ABSTRACT

Inhibition of TLR4 Minimizes Islet Damage due to Sterile Inflammation and Improves Islet Transplant Outcomes

Charles A. Chang, Ph.D.

Co-Mentor: Robert R Kane, Ph.D.
Co-Mentor: Bashoo Naziruddin, Ph.D.

Islet transplantation has emerged as an important treatment option for brittle type 1 diabetes and as an adjunct procedure after total pancreatectomy to prevent brittle diabetes. The efficacy and long-term function of islet transplantation have significantly improved over the last two decades. However, transplant outcomes are still largely compromised due to inflammation mediated prior to and after transplantation which results in the loss of as much as 50% of the islet graft. Toll-like receptor 4 (TLR4) has been identified as a major pro-inflammatory mediator of sterile inflammation by sensing damage-associated molecular patterns (DAMPs) and compromising graft function, making it a putative therapeutic target. Here, we study the effects of TLR4 blockade during the peri-transplant period on islet transplant outcomes using TAK-242, a small molecule inhibitor of TLR4, and a combination of basic biological assays as well as in vivo transplant models in mice. The results of early TLR4 blockade during islet isolation demonstrate a markedly reduced inflammatory profile in islets post-isolation which translated to reduced islet damage post-transplant and overall improved transplant outcomes with a cure rate of 75% for treated

islets and 29% for untreated islets. Next, we developed a TLR4-antagonist prodrug and a chemical conjugation method to link the prodrug to the surface of islets which is slowly released, creating drug-eluting islets. Transplantation of a marginal dose of 100 modified islets into the kidney subcapsular space resulted in a cure rate of 100% compared to 0% for unmodified islets. In conclusion, we demonstrate that TLR4 is a major mediator of islet graft loss during the peri-transplant period. Therapies directed to inhibit this receptor, before and after transplant, are a promising avenue for improving islet transplant outcomes. The addition of TAK-242 to media during the isolation process is a rapidly translatable approach to clinical use, while the surface modification technique opens a broad range of possible transplant applications.

Inhibition of TLR4 Minimizes Islet Damage due to Sterile Inflammation and Improves Islet Transplant Outcomes

by

Charles A. Chang, B.S.

A Dissertation

Approved by the Institute of Biomedical Studies

_____

Robert R. Kane, Ph.D, Director

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

Approved by the Dissertation Committee

_____

Robert R. Kane, Ph.D., Co-Chairperson

_____

Bashoo Naziruddin, Ph.D., Co-Chairperson

_____

Michael C. Lawrence, Ph.D.

_____

Alex Tong, Ph.D.

_____

Patrick J. Farmer, Ph.D.

Accepted by the Graduate School

August 2018

_____

J. Larry Lyon, Ph.D., Dean

Page bearing signatures is kept on file in the Graduate School.

TABLE OF CONTENTS

LIST OF FIGURES v

ACKNOWLEDGEMENTS viii

CHAPTER ONE 1
Introduction 1

CHAPTER TWO 6
Literature Review 6

CHAPTER THREE
Preliminary Works.....22

CHAPTER FOUR 33
Early TLR blockade attenuates sterile inflammation-mediate stress in islets during isolation and promote successful transplant outcomes 33
Introduction 34
Materials and Methods 36
Results 41
Discussion 53

CHAPTER FIVE 57
Ex-Vivo Generation of Drug-Eluting Islets Improves Transplant Outcomes by Inhibiting TLR4-Mediated NFkB Upregulation 57
Introduction 58
Materials and Methods 60
Results 67
Discussion 86

CHAPTER SIX 91
Conclusions 91

REFERENCES 95

LIST OF FIGURES

Figure 2.1. TLR4 intracellular signaling pathway 17

Figure 2.2. TLR4 plays a role in multiple pathways to compromise graft survival 18

Figure 3.1. Assessment of IBET762 toxicity in MIN6 cells 23

Figure 3.2. IBET762 inhibition of inflammation in hPBMCs 24

Figure 3.3. Surface labelling of islet with CD47 peptide 26

Figure 3.4. Neutrophil infiltration of islet grafts 26

Figure 3.5. CD47 peptide conjugation to DBCO-beads 27

Figure 3.6. Structure of TAK-242 28

Figure 3.7. Cytokine expression in samples with or without TAK-242 treatment 29

Figure 3.8. Mean blood glucose in mice after islet transplant with or without systemic TAK-242 treatment 29

Figure 3.9. Islets after reaction with high dose NHS 31

Figure 3.10. Time-dependent fluorescent labelling of cells 32

Figure 4.1. Islet transplant outcomes 43

Figure 4.2. Blood glucose graphs of individual islet recipients 44

Figure 4.3. Serum markers of islet damage and inflammation post-transplant 45

Figure 4.4. Islet kidney graft histological analysis 46

Figure 4.5. Inflammatory gene expression analysis after islet isolation 48

Figure 4.6. Gene expression levels of inflammasome proteins 49

Figure 4.7. Proinflammatory gene expression in islets treated with TAK-242 before or after isolation 50

Figure 4.8. Proinflammatory chemokine expression in islets isolated with Liberase TL 50

Figure 4.9. Semiquantitative western blot analysis of MAPKs and P65 52

Figure 4.10. Assessment of islet viability and function 53

Figure 5.1. TAK-242 is non-toxic to beta cells and dose-dependently inhibits TLR4-mediated NFkB upregulation 69

Figure 5.2. TAK-242 inhibits TLR4-mediated inflammation in murine pancreatic islets 70

Figure 5.3. Protection of mouse islets against rHMGB1-mediated TLR4 inflammation with TAK-242 71

Figure 5.4. Cleavable prodrug chemistry 72

Figure 5.5. Verification of linker chemistry 74

Figure 5.6. Chemically modified kidneys 75

Figure 5.7. Assessment of linker kinetics 77

Figure 5.8. Modified islet viability and functionality assays 79

Figure 5.9. Assessment of drug-eluting islet protection in vitro 81

Figure 5.10. Islet dose titration with TAK-242 pretreated islets 83

Figure 5.11. Islet transplant outcomes and function in a syngeneic model 85

Figure 5.12. Blood glucose of mice after islet graft removal by nephrectomy 86

ACKNOWLEDGMENTS

I thank the following for training me during my studies, for imparting their graduate wisdom, and for their friendship: Gumpei Yoshimatsu, M.D.-Ph.D., Mazhar Kanak, Ph.D., Babatope Akinbobuyi, Ph.D., Joshua M. Horton, Ph.D., Matthew R. Byrd, M.S., and Zacharie J. Seifert, M.S.

I thank the following for directly assisting me in completing projects and writing manuscripts: Babatope Akinbobuyi, Ph.D., Jeremy M. Quintana, Gumpei Yoshimatsu, M.D.-Ph.D., Waqas Z. Haque, Prathab S. Balaji, Ph.D, and Kayla Murphy.

I thank the following for training me in clinical pancreas procurement, islet isolation, and transplantation: Gumpei Yoshimatsu, M.D.-Ph.D, Rauf Shahbazov, M.D.-Ph.D, and Mazhar Kanak, Ph.D.

I thank Ana Rahman, Yoshiko Tamura, and Yang Liu for training me in cGMP facility aspects and for technical assistance with experiments.

I thank Betsy Stein, Nicolas Onaca, M.D., and Ernest Beecherl, M.D., for administrative and clinical support of the Clinical Islet Transplantation department at the Baylor University Medical Center.

I would also like to thank Robert R. Kane, Ph.D, Michael C. Lawrence, Ph.D, and Bashoo Naziruddin, Ph.D, for their friendship, guidance, and help over the past several years. They have been critical in achieving this success for me.

Financial support from Baylor University Medical Center, Baylor Scott & White Research Institute, and Baylor University is gratefully acknowledged

CHAPTER ONE

Introduction

Organ and tissue transplantation is an important and often life-saving treatment option for various diseases and disorders ranging from various cancers to organ failure due to chronic inflammation, genetic mutations, autoimmunity, or viral infection. The successful outcomes of transplantation have improved greatly over the past few decades and are now performed routinely as standard of care [1,2]. Despite the advancements made, short- and long-term graft function and survival remain suboptimal and a challenge to be overcome [3,4].

Islet transplantation is an archetypal model of transplantation [5]. It is performed as an alternative to pancreas transplantation or to exogenous insulin injections for the treatment of brittle type 1 diabetes (T1D) complicated by problematic hypoglycemia, and as an adjunct procedure after pancreatectomy to restore glycemic awareness and control. The success of islet transplantation, while it has made significant improvements in the past two decades [6], remains impaired due to islet graft loss post-transplant and long-term rejection.

In most cases, an allogeneic donor is utilized as the source of organs and tissues to be transplanted. Standard protocols for using allogeneic tissue requires the systemic administration of immunosuppression to the recipient to prevent graft rejection [7]. Though immunosuppression is necessary to facilitate long-term graft function, it also acts as a double-edged sword in that it carries increased infection and cancer risk and is cytotoxic

to some degree [8-11]. To avoid the risks of immunosuppression, sub-optimal drug doses are used, compromising the anti-rejection potency. Therefore, finding ways to localize and minimize or eliminate the need for long-term systemic immunosuppression is a critical goal to reduce off-target side effects and improve the efficacy of treatment.

