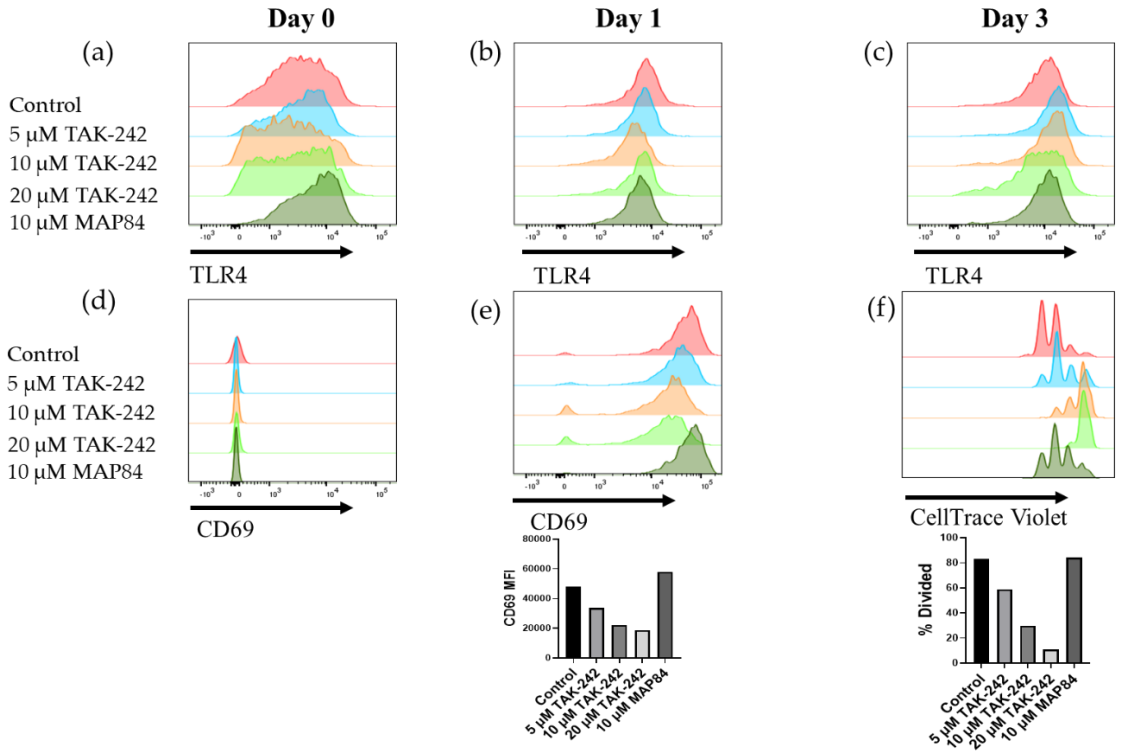

Figure 4.5. TLR4 expression on CD8+ T cells undergoing polyclonal CD3/CD28 stimulation (a) at the beginning of the experimental period, (b) after 24 h, and (c) after 3 days. CD69 expression was monitored on CD8+ T cells (d) at the beginning of the experiment and (e) 1 day after beginning the experiment. (f) Proliferation was monitored 3 days after stimulating CD8+ T cells with CD3/CD28-coated beads.

3.5 T Cell Activation and Islet Damage Is Inhibited by the Presence of TAK-242

The mixed lymphocyte reaction was repeated utilizing islets instead of splenocytes. Soluble TAK-242 as well as islets modified with NHS-Linker-TAK-242 showed significantly less CD69+ CD8+ T cells (control = 47.8 ± 3.2%, TAK-242 = 35.35 ± 1.35%, MAP84 = 49.35 ± 7.15%, TAK-242 modified = 32 ± 0.2, $p < 0.05$) after 24 hours of co-culture (Figure 6). This decreased activation was accompanied by decreased stress and damage miRNA-375 (control = 6.60±1.13 fM, TAK-242 = 0.927 ± 0.170 fM, MAP84 = 10.413 ± 4.881 fM, TAK-242 modified = 0.258 ± 0.0570 fM, $p < 0.05$) and miRNA-200c (control = 2.266 ± 0.307f, TAK-242 = 0.148 ± 0.0634 fM, MAP84 = 2.111 ± 0.232 fM, $p < 0.05$) (Figure 6). There was also a significant decrease in IL-1β (control = 1,310 ± 32

pg/mL, TAK-242 = 37 ± 2.9 pg/mL, MAP84 = 1,090 ± 45.5 pg/mL, $p < 0.05$), IL-6 (control = 11,792 ± 81 pg/mL, TAK-242 = 5,592 ± 825 pg/mL, MAP84 = 12,216 ± 419 pg/mL, $p < 0.05$) , and TNFα (control = 1,558 ± 254 pg/mL, TAK-242 = 649 ± 172 pg/mL, MAP84 = 1,298±211 pg/mL, $p < 0.05$) present in the supernatant of TAK-242 treated samples (Figure 4.6). Islets extracted from control and TAK-242 treated co-cultures showed improved islet structure in cultures containing TAK-242.

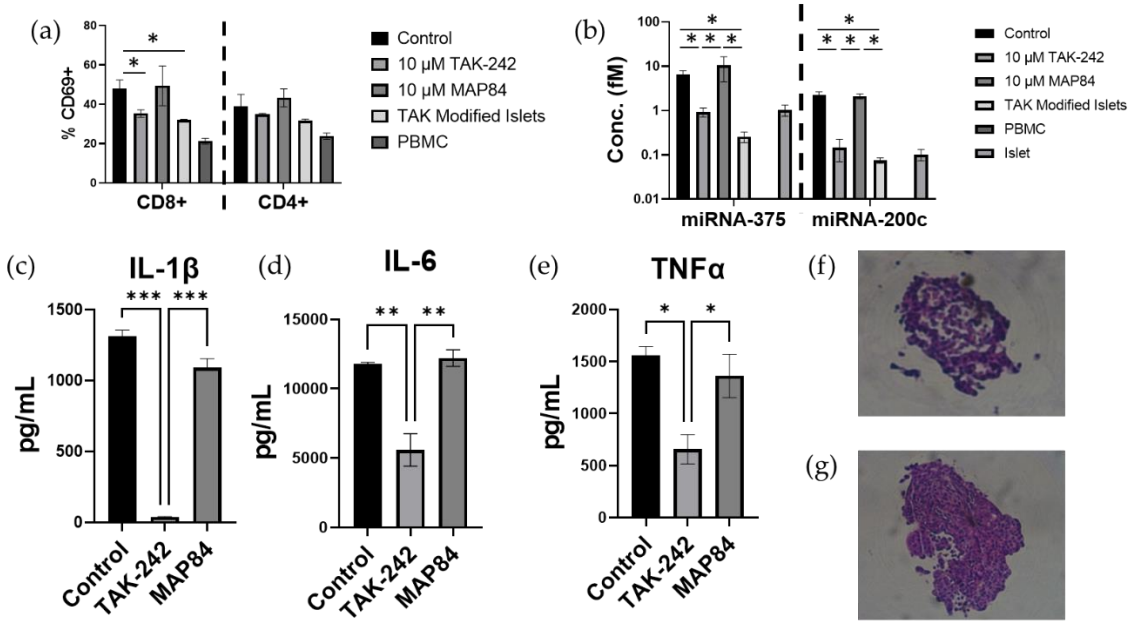

Figure 4.6. Activation of (a) CD8+ and CD4+ T cells 24 h after incubation with allogenic islets. Concentrations of (b) miR-375 and miR-200c in the supernatant 24 h after the mixture of allogenic islets and PBMCs. Expression of (c) IL-1β, (d) IL-6, and (e) TNFα after 24 h of incubation of PBMCs and islets. Representative pictures of (f) control mixed culture and (g) TAK-242 mixed culture. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

4. Discussion

In this study we have shown that inhibition of TLR4 using small molecule, TAK-242, attenuates damage to islets due to IBMIR and other innate immune reactions. Based on previously published research, it is known that islets release increased amounts of miRNAs in response to stress and damage. Our previously published work demonstrated

that miR-375 and miR-200c were among the most abundant islet-specific exosomal miRNAs when islets were subjected to cytokine and hypoxic stress as the islet viability decreased (Saravanan et al. 2019). Using this information, we first sought to investigate whether these miRNAs can be used as diagnostic biomarkers to assess islet graft damage and functional outcomes. Using plasma samples obtained prior to and after islet infusion during TPIAT, I determined that increases in miR-375 and miR-200c were correlated to increased HbA1c and increased use of insulin 3 months after transplant suggesting that early damage to the autologous islet grafts results in poor functional outcomes in TPIAT. We then used these markers in an in vitro model of IBMIR to evaluate whether TAK-242 could lessen the damage to islets as measured by release of these biomarkers. We observed that there was significantly reduced miRNA-375 and miRNA-200c in mixtures of whole allogenic blood in the presence of TAK-242 than in control conditions or in the presence of inactive TAK-242 analog, MAP84 (Figure 4.1). Although miR-375 and miR-200c have proven to be very sensitive biomarkers for assessing islet damage, these markers to do not provide real time results of islet stress and damage as sample processing takes time, and these markers are differentially expressed after islet damage has occurred. A previous study carried out focusing on the inhibition of NF-KB showed that inhibition of NF-κB was able to attenuate IBMIR in the form of increased viability, decreased cytokine expression, and decreased neutrophil infiltration (Kanak, Takita, Itoh, et al. 2014). Because one of the major targets of TLR4 is NF-κB and TLR4 is one of the major receptors for DAMPs, it can be assumed that TLR4 can play a role in the inflammation and infiltration of leukocytes associated with IBMIR.