Our method for addressing the issue of systemic immunosuppression has progressed first through choosing a drug candidate in which to design a pro-drug and linker platform around. Using an inhibitor of a pro-inflammatory innate immune receptor, we first described and demonstrated the chemistry and islet surface modification methods to make it possible for further work. This was an extensive process that required a steep learning curve of all the assays required in order to produce reliable data. While no experiments were performed with immunosuppressive drugs, such as FK506, in the context of allotransplant with our surface modifies islets, the data and methods described in the following chapters paves the way for allogeneic transplant studies with a pro-drug form of FK506 or other immunosuppressive compound.

Short-term graft survival is influenced by organ procurement and storage conditions [12], and post-transplant factors including innate immunity [13], which may negatively affect graft function. Islet transplant success is significantly compromised due to inflammation during the peri-transplant period and has a high rate of primary graft dysfunction. The instant blood-mediated inflammatory reaction (IBMIR) and sterile inflammation are responsible for the loss of as much as 50-70% of islets within a brief period post-transplant [14-16]. Loss of this islet mass often fails to facilitate single-donor insulin independence, and multiple donor pancreases are required to achieve long-term

insulin independence [17]. Therefore, ameliorating islet inflammation and damage during the peri-transplant should be a priority in improving islet transplant efficacy.

The mechanisms behind acute graft inflammation and injury are of intense interest in order to develop effective therapies. In recent years, the importance of innate immune receptors such as TLRs have become understood to play an important, if not integral, role in transplant outcomes. Of particular interest to this study was TLR4, the canonical receptor for gram-negative bacterial lipopolysaccharide (LPS) [18]. TLR4 has been elucidated as major mediator of sterile inflammation by acting as a promiscuous receptor for DAMPs [19]. Studies in animal models of islet transplantation using α-TLR4 mAbs and TLR4-/- mice have demonstrated the positive effects role of inhibiting this receptor in transplantation outcomes [20,21], making it an attractive therapeutic target. A recently developed and clinically-tested small molecule inhibitor of TLR4, TAK-242 [22], was identified and used as a basis for further study here.

Since islets are inflamed due to the procurement and isolation process prior to transplant [23], we were interested to see if early TLR4 blockade during the isolation process would reduce inflammation in islets prior to transplant by reducing TLR4 activation. The results demonstrated that early TLR4 blockade in islets by the addition of TAK-242 to the collagenase solution is sufficient to significantly reduce the expression of key inflammatory proteins, reduce islet damage post-transplant, and improve transplant outcomes. This treatment protocol is attractive since it avoids direct administration of a potentially immunosuppressive drug and has the potential for translation to clinical use due to the simplicity of use and previously demonstrated non-toxic profile.

Additionally, since the majority of islet damage is due to sterile inflammation and IMBIR after transplant, we sought to inhibit this inflammation with TAK-242 using a localized drug delivery method to avoid off-target effects of systemic administration. We developed a pro-drug that can be conjugated to live tissues and releases TAK-242 at a tunable rate. We then demonstrated *in vitro* that when modified with the pro-drug, islets are protected from TLR4 stimulation for multiple days. In an animal transplant model experiment, we were able to demonstrate that, with a marginal dose of just ~100 islets, we can reverse diabetes in 100% of recipients with modified islets compared to a cure rate of 0% in recipients receiving unmodified islets.

The results from these experiments demonstrate that TLR4 plays a role as a sterile inflammation receptor in islet transplantation and suggest that TAK-242 has the potential to be translated to clinical usage to reduce sterile injury in islets pre- and post-transplant. Additionally, our novel pro-drug and conjugation chemistry holds great promise in islet transplantation and may be translatable to solid organ transplant as well.

Although this dissertation is presented in a logical developmental order to simplify reading, it is not a chronological record of the experiments. First, in vivo and in vitro assays were performed with TAK-242, demonstrating the potential of this compound. After the TAK-linker compounds were synthesized, the in vitro assays necessary to study them were developed followed by in vivo transplant models. While the surface modification paper was being prepared and submitted, TAK-242 was evaluated in a relevant islet isolation-transplant model of total pancreatectomy with islet autotransplant (TPIAT). These experiments (early TLR4 blockade) began by simply looking at pro-inflammatory gene expression in islets treated with TAK-242 during the isolation process, during which islets

are stressed. After observing that early TLR4 blockade reduced the expression of key inflammatory proteins, we began an in vivo transplant model in which we monitored mice for 60 days post-transplant. During the transplant experiment, we filled in the intracellular signaling and other mechanism gaps with various in vitro assays.

As for project contributions, the early TLR4 blockade project was my own design with minor experimental input by my mentors. The surface modification project has been a long effort in the Kane and Naziruddin groups. Jeff Sorelle and Mazhar Kanak laid much of the foundational groundwork regarding chemical modification of islet surfaces, aided in part by Joshua Horton. All the work involving TLR4 as a target occurred during my PhD studies. Joining a transplant research lab after working in an organic chemistry lab left a steep learning curve to overcome in order to develop the proper assays for studying islet surface modification and its effects. The prodrug chemistry was designed and compound synthesis were performed in Dr Kane's lab, while all islet work was performed by me in collaboration with other scientists in Dr Naziruddin's lab.

CHAPTER TWO

Literature Review

The pancreas is an organ responsible for producing and secreting zymogens, such as trypsinogen, which are necessary for the proper digestion of food [24]. Zymogens are produced in acinar cells, which makes up the bulk of the pancreatic parenchyma and is referred to as the exocrine portion of the pancreas since the produced zymogens are secreted into the pancreatic duct and eventually into the duodenum [25]. The pancreas is also home to the islets of Langerhans, which are micro-organs that make up approximately 1-2% of the pancreas mass and are responsible for the pancreas endocrine function. Islets are comprised primarily of α, β, δ, ε, and PP endocrine cells, in addition to harboring resident macrophages and fibroblasts, and are also vascularized and innervated [26]. α-cells secrete glucagon, a hormone peptide that is produced in response to hypoglycemia and inhibited by hyperglycemia [27]. β-cells secrete insulin in response to increased blood glucose to promote glucose uptake into cells and the conversion of glucose to glycogen [28]. δ-cells secrete somatostatin to regulate the function of α- and β-cells [29-32]. ε-cells secrete the growth hormone ghrelin which plays a role in digestion and glucose metabolism [32,33]. PP cells secrete islet amyloid pancreatic polypeptide/amylin which promotes the function of pancreas and islet cells, insulin, and has apparent neuro-protective effects [34-36]. All these cells must work together to maintain a fragile yet critical balance of blood glucose levels. Of the different islet cell types, β-cells are the most numerous cells of an islet, followed by α-cells [37]. The remaining cells make up just 10-20% of an islet, emphasizing the β-cells primary role in glucose homeostasis. B-cells are the most studied and arguably the most

most important cells within an islet. Dysregulation or damage of β-cells leads to diabetes mellitus (DM).

Diabetes Mellitus is a disease defined by chronic hyperglycemia due to impaired β-cell and insulin function [38]. This disease is a major cause of morbidity and mortality in the U.S. and is considered a "gateway" disease that leads to cardiovascular and kidney disease [39]. The economic burden of DM is considerable, estimated to be greater than $300B annually in the U.S. due to medical costs and lost productivity [40]. In severe cases of DM, patients can present with "brittle" diabetes, which is when there is severely compromised glycemic control, hypoglycemic unawareness, severe hypoglycemic events, and frequent hospitalization [41].

The pathologies of DM can be grouped into three main classifications: Type 1, Type 2, and Type 3c [42]. Type 1 diabetes mellitus (T1D) is the autoimmune destruction of β-cells primarily mediated by immune cells such as B- and T-cells [43,44]. Originally referred to as "juvenile diabetes" due to its early onset in most cases, T1D often sets in by puberty and affects 5-10% of all diabetics [45,46]. To-date, there is no known cure or preventative therapy [47]. While there are suspected genetic and epigenetic links, these alone cannot explain the increasing incidence worldwide [48], therefore the causes of T1D remains incompletely understood but environmental influences are thought to contribute to increasing incidence worldwide [49]. The only standard of care for T1D in the U.S. is exogenous insulin injections, either via manual multiple daily injections or through insulin pumps which may be augmented with continuous glucose monitoring [50,51]. Although these treatments are somewhat effective in the majority of T1Ds, there is a sub-population of

non-responders who continue to suffer from severe glycemic lability, diabetic ketoacidosis (DKA), and severe hypoglycemic events, despite aggressive treatment.

Type 2 diabetes mellitus (T2D) is the most common form of DM, making up 80-90% of all diabetics and affecting 12-14% of all US adults [52]. 40% of Americans are considered prediabetic [53]. T2D is defined as impaired insulin sensitivity leading to impaired and inadequate compensatory insulin activity [54]. T2D is a heterogenous disease that may affect any combination of the secretion of insulin from β-cells, the production of insulin, tissue sensitivity to insulin, and the activity of insulin. Current treatment options include medications such as metformin, diet and exercise, bariatric surgery, and exogenous insulin in severe cases [55-57].

Type 3c diabetes mellitus (T3cD) is the least commonly known or recognized form of DM [58,59]. This form of DM is not caused by direct injury or insult to the β-cells, but rather as a secondary or bystander effect. T3cD is DM that occurs as a result of injury to, or disease of, the exocrine pancreas that causes islet inflammation and damage [60]. The most common cause of T3cD is chronic pancreatitis (CP) which accounts for ~80% of cases [61]. CP itself is a heterogenous disease with many possible causes and factors. The etiology of CP is still not fully understood, though it is accepted that it progresses first through acute pancreatitis (AP) and recurrent acute pancreatitis (RAP) before being diagnosed as CP [62]. The prevalence of T3cD is thought to be as high as 9-10% of diabetics although it could be higher due to limitations of reliable lab tests or lack testing at all, and misclassification occurs frequently [59,63]. Genetic mutations exacerbated by alcohol and smoking are the most common cause of CP, followed by pancreatic ductal strictures [62,64]. The treatment options for CP vary depending on the diagnosed etiology on a case-by-case basis. Oftentimes, duct

stenting or removal of gallstones is sufficient to prevent RAP. For CP with intractable pain, removal of the whole or parts of the pancreas is performed. Damage to the pancreas due to CP and removal of the pancreas leads to exocrine insufficiency which can be treated by supplemental pancrelipase. In the cases where there is significant islet loss, either through inflammation and fibrosis or pancreatectomy, insulin injections are necessary to maintain glycemic control.

Of the three types of DM, only T2D leads to relative insulin insufficiency, while both T1D and T3cD lead to absolute insulin insufficiency. As mentioned above, T2D can almost be completely managed with medications and lifestyle changes. T1D and in some cases, T3cD, on the other hand require supplemental insulin for survival. In the U.S., currently exogenous insulin is currently the standard of care for insulin insufficiency. However, there are two main fields of study for treating insulin insufficiency: 1. Developing an artificial pancreas, and 2. β-cell replacement therapy.

The original sensor-augmented closed-loop insulin delivery system, or artificial pancreas, was designed to continuously infuse a low bolus of insulin and respond to increases in whole blood glucose [65,66]. In the early years of development, and indeed even now, many technical problems arose such as developing pump algorithms and counterregulatory mechanisms to effectively manage glucose excursions while minimizing the risk of hypoglycemia [67-69]. Over the years, the artificial pancreas has become a fusion of technology and biomedical engineering, comprised of a body-mounted continuous glucose monitor (CGM), insulin pump, and a platform device such as a smartphone [70]. The artificial pancreas has made significant progress since the initial inception of the idea, primarily driven by technological advancements. Multiple trials published in recent years

report the findings from the first at-home studies with a modern device and found that it is able to improve glycemic control, but there were still some hypoglycemic events [71,72]. Nevertheless, the artificial pancreas continues to make significant progress as a complete replacement to the current standard of intensive insulin therapy. Commercial sale of these devices has recently been approved in the U.S. [73-75]. Future priorities for improving the artificial pancreas include developing a bi-hormonal pump that secretes both insulin and glucagon [76], improving pump algorithms to better control glycemia, improving the user-friendliness of the systems, and possibly translating the technology to younger users [77].

β-cell replacement therapy is another approach to controlling diabetes and can be performed by multiple methods. Currently, allogeneic pancreas transplantation is the most common procedure for β-cell replacement with over 40,000 registered cases [78,79]. For this procedure, a pancreas, and most often a kidney due to renal failure due to DM, from a deceased donor is procured and transplanted into someone with T1D who is then put on life-long immunosuppression. Pancreas transplantation is successful in regards to patient survival (>95% at 1 yr) and long-term graft function (~90% at 1 yr, 73% at 5 yr, and 56% at 10 yr) [80]. However, the procedure is invasive and results in a significant amount of post-transplant morbidity and mortality [81,82]. Despite the high percentage of patient survival and demonstrated efficacy, the number pancreas transplants for the treatment of T1D has gone down in recent years, possibly due to a combination of improved insulin pumps and medical DM management as well as insufficient advertising and referrals to specialized transplant centers [83]. Given the high rate of morbidities associated with whole-pancreas transplantation, high cost, and the emergence of less invasive effective treatment options, the popularity of pancreas transplant may continue to decline.

Islet transplantation is emerging as an effective, minimally invasive treatment option for brittle diabetes secondary to T1D or T3cD [84]. The first islet transplant in humans for the treatment of T1D dates back to the 1980s [85]. Similarly, the first human autologous islet transplants after pancreatectomy were performed in the 1980s [86]. The early allo-islet transplants were plagued by poor long-term outcomes due to peri-transplant inflammation and ineffective cytotoxic immunosuppression [87,88]. Efficacy of islet transplantation may also be affected by poor organ allocation for whole pancreas vs islet transplant since the best organs are used for whole organ transplants [89]. In 2000, the clinical islet program at the University of Alberta in Edmonton, published their results on islet transplants into 7 patients using a corticosteroid-free immunosuppression regimen who remained insulin-free after 1 year [90]. This landmark publication introduced an important paradigm shift in islet transplantation that brought focus onto improving induction immunotherapy, steroid-free immunosuppression, and the multiple donors needed to achieve the islet dose necessary to achieve insulin independence. The follow-up publication dampened enthusiasm for islet transplantation due to poor long-term islet graft function [91]. In the meantime, the focus of islet transplantation shifted to improving collagenase blends to increase islet yields and function [92,93], anti-inflammatory induction therapy and T-cell depletion therapy [6,94], and islet immunoisolation and encapsulation [95-97].

Even with modern protocols, achieving single-donor insulin independence with islet transplantation remains a challenge. An additional challenge is the shortage of donor organs available for transplant [89]. To this end, there is great interest in both xenotransplantation and stem cell-derived islet cells. In xenotransplantation, islets from one species is transplanted into a recipient of another species. The most promising source

of xenogeneic islets for humans are islets from pigs which, if clinical trials are successful, would allow for a virtually limitless supply of islets [98]. The primary problem with using porcine islets is that they are more immunogenic than human islets, therefore requiring more immunosuppression. Additionally, sourcing designated pathogen-free animals is difficult, requiring costly specialized facilities [99].

Stem cell-derived islet cells can come from multiple sources: 1. Embryonic stem cells (ESCs), 2. Induced pluripotent stem cells (iPSCs), and 3. Adult stem cells [100-103]. Like xenotransplantation, stem-cell derived islet cells may provide a virtually limitless supply of endocrine cells for transplant. Current protocols are able to efficiently mature stem cells into glucose-responsive, insulin-secreting β-cell-like cell [104]. However, these cells face by similar challenges to both allogeneic and xenogeneic islets in that they remain immunogenic and do not function as well as native islets [105]. If xenotransplantation or stem cell derived islet cells are to be successful, it is anticipated that they will have to be encapsulated to prevent rejection or aggressive immunosuppression [106].

Islet encapsulation is the process of surrounding islets inside a protective boundary for the purpose of minimizing immune cell infiltration. Islets can be macro-encapsulated inside a device [107], micro-encapsulated inside a polymer sphere [108], or nano-encapsulated/surface modified with polymers or bioactive compounds [95,109,110]. Macro-encapsulation devices have demonstrated limited efficacy in trials, likely due to poor nutrient transport, poor device vascularization, suboptimal transplant sites, and the limited doses of islets or stem cell-derived β-cells being transplanted due to size limitations [97,111]. Micro-encapsulated islet cells have shown some promise in clinical trials, but run into the same limitations in long-term outcomes as macro-encapsulated islets and require very-high

doses of islets [97,112,113]. Additionally, the polymers and gels used for macro-encapsulation are often immunogenic and become compromised due to fibrotic attack, limiting the long-term protection afforded by encapsulation [114].

Nano-encapsulation of islets has led to the most successful transplant outcomes in animal models [95,115], but has not been studied in humans. The advantages of surface modification vs macro- and micro-encapsulation are the negligible increase in graft volume and the negligible impact on nutrient transport. The minimal increase in graft volume is critical since islets are traditionally infused into the hepatic portal vein which can only take a limited mass of tissue before portal vein thrombosis or total occlusion occurs [116]. The liver as a transplantation site for islets remains the most effective for islet function, however it does have drawbacks such as decreased pO_2 [117], increased toxicity [118], and poor glucagon activity [119], therefore alternative sites for transplant are being explored [120].

While islet encapsulation is a promising method for protecting islets against immune attack after transplant, further challenges to islet graft function and survival are inflammation due to sterile stressors such as hypoxia, ischemia, and endogenous DAMPs [121]. In an attempt to address these insults, researchers have used oxygenation drugs or devices [122], anti-inflammatory drugs targeting key inflammatory proteins and pathways [123-125], and DAMP-neutralizing antibodies [20].

The innate immune system is known to play a major role in organ inflammation and dysfunction after organ transplantation [126]. Activation of these innate immune pathways upregulate inflammatory pathways through NFkB, MAPKs, AP-1, and IRFs, which mediate the production of pro-inflammatory proteins and may recruit immune cells,

triggering an adaptive immune response [127]. Toll-like receptors (TLRs) have been heavily implicated for their role in compromising organ grafts [128].