Three major components of IBMIR reaction are coagulation, complement activation (mostly in allogenic and xenogenic models) and activation of inflammatory response. It has been shown that targeting coagulation and complement activation with dextran sulfate results in improved islet transplant outcomes in an in vitro model and a nonhuman primate in vivo model (Johansson et al. 2006). These results have shown that TAK-242 does not inhibit the coagulation (Figure 4.1c). Complement activation has been shown to trigger TLR4 signaling mainly through C5a resulting in increased NF-κB activity and cytokine production in monocytes (Gong et al. 2019). Furthermore, C5a addition to neutrophils showed an upregulation of CD14 and TLR4 activated mRNAs such as IL-8 (Stevens et al. 2011). However, TLR4 has not been shown to influence activation of complement pathways. Islets are known to express TLR4, and future studies should investigate whether TAK-242 can mitigate the damage to islets caused by complement activation.

Previous work carried out in our laboratory validated that TAK-242 treatment to islets as well as modifying the surface of islets with TAK-242 does not affect glucose-stimulated insulin secretion or viability (Chang, Akinbobuyi, et al. 2018). Further, in a syngeneic diabetic mouse model of islet transplantation, administration of soluble TAK-242 or use of TAK-242 surface conjugated islets significantly improved achievement of normoglycemia. This same study also evaluated the use of TAK-242 in a syngeneic model of islet transplant resulting in significantly improved transplant outcomes for islets surface modified with TAK-242 (Chang, Akinbobuyi, et al. 2018) . We do acknowledge that this transplant model was carried out in the kidney capsule of mice where there is less exposure to immune and circulatory factors which contribute to islet loss. Therefore, future

experiments utilizing TAK-242 should focus on preclinical in vivo experiments utilizing the hepatic portal vein for islet infusion in order to assess the effect of TAK-242 on long term islet transplant outcomes.

After showing that the presence of TAK-242 had no significant changes in clotting times following mixture of platelet poor plasma and islets (Figure 4.2c.), I next focused on the activation of innate immune cells. As the surface modification strategy of islets utilized copper free click chemistry with a short chain PEG which is smaller than proteins, lipids, and carbohydrates of receptors on the cell surface, the surface modification strategy would not prevent receptor or complement binding to the islet surface. Therefore, the most drastic effects of our surface modification would be the effects of TAK-242 following β elimination and release from the islet surface. We then demonstrated that the presence of TAK-242 inhibits immune responses in a one-way mixed lymphocyte reaction experiment as dendritic cell activation was decreased in the presence of TAK-242 (Figure 4.2). This shows that not only could TLR4 stimulation affect transplanted donor islet tissue, but it could inhibit development of recipient immune responses as well. Antigen presentation and dendritic cell cross dressing play a major role in the activation of T cells following transplantation leading to rejection (GILLA 1999; Hughes et al. 2020). TLR4 plays a significant role in the process of antigen processing and presentation within monocytes as it has been shown that TLR4 stimulation leads to decreased antigen degradation as well as increased anterograde transport of endosomes (Alloatti et al. 2015; Weimershaus et al. 2018). For this reason, future studies should be investigating whether donor antigen presentation is inhibited in monocytes in the presence of TAK-242, which could be a possible reason for decreased CD8+ T cell activation.

This study has also shown that inhibition of TLR4 using TAK-242 results in significantly lower activation of CD8+ T cells (Figure 5). To validate this finding, we decided to test whether TAK-242 specifically inhibits antigen presentation to T cells or whether TAK-242 is able to directly inhibit T cell activation. we tested this by using polyclonal stimulation of CD8+ T cells with CD3/CD28 antibody-coated beads. In this experiment we noted a dose dependent decrease in T cell activation as well as proliferation in response to TAK-242. We also noticed an increase in TLR4 expression on these cells as they became activated and proliferated (Figure 5). Previous research has confirmed the presence and responsiveness of TLR4 to LPS (Tripathy et al. 2017). However, to our knowledge, this is the first report in which inhibition of TLR4 was shown to interfere with TCR signaling. Therefore, a focus of future research should be to investigate how TCR signaling is supported by TLR4 in the CD8+ T cells.

Previous work has shown that TAK-242 is able to decrease proinflammatory cytokine production in LPS stimulated macrophages (Sha et al. 2007). TAK-242 has also been shown to decrease neutrophil extracellular trap production by neutrophils during acute rejection of liver transplants (Liu et al. 2022). In this study we demonstrate that TAK-242 decreases production of IL-1β, IL-6, IL-8, IP-10, MCP-1, and TNFα in an allogenic co-culture of PBMCs and splenocytes (Figure 4.3). The presence of these cytokines in the supernatant of these reactions can be taken as indirect signs of reduced macrophage and neutrophil infiltration and activation. It is well known that activated macrophages produce IL-1β, IL-6, IL-8, and TNFα, which further contributes to immune infiltration and activation (Leopold Wager and Wormley 2014). Neutrophil migration is also strongly influenced by the presence of IL-8 (Kunkel et al. 1991). MCP-1 is recognized as one of the

key chemokines regulating macrophage and monocyte recruitment and infiltration (Deshmane et al. 2009). IL-1β has been found to be a key marker of β cell damage and death in islet transplantation (Montolio et al. 2007). IL-6, IL-8, and IP-10 were all found to be increased immediately following transplant in human autologous islet transplants into the portal vein (Naziruddin et al. 2014). Blockade of IL-6 and TNFα resulted in reduced production of IL-6, IL-8, and MCP-1 with improvement of islet function following transplant (Naziruddin et al. 2018). Although it has been shown that β cells are capable of secreting IP-10, our observation of a one-way mixed lymphocyte reaction showed that responder cells are also capable of producing IP-10 (Yoshimatsu et al. 2017). This expression of IP-10 could be inhibited through the inhibition of TLR4 with TAK-242. Previous research has shown that blockade of IP-10 in a mouse islet transplant model resulted in significantly less lymphocytic infiltrate and longer graft survival compared to wild type, showing that inhibition of TLR4 is an indirect mechanism of attenuating IP-10 production and immune infiltration to transplanted islet tissue (Baker et al. 2003). Low secretion of MCP-1 was found to be the most relevant factor of long-lasting insulin independence in islet transplantation and is produced by islets even in the absence of inflammation (Piemonti et al. 2002). Although stimulation of TLR4 using LPS did not lead to statistically significant increases in either IP10 or MCP-1, the presence of TAK-242 significantly decreases the production of these cytokines in these one-way mixed lymphocyte reactions showing that TLR4 plays a supporting role in the production of these cytokines. Therefore, we believe that inhibition of TLR4 affecting the production of all of these cytokines will result in improved outcomes in allogenic islet transplantation through modulation of islet inflammation as well as innate immune reactions. This hypothesis is

supported by representative images showing improved islet structure following co-culture of islets with PBMCs (Figure 4.6f and 4.6g).

As the IBMIR reaction is a multifaceted response with more than a single cause of inflammation, future studies may focus on the other mechanisms of TLR4 inhibition and how that contributes to the overall inflammation response to islets and IBMIR. TLR4 is expressed on immune cells not covered in this paper such as neutrophils, B cells, and macrophages, which can contribute to loss of islet graft. Figure 4.7 shows the effects of TAK-242 inhibition of TLR4 signaling and its modulation of innate immune responses in the context of transplants. TLR4 also plays a significant role in the activation of platelets and their interactions with neutrophils to produce neutrophil extracellular traps (Clark et al. 2007). Therefore, future studies could also focus on these diverse populations to investigate how targeting of TLR4 could attenuate inflammation within these cells and particles. We also acknowledge a shortcoming of this study was failing to investigate specific T cell populations such as naïve, memory, effector, and regulatory T cells which play a significant role in the long-term success or failure of islet transplants. As the focus of this study was on innate responses following transplant, our focus was on short-term activation markers on monocytes and T cells. Future work investigating long-term transplant outcomes with TAK-242 should focus on altered T cell phenotypes following transplant. We would also like to point out the limitations of this study such as limited availability of qualified patient samples. Although we did meet the minimum of 10 patient samples computed in the power calculation, a larger patient cohort would have strengthened the results of our experiment. These results are also limited in that we did not obtain histological images of islets following exposure to blood with and without TAK-

242, which could provide additional insight into the infiltration of macrophages and neutrophils. As much of the work carried out focused on in vitro experiments, the next logical step for future studies should be to apply TAK-242 to an allogenic islet transplant model in mice to examine the effects of TAK-242 on allogenic islet transplant outcomes in the portal vein.