TLRs were first described in fruit flies and since then have been well studied as innate pathogen recognition receptors in mammals [129,130]. There have been 10 TLRs identified in humans and 12 in mice [131]. TLRs are part of the Toll/interleukin-1 receptor superfamily which also includes the interleukin-1 receptors due to the shared homology of their intracellular domains [132]. The hook-shaped extracellular domains of TLRs are comprised of tandem repeats of leucine-rich regions which confers their specificity for different ligands. TLRs 1, 2, and 6 bind gram positive bacterial lipoproteins and peptidoglycans, and form TLR1/TLR2 and TLR2/TLR6 heterodimers when activated. TLR4 is the canonical receptor for gram negative lipopolysaccharides and certain endogenous ligands. TLR3 binds viral dsRNA, TLR5 binds flagellin, TLR7/8 binds ssRNA, and TLR9 binds CpG DNA motifs [133]. TLRs 3/4/5/7/8/9 form homodimers when activated. The function of TLR10 remain unknown, but it may act as a regulatory receptor for cell-surface TLRs [134]. TLRs 1/2/4/5/6/10 are found on the cell surface while TLRs 3/7/8/9 are found in intracellular compartments.

The recognition of a PAMP or DAMP by their corresponding TLRs results in the activation of signaling pathways that promote the upregulation of pro-inflammatory cytokines, chemokines, and inflammation-associated proteins via MyD88-dependent or MyD88-independent pathways [135]. The MyD88-dependent pathway, utilized by all TLRs except TLR3, is known to activate the NFkB and the c-fos/c-jun AP-1 pathway via MAPKs, both of which are involved in inflammatory responses [136]. On the other hand, the MyD88-independent pathway is utilized by TLR4 and the endosomal TLRs 3/7/8/9. The

MyD88-independent pathway signals via IRFs recruited to TRIF domains on TLRs. MyD88-independent activation of IRFs through TRIF, and thus TLRs 3/7/8/9, is predominantly responsible for anti-viral responses by upregulation of type I interferons [135].

While TLRs were initially believed to only respond to pathogens such as bacterial cell wall components or viral genomic particles, it was soon discovered that certain TLRs also bind and respond to endogenous ligands and DAMPs [137]. Endogenous ligands include damage or "danger" associated molecular patterns such as heatshock protein fragments, extracellular matrix fragments like fibrinogen, heparan sulfate, hyaluronan, HMGB1, and even mRNA [138]. The ability of endogenous ligands to trigger not only the innate immune system but also the adaptive immune system suggests that pathogen recognition receptors such as TLRs may have evolved to sense "danger" rather than act as self-nonself surveillance receptors [139]. Various TLRs have been implicated in playing a role in autoimmune diseases such as encephalomyelitis (TLR 2) [140], autoimmune arthritis (TLRs 3/4) [141,142], lupus (TLRs 7/9) [143], and many other diseases [144]. The mechanisms by which TLRs play a role in autoimmunity include aberrant activation or dysregulation of endosome-restricted TLRs 3/7/8/9 which causes immune cells such as DCs to be activated by self-nucleic acids and drive an adaptive B- and T-cell response [145], or chronic inflammation triggering the release of DAMPs and continuing a cycle of TLR4-mediated autoimmunity [146].

TLRs have also been implicated in mediating transplant graft failure [147]. While both TLR2 and TLR4 TLR4 are often implicated, TLR4 is the most commonly studied in affecting transplant outcomes [126]. TLR4 was the first TLR identified in mammals and is the most well-studied TLR overall [148,149]. TLR4 activation by a ligand induces

homodimerization and signals via both the MyD88-dependent, which leads to AP-1 and NFkB nuclear translocation, and the MyD88-independent pathway which activates IRFs (Figure 2.1). TLR4 has been shown to play a role in ischemia/reperfusion injury by acting as a promiscuous receptor for DAMPs, such as HMGB1, released by stressed and damaged cells [150,151]. Studies using TLR4$^{-/-}$ models and α-TLR4 antibodies have demonstrated reduced ischemia injury and improved graft function [20,152-154]. Importantly, a TLR4$^{-/-}$ model using recombinant HMGB1 did not alter IRI in mice, demonstrating a critical HMGB1/TLR4 interaction [155]. Additionally, TLR4 has been implicated in exacerbating allo-rejection, resulting in poorer allograft success [156,157].

Although simply targeting TLR4 may not block the majority of sterile inflammation generated during transplant, it is an attractive target since it is a non-vital receptor and some inflammation is actually beneficial for survival [158]. As summarized in Figure 2.2, TLR4 has been implicated to play a role in several biological processes. In triggering IBMIR, TLR4 activation may activate innate immune effectors such as neutrophils, macrophages, and dendritic cells into a pro-inflammatory state and drive graft infiltration. Activation of TLR4 on platelets has been linked to thrombosis formation [159]. Ischemia/reperfusion injury is also driven by TLR4-mediated signaling in concert with the release of DAMPs from damaged cells to drive a strong sterile inflammation response [160].

Global inhibition of major inflammatory pathways such as NFkB or MAPKs may result in significant cell death [161,162]. Besides being a non-vital receptor, there is also a commercially available, clinically-tested TLR4 antagonist, TAK-242, allowing for rapid use in new trials [163]. TAK-242 is a small molecule that functions by binding to Cys747 in the intracellular domain of TLR4 [164]. Initially developed to treat sepsis, TAK-242 has been

shown effective in a range of settings including hyperplasia [165], autoimmune disorders [166,167], brain trauma [168], and reduction of pain [169]. It has also completed a double-blind, placebo-controlled phase 3 clinical trial for the treatment of sepsis and demonstrated good tolerability, but was unfortunately not effective against severe sepsis [163].

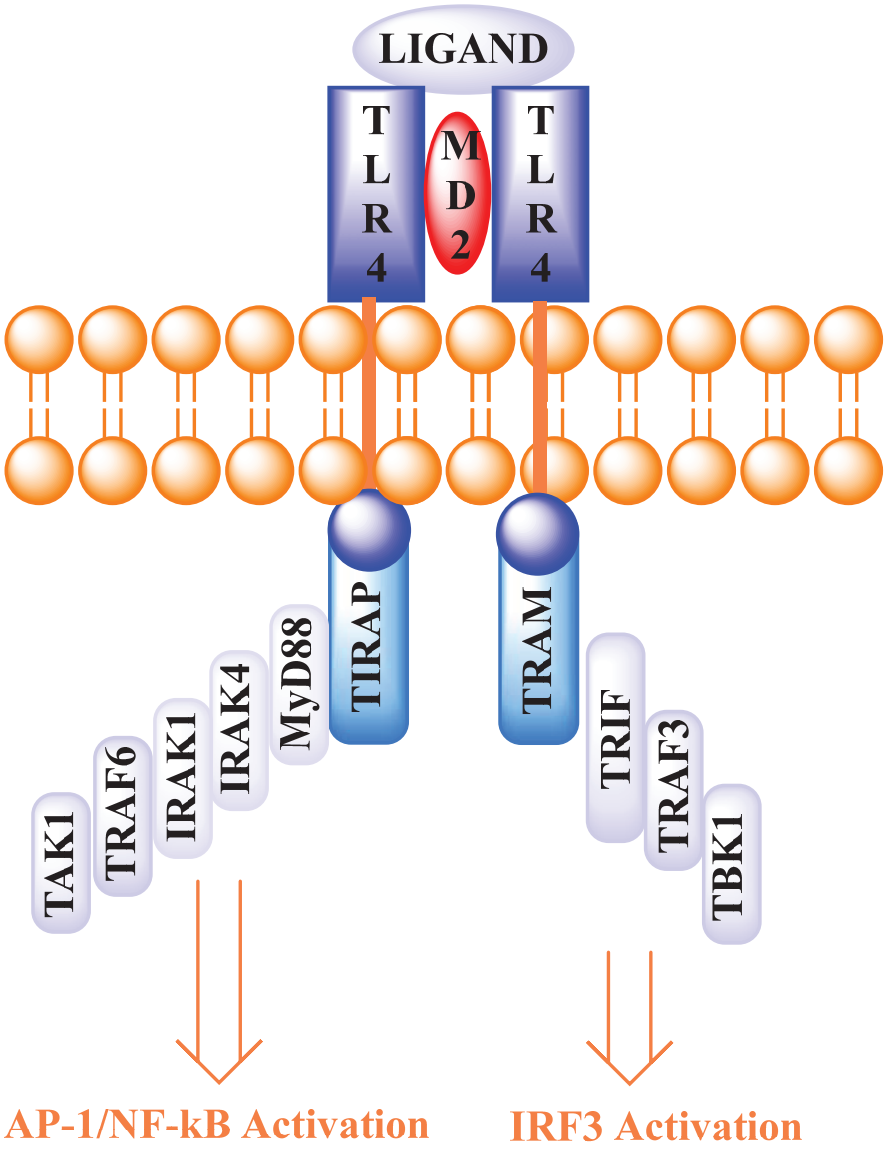

Figure 2.1. TLR4 intracellular signaling pathway.