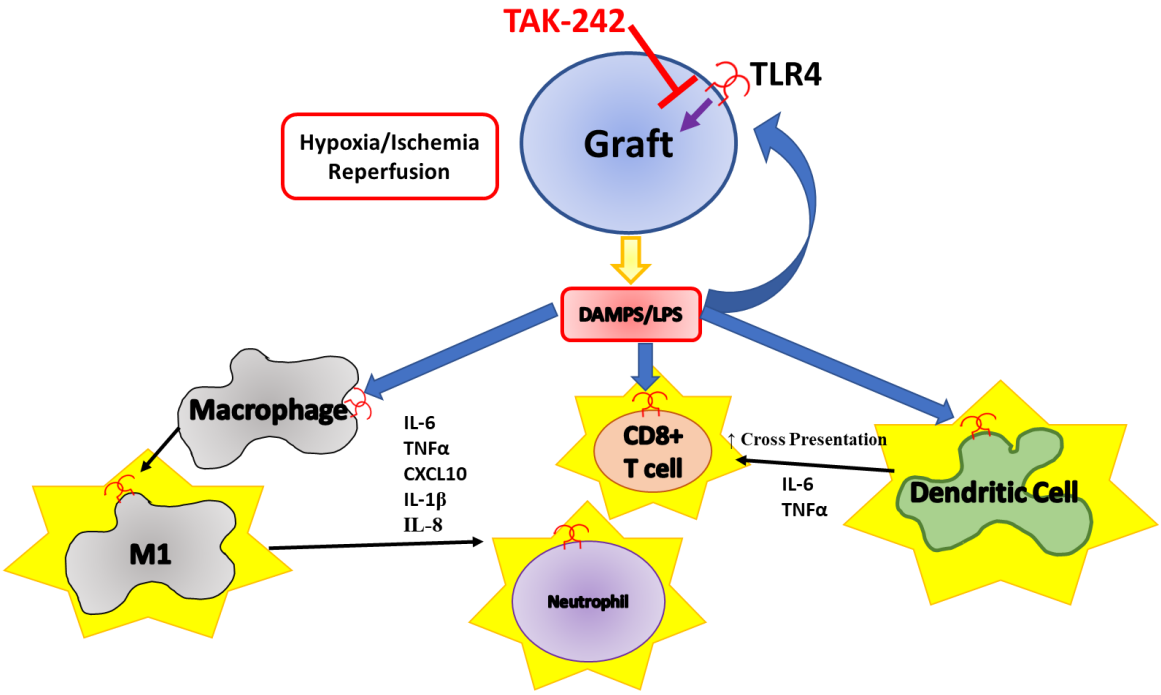

Figure 4.7. TLR4 plays a significant role in the initiation of innate immune responses that can result in inflammation and destruction of transplanted islet tissue.

5. Conclusions

Taken together, these results demonstrate TAK-242 as a viable therapeutic to help attenuate early innate graft damage due to host innate immune responses. As previous research has shown that inhibition of TLR4 can lead to prolonged graft survival but not tolerance, future research should utilize not only TAK-242, but also compounds such as

rapamycin shown to enhance Treg activity in order to promote complete graft acceptance and long term glycemic control in allogenic islet transplantation (N. Zhang et al. 2010).

CHAPTER FIVE

Exosomes in Islet Transplantation

Large portions of this chapter were previously published as: Jordan Mattke, Srividya Vasu, Carly M. Darden, Kenjiro Kumano, Michael C. Lawrence, Bashoo Naziruddin. Role of Exosomes in Islet Transplantation. 2021. Frontiers in Endocrinology. 12:681600.

Abstract

Exosomes are a classification of extracellular vesicle generated by all cells known for their ability to transport nucleic acid, lipid, and protein molecules, which allows for communication between cells and tissues. The cargo of the exosomes can have a variety of effects on a wide range of targets to mediate biological function. Pancreatic islet transplantation is a minimally invasive cell replacement therapy to prevent or reverse diabetes mellitus and is currently performed in patients with uncontrolled type 1 diabetes or chronic pancreatitis. Exosomes have become a focus in the field of islet transplantation for the study of diagnostic markers of islet cell viability and function. A growing list of miRNAs identified from exosomes collected during the process of isolating islets can be used as diagnostic biomarkers of islet stress and damage, leading to a better understanding of critical steps of the isolation procedure that can be improved to increase islet yield and quality. Exosomes have also been implicated as a possible contributor to islet graft rejection following transplantation, as they carry donor major histocompatibility complex molecules, which are then processed by recipient antigen-presenting cells and sensed by the recipient immune cells. Exosomes may find their way into the therapeutic realm of islet transplantation, as exosomes isolated from mesenchymal stem cells have shown promising

results in early studies that have seen increased viability and functionality of isolated and grafted islets in vitro as well as in vivo. With the study of exosomes still in its infancy, continued research on the role of exosomes in islet transplantation will be paramount to understanding beta cell regeneration and improving long-term graft function.

Introduction

Diabetes mellitus is characterized by chronic hyperglycemia due to partial or absolute insulin deficiency (type 1 diabetes, T1D) or pancreatic beta cell dysfunction and/or insulin inaction (type 2 diabetes, T2D) (Alam et al. 2014; Pearson 2019; Powers 2021). In T1D, autoimmune cells infiltrate pancreatic islets (insulitis) and target pancreatic beta cells for destruction, leading to loss of the beta cell mass necessary for maintenance of euglycemia. T2D is a heterogeneous disease, with impairments or abnormalities in synthesis/ secretion of insulin from islet beta cells, tissue sensitivity to insulin, or insulin action. Type 3c diabetes mellitus (T3cD), the least known or recognized form of diabetes mellitus (Hardt et al. 2008; Roeyen and De Block 2017), is caused by pathologies of the exocrine pancreas that cause islet inflammation and damage (Hart et al. 2016). Chronic pancreatitis (CP), the most common cause of T3cD, is treated based on the etiology on a case-by-case basis. For refractory CP, partial or total pancreatectomy is performed to alleviate severe pain associated with chronic inflammation and necrosis. Current therapeutic options vary based on the type of diabetes. T2D is typically managed through lifestyle changes and medications targeting insulin resistance and/or beta cell function (Courcoulas et al. 2014). For T1D and some cases of T3cD, exogenous insulin therapy is the standard of care. Even with aggressive therapy, a subset of diabetic patients present with glycemic lability, hypoglycemia unawareness, severe hypoglycemic episodes,

diabetic ketoacidosis, and other complications of diabetes, including cardiovascular and kidney disease (Gill 1992).

Pancreatic islet transplantation is an effective, minimally invasive treatment option for T1D or T3cD. In the 1980s, the first allogenic (for T1D) and autologous (after pancreatectomy) islet transplants in humans were performed (Najarian et al. 1980; Sutherland et al. 1980), with poor long-term outcomes due to peri-transplant inflammation and ineffective immunosuppression (Kendall and Robertson 1997; Robertson 2001). In 2000, the Edmonton protocol with improved induction immunotherapy, a corticosteroid-free immunosuppressive regimen, and optimal islet dose, was established (Shapiro et al. 2000). Despite initial success in achieving insulin independence 1 year after transplantation, these patients exhibited poor long-term islet graft function. Several reports including our own data suggest that 50% to 70% of transplanted islet cells are lost during the islet isolation process and in the peri-transplant period due to an innate immune response called instant blood-mediated inflammatory reaction (IBMIR). This acute response involves the complement and coagulation systems and activation of inflammatory mediators (Kanak, Takita, Itoh, et al. 2014; Naziruddin et al. 2014). Post-transplant inflammatory events lead to the recognition of the islet graft (either autologous or allogenic) by the host immune system (innate or adaptive) and to the eventual rejection of transplanted islet cells. In allogeneic islet transplantation, alloantigen presentation to the host immune system triggers immune cell infiltration of the graft tissue, resulting in graft rejection. Observations in independent CP and T1D cohorts suggest that an optimal islet yield (>5000 islet equivalents/kg), islet survival in the peri-transplant period and engraftment, and optimal immunosuppressive regimen are important for achieving post-

transplant insulin independence (Dunn et al. 2017; Bellin et al. 2019). Despite the advancements made, short- and long-term graft survival and function remain suboptimal and a challenge to be overcome (Starzl 2005; Morrissey and Monaco 2014).

Islet graft function and survival after transplantation are commonly assessed by glucose tolerance tests, C-peptide and hemoglobin A1c levels, exogenous insulin usage, and noninvasive imaging techniques (Sutherland et al. 2012b; Darden et al. 2020). However, these assessments tend to reflect the loss of islet function, offering little insight into islet stress, islet engraftment, and the ongoing loss of islet function. Establishing robust noninvasive methods to monitor islet survival and function after transplantation will help in the development of novel strategies to alleviate islet damage and improve transplantation outcomes. These challenges warrant further research on 1) delineating mechanisms of acute and chronic graft rejection, 2) monitoring islet stress and damage during and after transplantation, and 3) tailoring existing protocols to achieve improved short- and long-term outcomes.

In this regard, extracellular vesicles called ‘exosomes’ have emerged as prominent players in the assessment of islet function. Exosomes play an important role in donor-to-host cell communication, especially in presenting donor antigens to host immune cells and in horizontal transfer/dissemination of their content. Recent research also suggests a role for exosomes in carrying islet stress or damage molecular markers in circulation. In this chapter we provide an overview of the roles of exosomes in allogeneic and autologous antigen presentation to the recipient immune system, exosome cargo, and the utility of exosomes for diagnosis and therapy.