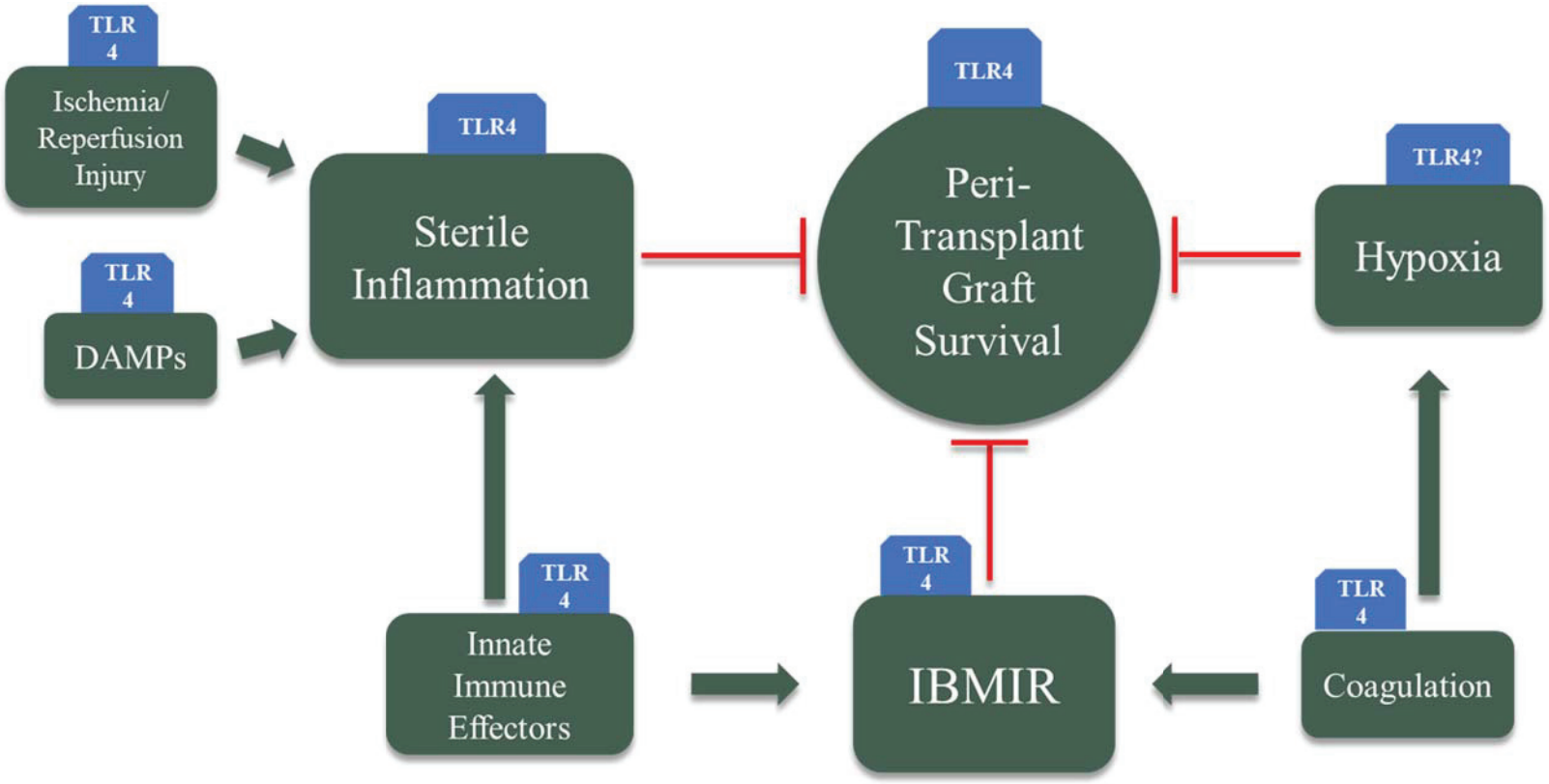

Figure 2.2. TLR4 plays a role in multiple pathways to compromise graft survival

Besides TAK-242, other TLR4 antagonists exist, which tend to be Lipid A derivatives. Lipid A is the hydrophobic region of gram negative bacterial LPS which anchors LPS to the outer bacterial membrane [170]. One such derivative, eritoran (E5564), has also demonstrated a good safety profile through a phase 2 clinical trial [171]. It should be noted that while TAK-242 binds to TLR4 in its intracellular domain, Lipid A derivatives bind to the extracelluar region in a similar fashion as LPS. Another factor that made TAK-242 an attractive drug for our purposes is its sulfonamide motif, which is common in medicine and allows for further chemical manipulation. Based on the demonstrated safety and potency of TAK-242, and it's easy of chemical manipulation, we chose this this drug for our studies in islet transplantation and surface modification.

Our islet surface modification chemistry takes advantage of the flexibility and biocompatibility of bioorthogonal "Click" chemistry [172]. Click chemistry is attractive for use on organic tissue and even inside living organs due to its rapid reaction in physiologically-compatible conditions and buffers [173]. This is a far cry from how chemical reactions were traditionally planned: by carefully choosing solvents, modulating reaction temperatures, excluding atmospheric gasses and moisture, adding catalysts, and also chemical protection and deprotection of reactive groups [174]. The value and importance of the development of Click chemistry can be best observed by examining how this has allowed scientists to interrogate biomolecules in vitro and in vivo, observing them in their native habitat, and elucidating molecular interactions in cells as well as in whole organisms [175]. One of the first examples of Click chemistry used metabolically-incorporated azide-labelled glycoconjugates to express azides on the cell surface which were then reacted with a biotinylated triarylphosphine to create cell-surface adducts [176]. Intracellular reporters

using azide-alkyne reactions with modified nucleic acids to track DNA synthesis [177], and metabolite accumulation [178] have also been reported, demonstrating the power of Click chemistry in answering basic questions in biology and aiding in diagnosing diseases. Previously, other groups have used Click chemistry to successfully conjugate biologically active and inert molecules to the islet surface. A popular inert molecule has been polyethylene glycol (PEG). Coating an islet with PEG has been shown to help reduce allosensitization post-transplant [95,179]. Active molecules, such as VEGF, have also been tested on islets [180]. Combinations of a steric barrier (PEG) with bioactive compounds (anticoagulants) have also been studied [181].

The concept of conjugating drugs to carriers to improve their half-lives and pharmacokinetics, and therefore improve their therapeutic potential is an important development in drug delivery [182]. Drug-macromolecule carriers have been described and FDA-approved for use [183]. However, the obvious limitations of drug-carriers are the loss of drug-potency due to conjugation and, depending on the carrier, restriction to extracellular spaces. Therefore, to overcome such limitations, developing a cleavable linker that can be covalently attached to the carrier was important. Cleavable pro-drug linkers have already been described and studied on inert polymer carriers [184,185]. This approach by Santi et al. utilizes a carrier bearing a succinimidyl carbonate group for attachment to an amine-containing drug, an electron-withdrawing tunable modulator to control the rate of β-elimination cleavage, and an azide for strain-promoted azide-alkyne cycloaddition to a carrier. Using exenatide as an example, they were able to increase its half-life 56-fold by conjugation to a PEG carrier. Their results demonstrate the feasibility of creating cleavable prodrugs which are effective in improving the pharmacokinetics of the therapeutic. In our

studies, we borrow their cleavable linker design to create out TAK-242 prodrug. To our knowledge, the combination of both Click chemistry on living tissues with a cleavable pro-drug carrier is novel to our studies here.

CHAPTER THREE

Preliminary Works

In focusing on the chemical modification of the surface of islets with therapeutic compounds, we first had to search for effective drugs for reducing inflammation and the effects of IBMIR in islets after transplantation. The hallmarks of IBMIR are inflammatory cytokine production, leukocyte infiltration, and coagulation [186]. To address these, we identified IBET762, CD47, and dabigatran, respectively. IBET762 is an anti-inflammatory inhibitor of bromodomain and extra-terminal domain proteins which act as nuclear transcription factors and activate T cells [187]. CD47 is known as the "don't-eat-me" signal that prevents immune cells from phagocytosing "self" cells [188]. Dabigatran is a potent direct thrombin inhibitor (DTI) which effectively inhibits thrombosis and is an attractive alternative to heparin since it has an effective antidote in the case of overdose [189]. Later, we identified TLR4 as a major mediator of sterile inflammation as well as TAK-242, a small molecule antagonist of TLR4 which was amenable to chemical modification [22,190]. The works and findings regarding these preliminary efforts are described here.

IBET762

IBET762 was first studied on MIN6 cells to assess the range of non-toxic doses. Briefly, MIN6 cells were plated in a 96-well plate at a density of 15,000 cells/well and cultured for 24 hours prior to treatment with IBET762 at various concentrations for up to 72 hours. Viability was measured by MTT assay. The experiment revealed that IBET762

was negligibly toxic to MIN6 cells up to 72 hours at a concentration of 500nM (Figure 3.1).