Exosomal Protein Cargo in Islet Transplantation

Exosomes contain distinct nucleic acid and protein profiles reflecting the phenotypes of their cell source and cellular state. Exosomes are enriched in endosome-associated proteins including flotillins and annexins owing to their origin from endosomes. Exosomes also contain tetraspanins (CD9, CD81, CD82, CD37, and CD63), ESCRT proteins, heat shock proteins (HSC70 and HSC90), Alix, and TSG101 and are commonly used as exosome markers for research purposes (Hessvik and Llorente 2018).

Exosomes share surface major histocompatibility complex (MHC) antigens with their lineage. For example, dendritic cell-derived exosomes express CD80, CD86, and MHC class II molecules (Segura et al. 2005; Montecalvo et al. 2008). In a human-to-mouse xenogeneic islet transplant model and allogeneic human islet transplantation, transplanted human islets released donor human leukocyte antigen (HLA, MHC class I)-specific exosomes into the recipient circulation. Acute and long-term follow up of these recipients revealed gradual reduction in circulating donor HLA-specific exosomes, and elevated recipient T cell-derived CD3-specific exosomes, reflecting graft rejection (Vallabhajosyula et al. 2017). In both a mouse model and human allogeneic islet transplant recipients, donor HLA-specific exosomes contained islet endocrine hormones including insulin, glucagon, and somatostatin (protein and mRNA), which decreased after immune rejection of islet grafts (Vallabhajosyula et al. 2017). Exosomes derived from mouse insulinoma clonal cells (MIN6) expressed insulin and glutamic acid decarboxylase (GAD65) (T1D-associated autoantigen) in addition to exosome markers (Li et al. 2020). Ex vivo, human and rat islets released exosomes containing GAD65 and IA-2, autoantigens in T1D (Cianciaruso et al. 2017). In an allogeneic human islet transplant recipient, GAD65 antigen was detectable in

donor HLA-specific exosomes at 455 days post-transplantation, with development of GAD65 autoantibodies at 1001 days post-transplantation (Korutla et al. 2019). Our recently published observations revealed that human islets exposed to proinflammatory conditions released exosomes containing cytokines including IL-6, IL-8, MCP-1, and IP-10 (Kumano et al. 2021). Proteomic profiling of MIN6-derived exosomes demonstrated enrichment of proteins involved in glycolysis, gluconeogenesis, citrate cycle, fructose and mannose metabolism, pyruvate metabolism, purine metabolism, and other metabolism-related pathways (Li et al. 2020).

Exosomal Nucleic Acid Cargo in Islet Transplantation and its Utility as a Biomarker

Apart from proteins, intra-exosomal cargo contains nucleic acids, including DNA, mRNA, and miRNA. Loading of miRNA into exosomes is not a random process, as the types and diversity of miRNAs vary by cell type and cellular state at the time of sampling (Yang et al. 2010; Goldie et al. 2014; Goldie et al. 2014; Stevanato et al. 2016; Saravanan et al. 2019). Following intraportal transplantation, islets are exposed to hypoxic and inflammatory conditions, leading to loss of islet mass (Figure 5.1). *Ex vivo* mRNA and small RNA profiling of human islet–derived exosomes reveal distinct profiles depending on the islet culture conditions. Exosomes derived from human islets exposed to proinflammatory cytokines and/or hypoxia contain significantly higher levels of specific miRNAs. The Venn diagram in Figure 5.2a depicts the numbers of islet-derived exosomal miRNAs with differential expression under a) hypoxic, b) cytokine stress, and c) hypoxic and cytokine-induced cellular stress. Time course analysis revealed that miR-29b-3p and miR-216a-5p were released in exosomes starting 6 hours after hypoxia and cytokine exposure, relating to islet stress. miR-375 and miR-148a-3p were released in exosomes

after 24 hours of hypoxic and cytokine-induced stress and damage, coinciding with onset of apoptosis (Figure 5.2). These early cellular stress and damage-induced exosomal miRNAs were also detected in plasma following islet transplantation in mice with streptozotocin-induced diabetes (Saravanan et al. 2019). During islet infusion and after transplantation in patients undergoing total pancreatectomy with islet autotransplantation, these miRNA markers were elevated in circulation, signaling islet stress and damage during transplantation and in the peri-transplant period (Figure 5.1) (Saravanan et al. 2019). In an independent study, human islets exposed to cytokines released exosomes containing 19 differentially expressed miRNAs, of which miR-155-5p, a well-known miRNA involved in inflammation, was the most upregulated miRNA (Krishnan et al. 2019). Apart from miRNAs, other small RNAs including piRNAs, lncRNAs, snoRNAs, and tRNAs were also identified in exosomes in this study (Krishnan et al. 2019). Among the small RNAs studied, exosomal miRNAs have potential as biomarkers of islet stress and damage in islet transplantation. Of particular interest are miR-375, miR-29b-3p, miR-148a-3p, miR-216a-5p, miR-200c-3p, miR-122-5p, miR-155-5p, and miR-221-3p, as these miRNAs have been identified consistently in an islet transplantation setting (Saravanan et al. 2019; Vasu et al. 2019). KEGG analysis revealed that miR-29b-3p, miR-216a-5p, miR-148a-3p and miR-375 target key molecules in PI3K/Akt, FOXO, mTOR signaling pathways and platelet activation (Saravanan et al. 2019). Further investigations are necessary to understand whether these islet stress and damage specific exosomal miRNAs alter key signaling pathways in immune cells including antigen presenting cells and lymphocytes in the context of islet transplantation.

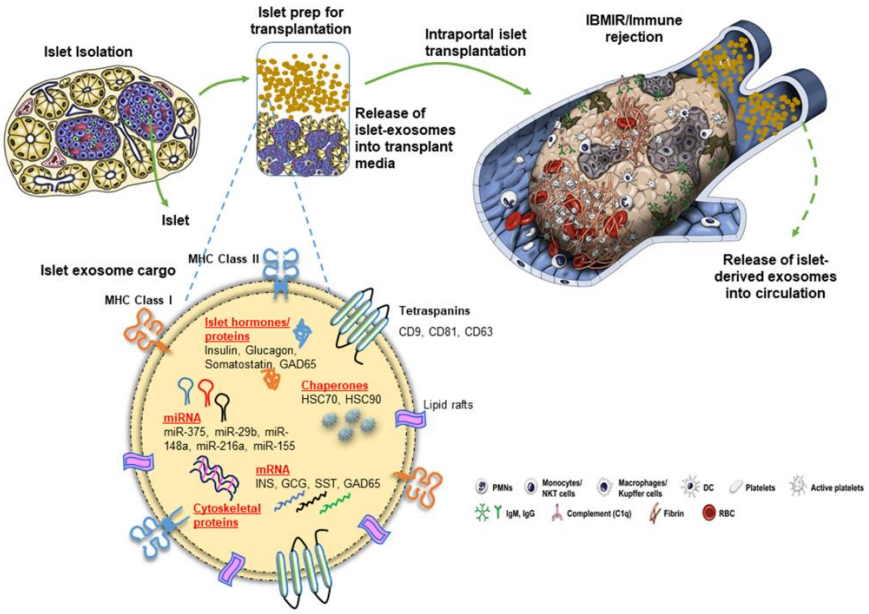

Figure 5.1. Major steps in the clinical islet transplant procedure when exosomes are released and the cargo of the exosomes is released from human pancreatic islets.

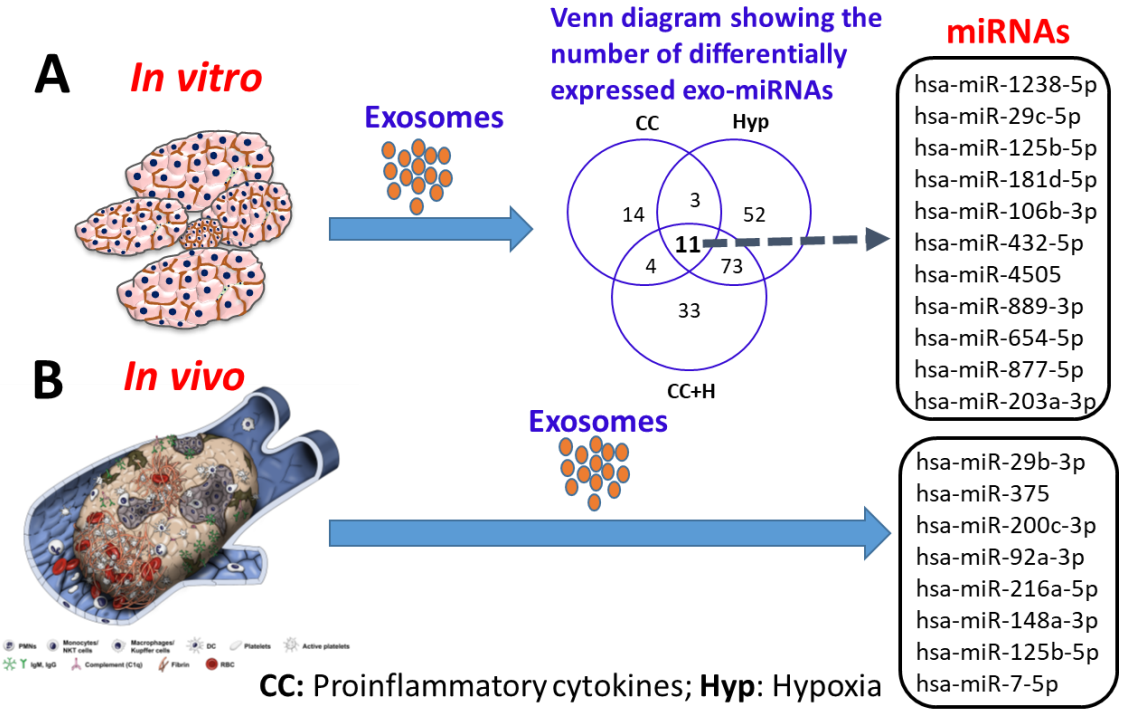

Figure 5.2. Release of exosomal microRNAs from human islets subjected to proinflammatory cytokines and hypoxic conditions [A] in vitro and [B] following intraportal transplantation of autologous islets in vivo. Source: (Saravanan et al. 2019). CC indicates proinflammatory cytokines; Hyp, hypoxia.

In the context of diabetes, several studies have shown elevated levels of circulating exosomal miRNAs including miR-25-3p in T1D (Garcia-Contreras et al. 2017) and miR-125a-3p, miR-99b-5p, miR-197-3p, miR-22-3p, miR-27b-3p, miR-200a-3p, and miR-141-3p in gestational diabetes (Nair et al. 2018). Although a number of circulating miRNAs have been reported as elevated or reduced in circulation in T1D, T2D, obesity, and gestational diabetes, these studies were performed using plasma or serum fractions (Vasu et al. 2019) and hence do not necessarily represent the exosomal miRNA content. Other types of extracellular vesicles including microvesicles may also contribute to the miRNAs in circulation.

Although it is still unclear, exosomal cargo containing metabolism-, inflammation-, and cellular stress-related molecular species (Table 1) may exert distinct biological actions in target cells. In the context of islet transplantation, apart from their utility as biomarkers of islet stress and damage, transplanted islet-derived exosomes may serve as auto- or alloantigens triggering an immune response. Future anterograde tracing studies focusing on the actions of islet-derived exosomes on target cells in physiological and pathophysiological conditions are necessary.

Table 5.1. Exosomal cargo in human islet transplantation

Exosome content	Exosome source	Comments	References
hsa-miR-375	Culture supernatant	"Damage-induced exo-miRNA" increased in response to hypoxia, streptozotocin, and cytokine stress after 24 h	(Sheng et al. 2011; Saravanan et al. 2019)
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/ human Serum	Elevated throughout islet isolation	

hsa-miR-216a-5p	Culture supernatant	"Stress-induced exo-miRNA" increased after 6 h cytokine and hypoxic stress	
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/ human serum	Elevated throughout islet isolation	
hsa-miR-148a-3p	Culture supernatant	"Damage-induced exo-miRNA" increased after 24 h cytokine and hypoxic stress	
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/ human serum	Elevated throughout islet isolation	
hsa-miR-29b-3p	Culture supernatant	"Stress-induced exo-miRNA" increased after 6 h ctyokine and hypoxic stress	
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/ human serum	Elevated throughout islet isolation	
hsa-miR-200c-3p	TPIAT supernatant/ human serum	Elevated throughout islet isolation	
hsa-miR-3613-5p	Xenotransplant mouse serum	Increased in normoglycemic xenotransplant mice	(Vallabhajosyula et al. 2017)
Angiopoietin-1	Xenotransplant mouse serum	Increase associated with normoglycemia following xenotransplant	
HSC70	Xenotransplant mouse serum	Increase associated with normoglycemia following xenotransplant	
Complement C3	Xenotransplant mouse serum	Increase associated with rejection following xenotransplant	

Hem+D22+A8:A8:C26	Xenotransplant mouse serum	Increase associated with rejection following xenotransplant

Exosome Recognition by Immune Cells

Transplant rejection by the host immune system is a complex process, initiated by a series of events starting from recognition of the allograft or autoantigens, stimulation of the host's immune system, and T cell-dependent rejection of the graft. Recent research has emphasized the roles of donor tissue-derived exosomes in recognition of the transplanted tissue by the host immune system. Purified allogeneic donor exosomes stimulate alloimmune responses by T cells *in vitro* and *in vivo*, emphasizing the significance of exosome recognition, uptake, and immune responses in transplantation (Marino et al. 2016). Exosomes weakly stimulate or fail to elicit immune responses in T cell lines and naïve T cells, respectively, and processing of exosomes by antigen-presenting cells (APCs) is required for T cell activation (Théry et al. 2002; Vincent-Schneider et al. 2002; Skokos et al. 2003).

After transplantation, recipient T cells can be stimulated through three pathways. The first is a *direct pathway,* where recipient T cells are stimulated by donor passenger APCs presenting an alloantigen on allogeneic MHC proteins (Boardman et al. 2016). If the recipient T cells are naïve, donor passenger APCs must migrate to secondary lymphoid organs to induce T cell responses. Structural differences in the donor MHC:alloantigen complex (multiple binary complexes hypothesis) (Matzinger and Bevan 1977) or each allo-MHC molecule on donor APCs (high-determinant density hypothesis) (Bevan 1984) may be recognized by T cells. The direct pathway is the driving mechanism behind acute graft rejection (Benichou et al. 2020). The second pathway is the *indirect pathway,* where T cells

recognize donor MHC/alloantigen-derived peptides processed and presented on self-MHC molecules by recipient APCs. T cells primed to recognize alloantigens are able to respond to peptides derived from allogeneic α and β chains of class II MHC molecules (Benichou et al. 1992). Skin grafts from MHC II knockout mice were detected and rejected by MHC I knockout mice even though the recipient MHC I knockout mice lacked $CD8^+$ T cells (Auchincloss et al. 1993). The indirect pathway is important for alloantibody production and chronic rejection leading to graft vasculopathy and fibrosis (Benichou et al. 2020). The third pathway is the *semidirect pathway:* Immediately after transplantation, donor passenger leukocytes do not travel to recipient regional lymph nodes for antigen presentation. Instead, recipient APCs take up donor exosomes containing donor MHC and antigens and process and present them on self-MHC molecules (cross-dressing), triggering T cell activation (Segura et al. 2005; Montecalvo et al. 2008; Morelli et al. 2017; Benichou et al. 2020). Recipient dendritic cells were able to acquire significant amounts of MHC molecules from both donor dendritic cells and endothelial cells (Jiang et al. 2004). Graft rejection was evident in recipients who lacked the indirect allorecognition pathway (Pimenta-Araujo et al. 2001). Following heart and islet transplantation in allogeneic mouse transplant models, recipient cells were cross-dressed with donor MHC antigens in draining and non-draining lymph nodes and spleen, with only a few passenger leukocytes at these sites (Marino et al. 2016). In a human-to-mouse xenogeneic islet transplant model, donor passenger leukocyte-derived exosomes were negligible in the donor HLA-specific exosome fraction, suggesting transplanted human islets as their source (Vallabhajosyula et al. 2017). Exosomes alone derived from rat mast cells only weakly stimulated specific T cells, and T cell activation increased by fixation of exosomes to latex beads in vitro

(Vincent-Schneider et al. 2002). MIN6-derived exosomes induced splenocytes isolated from NOD mice to produce proinflammatory cytokines including IL-6, IFN-γ, and TNF-α via the TLR-MyD88 signaling pathway. MIN6-derived exosomes increased CD86 expression on class II MHC-positive splenocytes and induced splenic T cell proliferation (Sheng et al. 2011). After pro-inflammatory cytokine exposure, human islet-derived exosomes induced mRNA expression of NOS2 and COX2 in THP-1 cells, a macrophage cell line (Kumano et al. 2021). The semidirect pathway is particularly important when recognizing donor peptides and MHC molecules by recipient immune cells. The involvement of the semidirect pathway in alloantigen presentation and stimulation of T cell responses is reviewed in detail elsewhere (Benichou et al. 2020).

Thus, exosomes containing donor antigens, MHC molecules, and miRNAs can elicit immune responses directly (mostly negligible due to suboptimal levels of exosomal cargo to activate T cells) or indirectly through recipient APCs. In the context of islet autotransplantation, exosomes carrying islet stress and damage markers, sequestered self-antigens, and MHC molecules may be important in inducing autoimmune responses.

Effects of Islet Exosomes on Innate Immune Inflammatory Responses

Previous Studies of Pro-inflammatory Exosomes Released from Islets

As exosomes have demonstrated the ability to stimulate alloimmune responses following transplantation, we have also sought to investigate whether exosomes and their cargo could also influence innate immune responses following transplantation. In a recent study published by Saravanan et al. it is demonstrated that islets exposed to cytokine stress and hypoxia released exosomes carrying inflammatory cargo. These same exosomes could be detected in islet infusion bags and correlated to increased insulin use and higher

hemoglobin A1c 1 year after TPIAT. These exosomes also exhibited pro-inflammatory properties when administered to monocytes through the toll-like receptor 3/7 : DAMP axis (Saravanan et al. 2024 Feb 15).