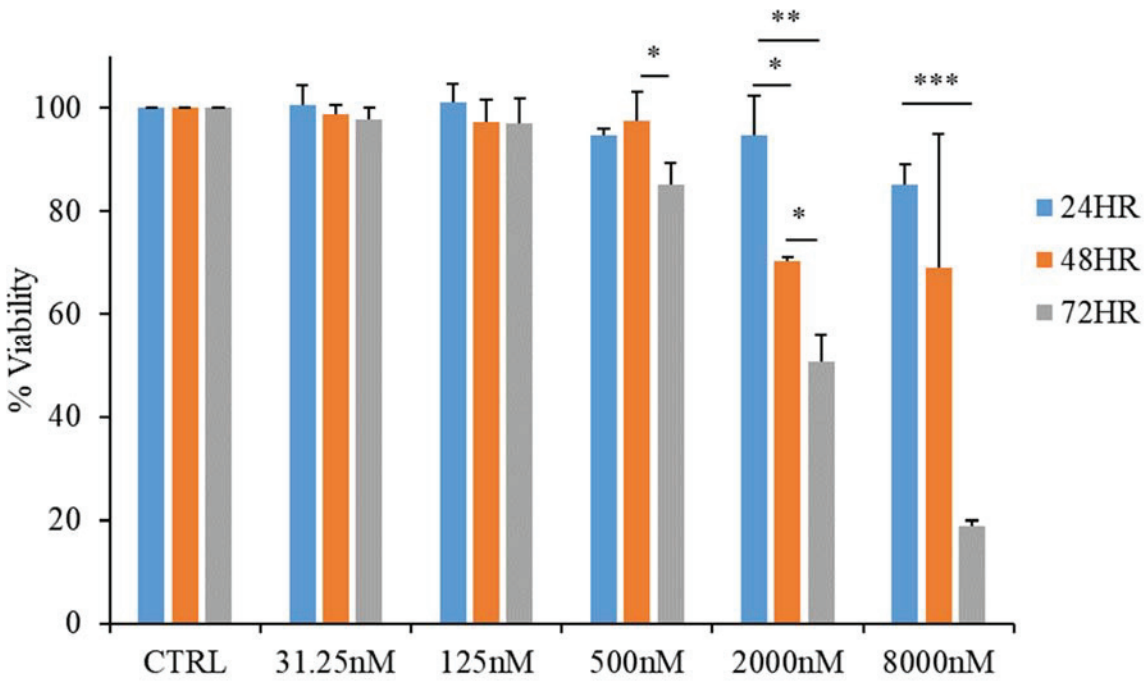

Figure 3.1. Assessment of IBET762 toxicity in MIN6 cells. MIN6 cells were treated with the indicated concentration of IBET762 for the indicated time and viability was determined by MTT assay. Statistical significance determined by Student's *t* test. Data represented as means±SD

The ability of IBET762 to protect human peripheral blood mononuclear cells (hPBMCs) for protection against inflammation was studied [191]. Briefly, whole blood was isolated from healthy human donors and the PBMCs isolated by treating it with ACK lysis buffer in order to lyse red blood cells. The isolated PBMCs were then treated with IBET762 and challenged with a standard PMA + Ionomycin protocol. Activation was measured by CXCL10 upregulation after 6 hours. The results show that IBET762 reduced the expression of CXCL10 in hPBMCs (P = 0.07; Figure 3.2). These results were quite promising,

demonstrating effective reduction in PBMC activation, and inhibitors of BET proteins are of great interest in the cancer field.

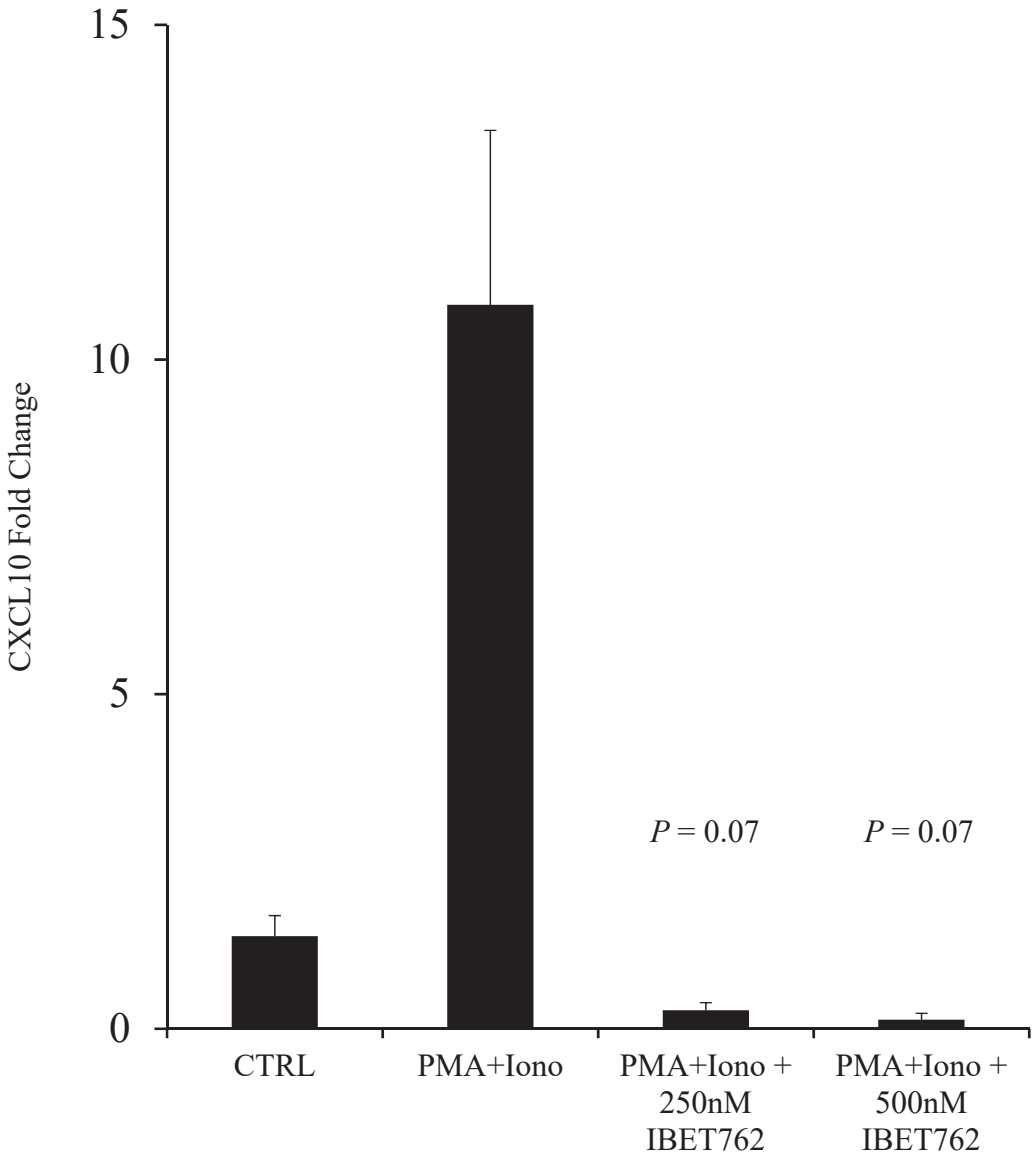

Figure 3.2. IBET762 inhibition of inflammation in hPBMCs. hPBMCs were treated with PMA + Ionomycin with or without the indicated concentration of IBET762 for 6 hours. CXCL10 expression was assessed by RT-PCR. Statistical significance determined by Student's t test. Data represented as means±SD.

CD47

The CD47 peptides was synthesize from the originally published sequences for human, mouse, and scramble control [192], modified with a terminal biotin and azide to make it amenable for conjugation to surfaces and fluorescent labelling. Initially we tried enzymatically conjugating the peptide to islets using microbial transglutaminas. However, the modification results were inconsistent and may have induced significant apoptosis in islets. Fortunately, we demonstrated it was possible to conjugate the peptide to the surface of islets using click chemistry, first priming the surface with a NHS-DBCO (Figure 3.3). When islets modified with the CD47 peptide were transplanted into the kidney subcapsular space, we observed a reduction in neutrophil infiltration, marked by a reduction in infiltrating Ly6-G+ cells compared to untreated islets (Figure 3.4). Later, it was confirmed that the N3 on the peptide would allow for rapid conjugation to DBCO-beads and was detectable by a streptavidin-488 fluorophore (Figure 3.5). While CD47 peptide conjugation was not thoroughly studied here, it remains an option of great interest and is currently being studied to coat transplantable devices and dialysis tubing to prevent adhesion and activation of coagulation and immune cells.

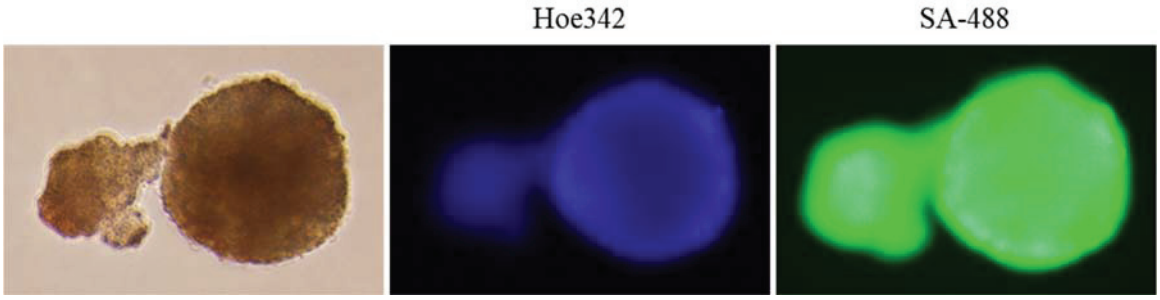

Figure 3.3. Surface labelling of islet with CD47 peptide. Human islets were surface-functionalized by reacting with NHS-DBCO, CD47 peptide, and streptavidin-488 sequentially for 1 hr each at room temperature.