As previous work has demonstrated blockade of TLR4 using soluble TAK-242 and releasable TAK-242 from islet surfaces promotes a less inflammatory state within islets, my research aim for this study was to investigate whether stimulation of TLR4 could result in the release of pro-inflammatory exosomes from islets (Chang, Murphy, et al. 2018; Chang, Akinbobuyi, et al. 2018).

Stimulation of TLR4 with LPS Does Not Alter Islet Viability or Function

To begin this study, human islets were obtained from the Integrated Islet Distribution Program. 1,000 IEQ of islets were then placed in a 12-well, flat bottom tissue culture plate (Genesee Scientific, Morrisville, NC, USA) suspended in Roswell Park Memorial Institute media (Gibco, Waltham, MA, USA) containing penicillin-streptomycin (Gibco, Waltham, MA, USA) and 10% exosome depleted fetal bovine serum (Gibco, Waltham, MA, USA). 5 μg/mL of ultrapure LPS (Invivogen, San Diego, CA, USA) was added to designated cultures as well as 10 μM TAK-242 (Tocris Bioscience, Bristol, United Kingdom).

After 24 hours, the supernatant was removed from each culture. Islets were then collected, and viability was assessed by staining with fluorescein diacetate and propidium iodide followed by image analysis using ImageJ (n = 20). Results of this experiment revealed no significant differences between control, LPS exposure, and LPS exposure with TAK-242 (Figure 5.3).

Islet function was assessed using a glucose stimulated insulin secretion assay. Approximately 300 IEQ of islets from each culture (n = 3) were suspended in Krebs Ringer Bicarbonate buffer containing 1.67 mM glucose and then transitioned to Krebs Ringer Bicarbonate buffer containing 16.7 mM glucose. The supernatant was collected from each culture, and the insulin secretion was quantified using human insulin ELISA (ALPCO, Salem, NH, USA). When comparing insulin released in the high glucose conditions to insulin released in the low glucose conditions, no significant differences were observed between the insulin secretion between the 3 treatment groups (Figure 5.3).

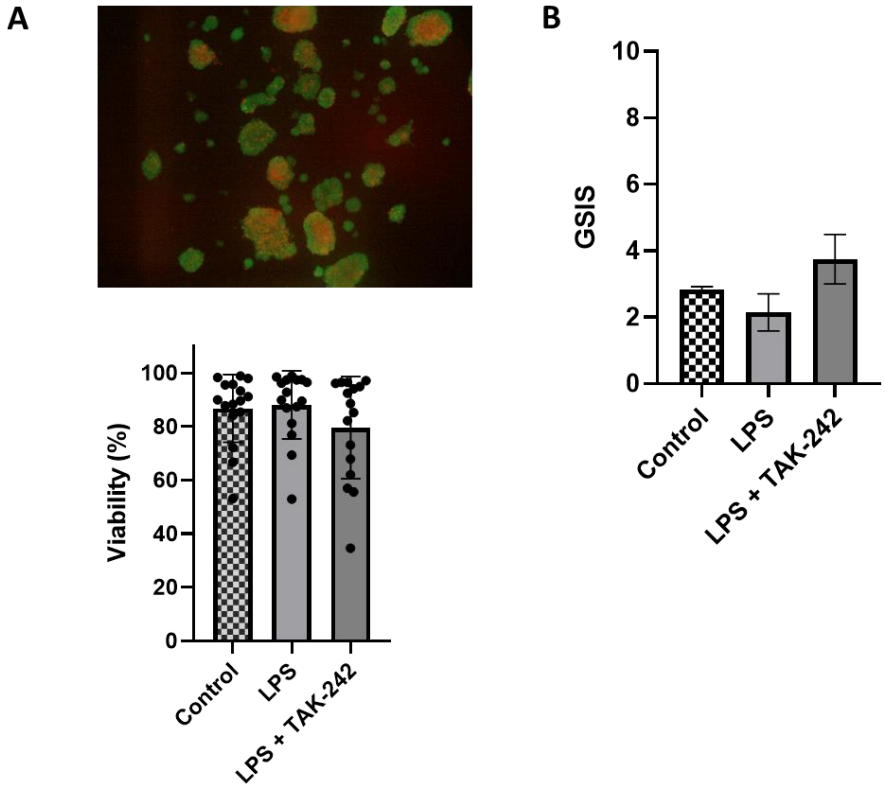

Figure 5.3. LPS and TAK-242 do not significantly alter [A] viability or [B] glucose responsiveness of islets.

Activation of TLR4 by LPS Promotes Expression of Pro-inflammatory Cytokines Within Islets

As no significant changes were noted in the viability or functionality of islets exposed to LPS or TAK-242, we next used real-time polymerase chain reaction to observe changes in mRNA within islets from each culture condition. mRNA from each islet culture (n = 4) was isolated using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) followed by chloroform extraction, isopropanol precipitation, and washes with ethanol. mRNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). cDNA was amplified and measured using RT^2 SYBR Green ROX™ qPCR Mastermix (Qiagen, Hilden, Germany) and probed with primers for HIF1α, IL-1β, IL-6, and NOS2 (Qiagen, Hilden, Germany). Data was collected on a QuantStudio™ 7 Flex instrument. Data was analyzed using the ΔΔCT method followed by fold change calculations. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison test.

The results of this experiment did not show significant changes in the mRNA expression of HIF1α or NOS2 indicating that the addition of LPS did not alter oxidative stress within islets. However, IL-1β (LPS = 4.494 ± 1.256 FC, LPS + TAK-242 = 2.204 ± 1.280 FC, $P = 0.0310$) and IL-6 (LPS = 5.951 ± 0.624 FC, LPS + TAK-242 = 3.783 ± 0.723, $P = 0.0087$), which are both mediated by NF-κB following TLR4 activation, were significantly increased by culture with LPS that decreased when TAK-242 was added (Figure 5.4).

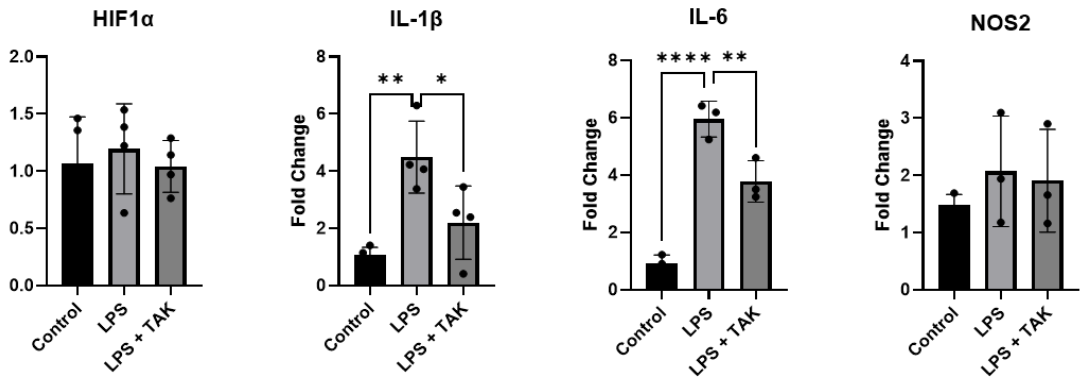

Figure 5.4. mRNA expression within islets following 24 hours of culture with LPS or LPS + TAK-242. (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$)

Exosomes Released by Islets Cultured with LPS can Activate THP-1 Derived Macrophages

Because islets from different treatment conditions expressed different amounts of inflammatory mRNA, we next investigated whether the exosomes released by these islets had the ability to affect innate immune responses. In order to do this, exosomes were isolated from the supernatant of each islet culture using Total Exosome Isolation (from cell culture media) (Invitrogen, San Diego, CA, USA) according to the manufacturers protocol. Exosomes were then resuspended in phosphate buffered saline and exosome concentration was determined by Pierce™ BCA Protein Assay (Thermo Scientific, Waltham, MA, USA). Exosomes were confirmed by size using a NanoSight NS300 instrument (Malvern Panalytical, Malvern, United Kingdom). Particle size distributions are presented in Figure 5.5.

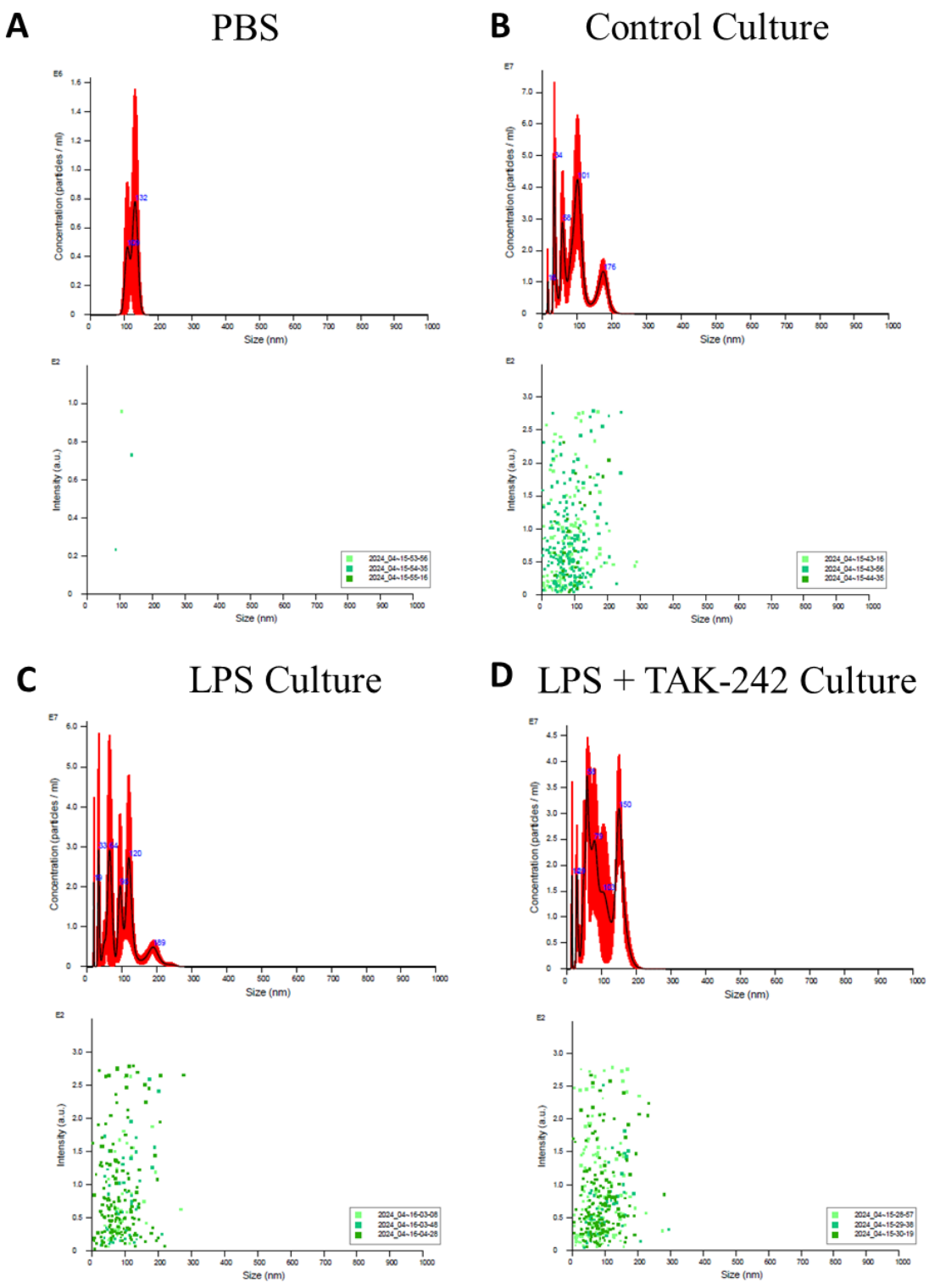

Figure 5.5. Nanotracking analysis of exosomes obtained from each islet culture condition. Size distribution of particles were analyzed for [A] sterile PBS, [B] control culture islet exosomes, [C] LPS islet culture exosomes, and [D] LPS + TAK-242 islet culture exosomes.

Macrophages used for this experiment were generated by culturing THP-1 cells (American Type Culture Collection, Manassas, VA, USA) to eBioscience™ Cell Stimulation Cocktail (Invitrogen, San Diego, CA, USA) with 10% fetal bovine serum for 48 hours followed by 24 hours of culture with no stimulation. Cells were then cultured in Roswell Park Institute Media (Gibco, Waltham, MA, USA) with 10% exosome depleted fetal bovine serum (Gibco, Waltham, MA, USA). Cultures were then supplemented with 2 μg/mL of exosomes from each islet culture condition (n = 3). 10 μM TAK-242 was then added to appropriate cultures.

After 24 hours of culture, cells were stained with Brilliant Violet 605™ anti-human CD14, APC anti-human CD86, Brilliant Violet 785TM anti-human CD83, BUV737 anti-human CD80 (Biolegend, San Diego, CA, USA) (BD, Franklin Lakes, NJ, USA). Cells were then analyzed on a BD LSRFortessa™ Cell Analyzer. Data was processed using FlowJo (FlowJo LLC, Ashland, OR, USA). Statistical significance was determined using two-way ANOVA followed by Tukey's multiple comparisons test.

After 24 hours of culture, exosomes isolated from control islet cultures did not show any significant changes in CD80 expression when compared to the control group with no exosomes (Control MFI = 1,011 ± 309, Exosomes MFI = 2,197 ± 2013). However, the CD83 expression was significantly increased when comparing these two groups (Control MFI = 2,189 ± 360, Exosomes = 3,473 ± 505, $P = 0.0045$) (Figure 5.6). When comparing the effects of control exosome samples to the effects of exosomes obtained from LPS or LPS + TAK-242 cultures, a large increase in CD80 expression was observed that was partially inhibited by adding TAK-242 to the LPS islet cultures (LPS Islet Exo MFI = 20,266 ± 336, LPS + TAK-242 Islet Exo MFI = 16,936 ± 2,238, $P = 0.0220$) (Figure 5.6).

This same trend was demonstrated when analyzing CD83 expression (LPS Islet Exo MFI = 6,413 ± 402, LPS + TAK-242 Islet Exo MFI = 4,969 ± 424, $P = 0.0013$) (Figure 5.6). Another trend of interest from this study was that although exosomes harvested from LPS islet cultures and LPS + TAK-242 islet cultures showed remarkable pro-inflammatory effects, the addition of TAK-242 to this mixture almost completely alleviated this effect.

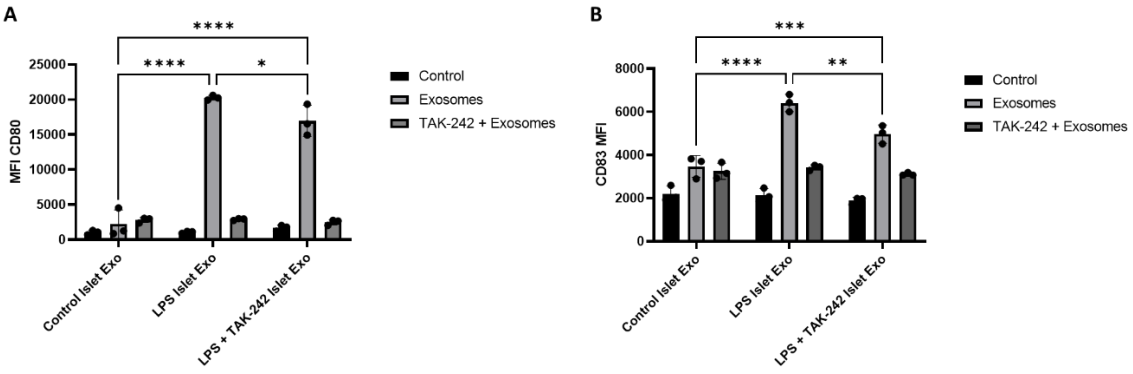

Figure 5.6. THP-1 derived macrophage activation following exposure to exosomes from different islet culture conditions. [A] CD80 expression is significantly increased when exosomes from islets exposed to LPS compared to control culture. This effect was significantly attenuated by the addition of TAK-242 to the LPS islet culture. [B] CD83 expression showed the same trends as exosomes harvested from LPS islet cultures cause significant increases in CD83 expression when compared to control islet exosomes. This effect was also significantly affected by the addition of TAK-242 to the LPS islet culture. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$)

Discussion

The results of this study demonstrate several interesting observations. First, we show that stimulation of TLR4 on islets does not affect the short-term viability or functionality of islets (Figure 5.3). Previous studies have noted that administration of LPS to islets can lead to impaired β-cell function as well as production and release of pro-inflammatory cytokines (Matsudaa et al. 2005; Amyot et al. 2012). However, to our knowledge, this is the first study that demonstrates inhibition of TLR4 signaling in islets

exposed to LPS with TAK-242 does not alter islet viability or function while reducing inflammatory mRNAs within islets (Figure 5.4).

This study is also novel in that we investigate the inflammatory properties of exosomes isolated from these culture conditions. Similar to results obtained to Saravanan et al., which used exosomes isolated from islets exposed to cytokine and hypoxic stresses, we also note that stimulation of islet TLR4 leads to production and release of pro-inflammatory exosomes as exosomes isolated from LPS or LPS + TAK-242 islet cultures demonstrated significant increases in CD80 and CD83 expression on THP-1 derived macrophages (Saravanan et al. 2024 Feb 15). As the liver has large numbers of macrophage-like cells referred to as Kupffer cells that phagocytose cells and cell debris as well as secrete inflammatory mediators and free radicals, we believe this same reaction is possible in the portal vein environment following islet transplantation (Delaune et al. 2017).

Taken together, these results indicate that not only can exosomes contribute to loss of islet grafts through allogenic immune responses, but they can also initiate innate immune responses during the peri-transplant period further contributing to their own demise resulting in islet graft failure following transplant.

As this was a preliminary study, there is still much work to do in this area. I believe the results of this study lay the foundation for the immunomodulatory abilities of exosomes in islet transplantation as well as other organ transplantation. Profiling the cargo and structure of these exosomes as well as their uptake could lead to the development of future interventions which could help alleviate innate immune responses following islet

transplantation. This would result in less islet loss and improved endocrine function leading to improved quality of life following this procedure.