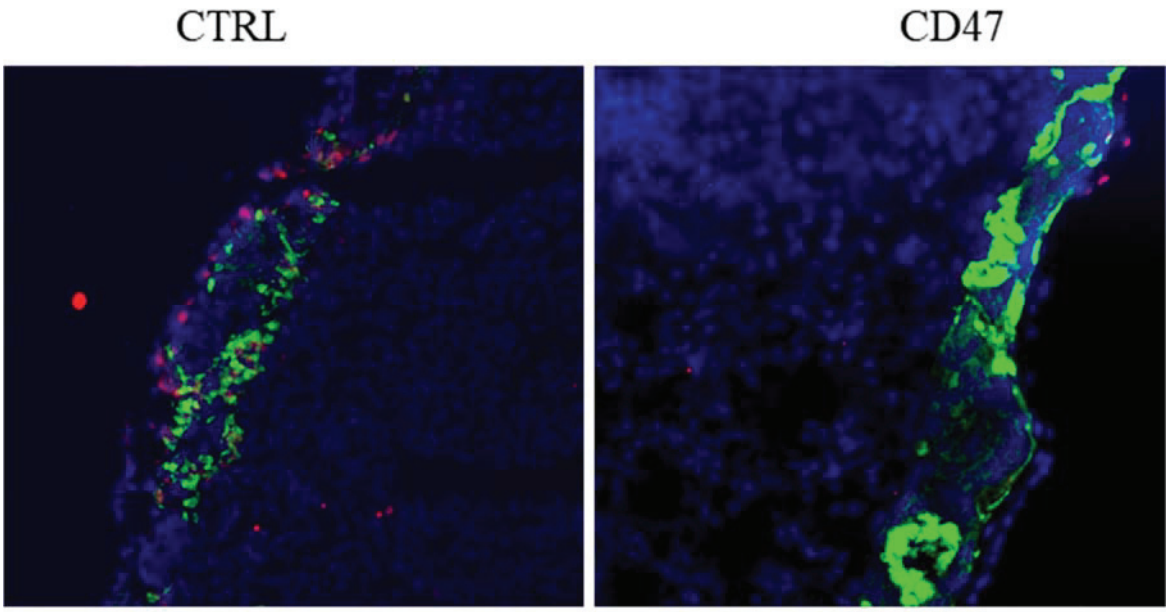

Figure 3.4. Neutrophil infiltration of islet grafts. C57BL/6 islets were modified with CD47 or left untreated then transplanted into the kidney capsule of diabetic syngeneic mice. 72 hours post-transplant, graft-bearing kidneys were recovered, and frozen sections prepared for immunofluorescent staining. Blue – Nuclei, Green – Insulin, Red – Ly6G.

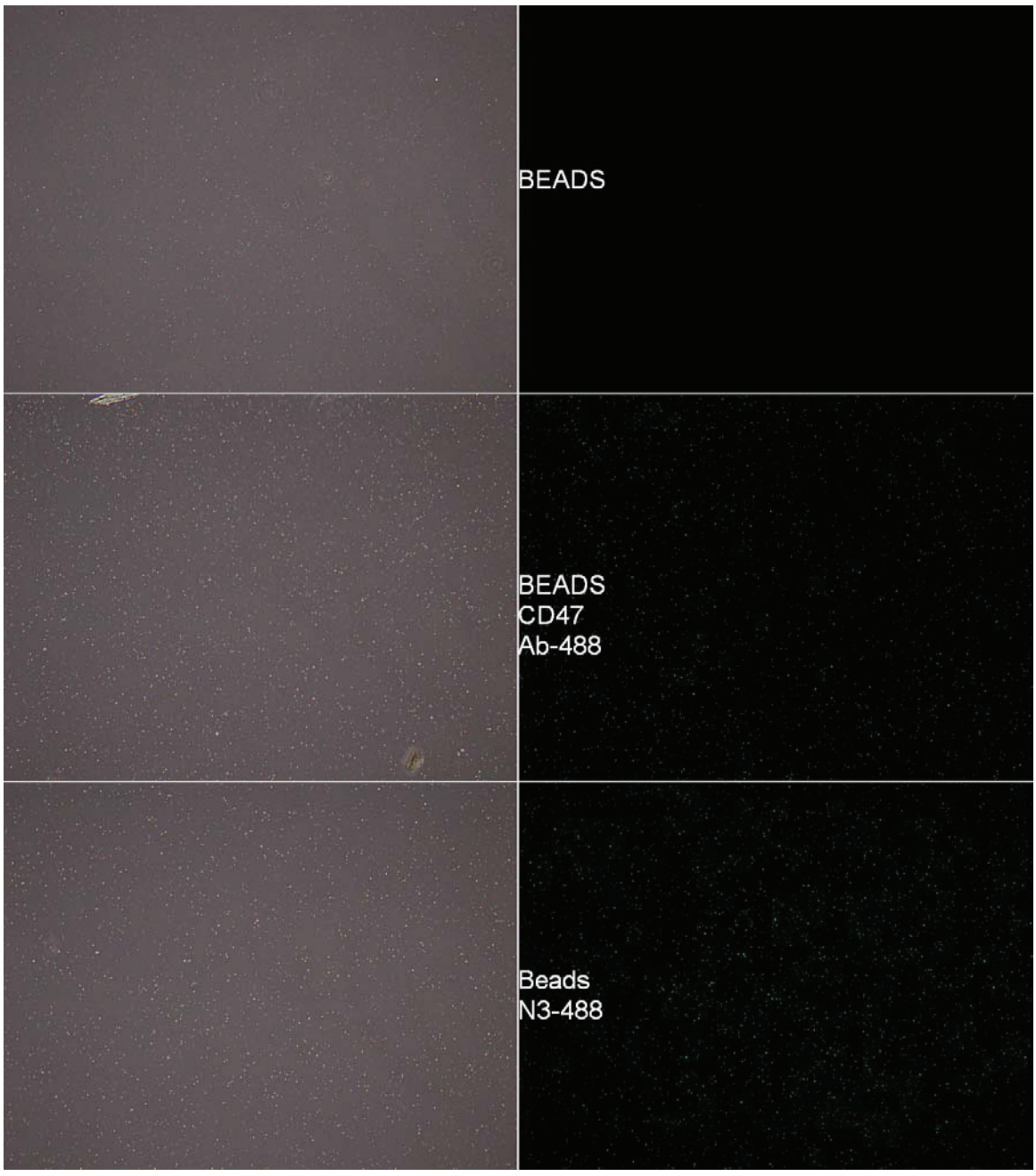

Figure 3.5. CD47 peptide conjugation to DBCO-beads. Top row – phase contrast and fluorescent image of beads alone. Middle row – phase contrast and fluorescent image of beads reacted with CD47 peptide and streptavidin-488. Bottom row – phase contrast and fluorescent image of beads reacted with azido-488.

TAK-242

Preliminary studies with TAK-242, a small molecule inhibitor of TLR4 (Figure 3.6), provided us with surprising and exciting results. In addition to proving to be very effective in inhibiting LPS-mediated TLR4 activation, it was also effective against non-specific sterile inflammation in our models. In an in vitro blood loop model, islets pre-treated with TAK-242 were able to reduce the upregulation of important cytokines in islets when mixed with blood (Figure 3.7A-D). In a basic in vivo syngeneic transplant mode, we found that systemic administration of TAK-242 (3ug/kg) to the recipient prior to islet transplantation was sufficient to significantly improve transplant outcomes with a marginal dose of 200 islets (Figure 3.8). However, it should be noted that what is defined as a "marginal" dose may vary quite a bit depending on transplantation skill, culturing conditions, and counting method. Here, a marginal dose is defined as an untreated islet dose at which recipient animals typically do not achieve euglycemia or non-fasting blood glucose <200mg/dL.