Therapeutic Potential of Exosomes in Islet Transplantation

Of particular interest is induction of allogeneic tolerance or tolerance to sequestered self-antigens in autotransplantation. The immunogenicity of allogeneic or self-antigens depends on the dose, presentation site, other signals, and nature/state of APC activation. The ability of exosomes to induce immune tolerance is still unexplored, but exosomes as therapeutic delivery vehicles have been studied recently. Human bone marrow mesenchymal stem cells transfected with si-Fas and anti-miRNA-375 were co-cultured with peripheral blood mononuclear cells. Exosomes were collected from co-culture supernatant and transplanted into NOD-SCID gamma mice, resulting in decreased immune response and rejection by enhancing regulatory T cell function (Wen et al. 2016). In living donor liver transplant recipients, infusion of donor regulatory dendritic cells resulted in cross-dressing of recipient immune cells with surface expression of PD-L1, which was most likely transferred through donor exosomes containing PD-L1, CD73, and CD39. There was an increase in recipient regulatory $CD4^+$ T cells as well as decreased activated $CD8^+$ T cells after the infusion of donor regulatory dendritic cells (Macedo et al. 2020).

More importantly, exosomes exhibit low toxicity, do not present a risk for tumor formation, and easily diffuse across biological barriers owing to their small size, which enables them to be used as injectable therapeutics (Zazzeroni et al. 2017). *In vitro*, mesenchymal stem cell-derived exosomes alleviated the detrimental effects of hypoxia-induced DNA damage, resulting in increased viability of porcine islet cell clusters in hypoxic conditions (Nie et al. 2018). Mouse islets cultured with mesenchymal stem cell-

derived exosomes showed reduced expression of the pro-apoptotic genes *BAD* and *BAX* and increased expression of prosurvival genes *PI3K* and *BCL-2,* as well as increased production of vascular endothelial growth factor in mouse islets, resulting in increased production of insulin mRNA (Keshtkar et al. 2020). Mesenchymal stem cell-derived exosomes protected β-cells from apoptosis via the actions of miR-21 on ER stress and inhibition of phosphorylation of p38 (Chen et al. 2020). In streptozotocin diabetic mice, infusion of exosomes derived from bone marrow–derived mesenchymal stem cells induced regeneration of pancreatic islets (Sabry et al. 2020). Administration of adipose tissue-derived exosomes into streptozotocin diabetic mice increased the regulatory T cell ratio in splenic mononuclear cells and improved insulitis (Nojehdehi et al. 2018). Exosomes isolated from lean adipose tissue explants also increased viability and functionality of isolated pancreatic β cells (Gesmundo et al. 2021). These observations are proof of concept that stem cell-derived exosomes may improve islet engraftment and functional outcomes of islet transplantation. Transplantation of MIN6-derived exosomes improved median survival time, glucose tolerance, insulin content, and islet architecture and reduced macrophage infiltration in streptozotocin diabetic mice (Sun et al. 2021). However, in diabetes-resistant NOR mice, immunization using MIN6-derived exosomes accelerated insulitis (Sheng et al. 2011), highlighting that exosomes may either induce immune tolerance or induce a response to allogeneic or autologous antigens.

Conclusions and Future Research Directions

Exosomes have emerged as important players in transplant rejection, as they carry donor antigens, which can weakly activate alloimmune responses through the direct or indirect allorecognition pathways. However, their major contribution to allorecognition

comes in the form of cross-dressing recipient immune cells, leading to rejection of allogeneic transplanted tissue. Exosomal protein and nucleic acid cargoes also have potential for use as biomarkers for monitoring graft function and survival. Recent research highlights the utility of mesenchymal stem cell–derived exosome therapy during transplantation to improve islet survival and function, induce immune regulatory responses, and improve transplantation outcomes. Although exosome biology has been studied extensively, exosome research has its limitations. Exosome yield and quality vary between different isolation methods including ultracentrifugation, ultrafiltration and precipitation. Although exosomal miRNA, mRNA isolation and qPCR methods are robust, lack of exosomal housekeeping controls for normalization may influence experimental observations across laboratories. Additionally, there is no commercially available kit to measure very small amounts of exosomal miRNA/mRNA content accurately. Thus, there is a need for optimization and standardization of exosome research methods. Future studies should also be carried out using larger sample cohorts to validate and append the growing list of biomarkers for use in diagnostics. Exosomes in inducing transplant tolerance is an interesting area of research and may open up exciting and novel avenues in post-transplant immunosuppressive regimen. Given the contribution of islet-derived exosomes to graft rejection (Vallabhajosyula et al. 2017), future studies should focus on the fine line between induction of rejection and tolerance. With several studies demonstrating exosomes as safe therapeutic agents, continued studies into engineering and administration of exosomes in order to attenuate immune responses and prolonging graft survival will be of vital importance to the field. As exosome research is still in its infancy, its utility as biomarkers of islet stress and damage, inflammation or immune response and/or as therapeutics in the

context of clinical islet transplantation should be validated and well-established independently across institutions.

CHAPTER SIX

Conclusions

In this dissertation, I have explored the roles of pattern recognition receptor, TLR4, and how inhibiting TLR4 signaling using small molecule TAK-242 can result in improved outcomes in the case of innate inflammation of pancreatitis or islet transplantation.

First, I addressed how inhibiting TLR4 signaling using TAK-242 resulted in a reduced inflammatory state within the pancreas following administration of cerulein injections by looking at mRNA expression within the pancreas. This also resulted in improved histology scores and reduced macrophage infiltration shown by immunofluorescent staining. Flow cytometric analysis of pancreatic single cells revealed a reduced population of $F4/80^+$ $CD86^+$ macrophages within mice treated with 3 mg/kg TAK-242 compared to cerulein-only controls. Flow cytometric analysis also showed reduced presence of $CD14^+$ monocytes in the pancreases of mice treated with cerulein and TAK-242 compared to cerulein-alone treatment. These results were echoed following repeated insult of cerulein injections with or without TAK-242 showing the added benefit of significantly reduced serum HMGB1 levels when TAK-242 was given at 10 mg/kg. Taken together, these results demonstrated TAK-242 as a viable therapeutic option to lessen complications of acute and recurrent acute pancreatitis.

Islet transplantation is a promising option for patients suffering from type 1 or type 3c diabetes. However, because of significant islet graft loss during the peritransplant period, patients often require substantial islet mass in order to achieve full endocrine function following islet transplantation. In order to address this issue, I focused on how

TAK-242 could alter inflammatory processes contributing to islet graft loss during the early transplant period. First, I validated miR-375 and miR-200c increases following intraportal transplantation as reliable biomarkers for short-term endocrine function following islet transplantation. I then show these markers are reduced in serum by soluble and surface released TAK-242 in an *in vitro* model of IBMIR without affecting clotting of islets. When focusing on early immune responses, I demonstrated that TAK-242 resulted in a reduction of dendritic cell activation markers CD83 and CD80 as well as attenuated cytokine expression during a one-way mixed lymphocyte reaction. This reaction also demonstrated a dose-dependent response in the activation and proliferation of CD8+ T-cells. I also found that TLR4 inhibition could affect polyclonal stimulation of CD8+ T-cells, which were found to express TLR4. Finally, repeating this one-way mixed lymphocyte reaction with islets showed reduced CD8+ T-cell activation and reduced expression of miR-375 and miR-200c in the supernatant of the mixed cultures. TAK-242 also significantly inhibited the secretion of pro-inflammatory cytokines in the culture supernatant and better histological structure of islets extracted from the cultures. As previous work demonstrates beneficial outcomes by targeting TLR4 of islets, these results show that TAK-242 may also be utilized to target innate immune responses during the peritransplant period (Chang, Murphy, et al. 2018; Chang, Akinbobuyi, et al. 2018).

As exosomes have generated much enthusiasm in recent years, I have also addressed the various roles of exosomes within the scope of islet transplantation. Exosomes contain useful biomarkers such as various RNA proteins. Exosomes are also widely recognized for their ability to stimulate alloimmune responses. Exosomes collected from various cell types have demonstrated various effects on β-cells and islets. However, a

recent study and our novel data also demonstrate that islets in a damaged or inflammatory state are able to secrete exosomes with a pro-inflammatory capacity, which could enhance innate immune responses leading to islet graft failure following transplantation. Islets cultured with LPS showed no significant changes in viability or function. However, exosomes secreted by islets treated with LPS produced pro-inflammatory phenotypes within THP-1 macrophages. This effect could be counteracted by the addition of TAK-242 to islets treated with LPS. Additionally, this study also showed that TAK-242 significantly reduced the ability of THP-1 derived macrophages to respond to exosomes indicating that TLR4 may play a supportive role in the activation of these immune cells in response to exosomes.

Taken together, these results warrant further study into the use of TLR4 inhibition via TAK-242 in pancreatitis and islet transplantation. In these studies, TAK-242 demonstrates an impressive immunomodulatory effect in pancreatitis and islet transplantation by targeting innate immune activation and infiltration. TAK-242 is also able to indirectly target immune cell activation by regulating the inflammatory state of islets which produces pro-inflammatory exosomes. As TAK-242 has been shown to be safe when given by intravenous administration for sepsis, this could also be implemented for use in islet transplantation (Rice et al. 2010). We also further validate the use of surface modification of islets with TAK-242 for localized release of TAK-242 to inhibit inflammation contributing to graft failure in islet transplantation.

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