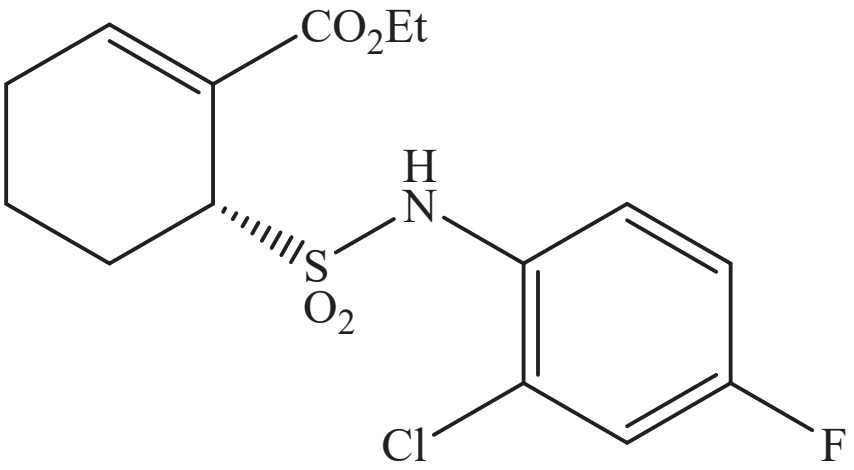

Figure 3.6. Structure of TAK-242

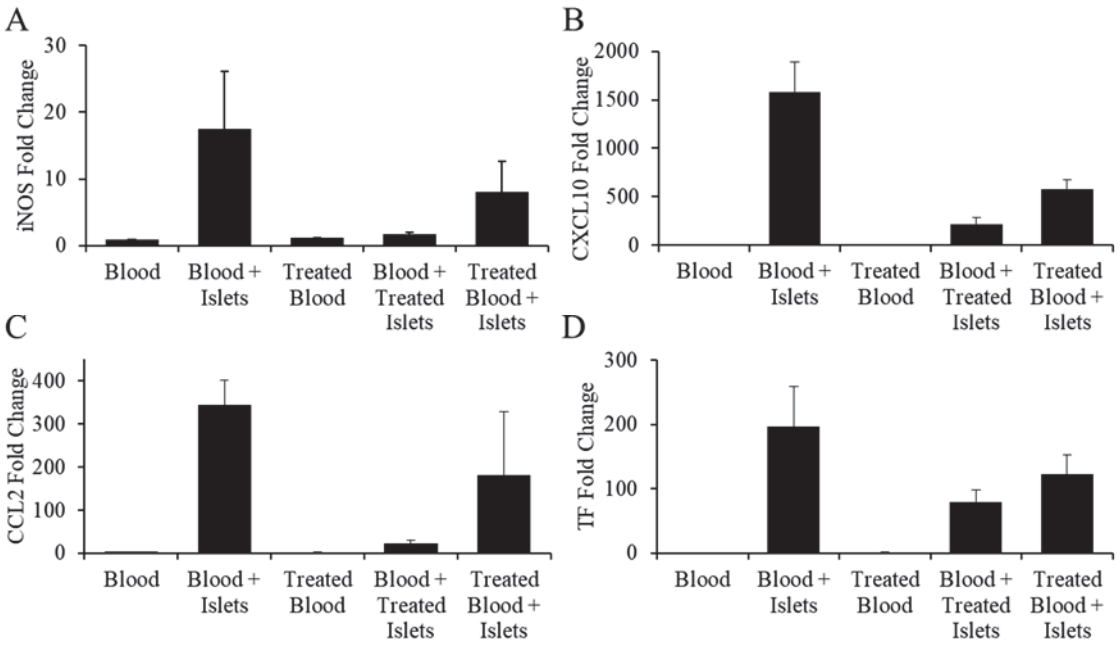

Figure 3.7. Cytokine expression in samples with or without TAK-242 treatment. Whole RNA collected from sample tubes after 6 hours incubation time were analyzed for iNOS (A), CXCL10 (B), CCL2 (C), and Tissue Factor (D). Statistical significance was not achieved in any of the treated islet samples likely due to the experiment being performed in duplicate, significantly reducing statistical power. Statistical significance was determined by Student's *t* test. Data represented as means±SD.

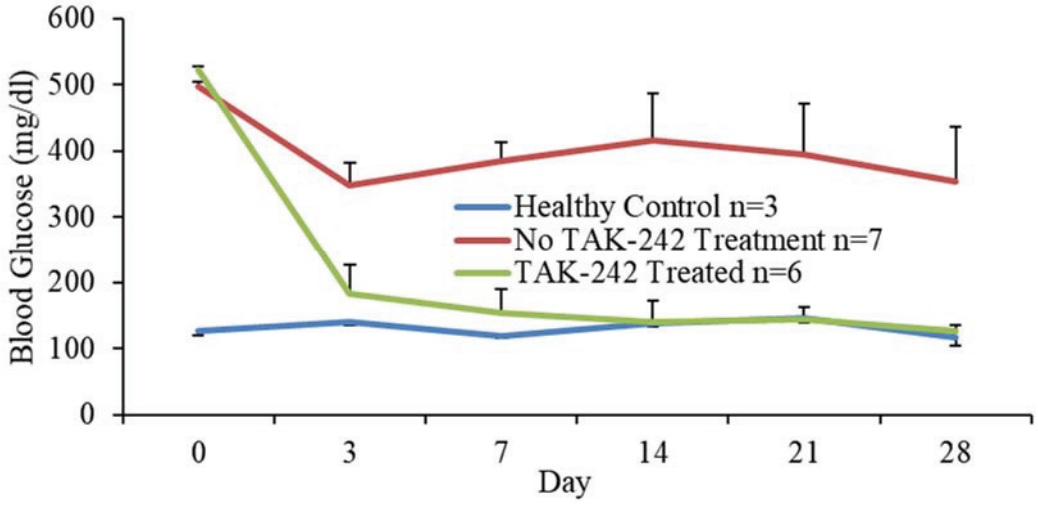

Figure 3.8. Mean blood glucose in mice after islet transplant with or without systemic TAK-242 treatment. 200 islets were transplanted into the kidney supcapsular space of diabetic mice with or without a 3µg/kg intraperitoneal injection of TAK-242.

Given the excellent results obtained from preliminary studies with TAK-242, we chose to target TLR4 for our proof-of-concept studies for islet surface modification.

Modification Chemistry

While the synthesis of the TAK-242 pro-drug was being developed, we began to examine effective modification methods. The bioorthoganol linker that was utlized had an N-hydroxysuccinimide (NHS) moiety on one end to react with primary amines on tissue surfaces, and a dibenzocyclooctyne (DBCO) on the other end which readily reacts with azides (N_3) to promote strain-promoted azide-alkyne cycloaddition (SPAAC).

There were 3 main hurdles in optimizing the modification chemistry. First, there were solubility issues with the NHS-DBCO at any concentration above 1μM in aqueous buffer. This challenge could not be remedied by prolonged vortexing or heating, as both would reduce the active compounds half-life. Instead, the aqueous buffer should be added directly and rapidly to the appropriate volume of stock solution, followed by pipetting and vortexing. While this method often works, it is still important to visually inspect the diluted solution for clarity to ensure proper dilution. Otherwise the preparation of the working solution should be repeated until the compound is appropriately dissolved.

Next, experiments were performed to determine an effective concentration of the compounds that would quickly label the surface while remaining non-toxic. Previous studies suggested 1mM as an effective dose for NHS modification of islets [193]. However, this concentration was not soluble with the NHS-DBCO we were using, likely because our linker contained a shorter hydrophilic PEG spacer (dPEG4 vs PEG_{3500}). A 1:10 dilution to 100μM was still very poorly soluble and resulted in significant islet death (Figure 3.9).

Fortunately, a further dilution to 25μM allowed for efficient surface modification with negligible toxicity.

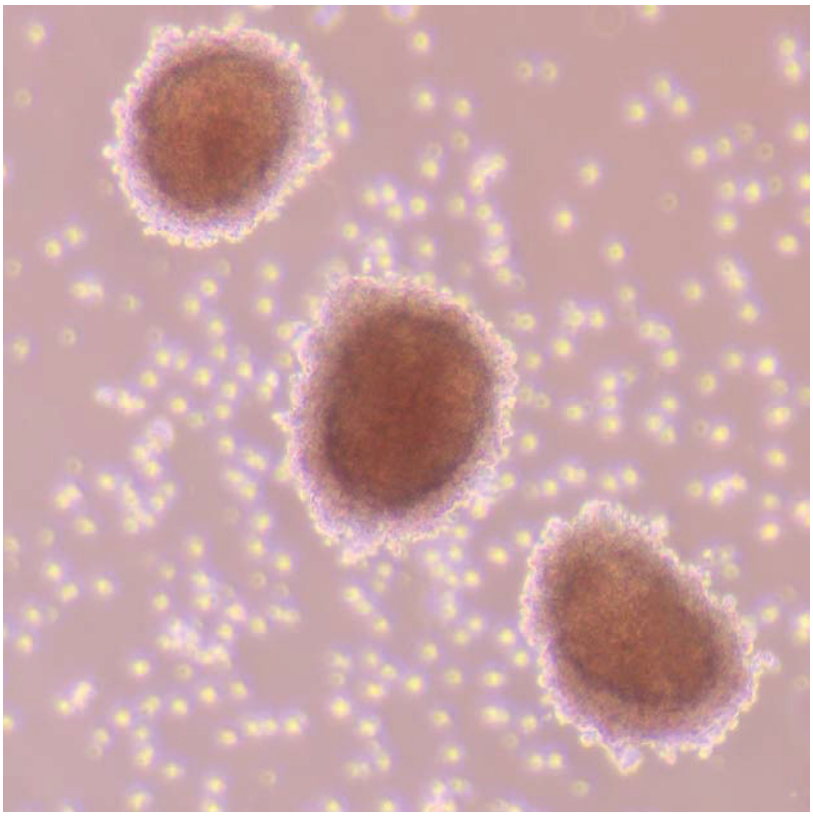

Figure 3.9. Islets after reaction with high dose NHS. Islets were reacted with 100μM NHS-DBCO and cultured overnight. The next day, islets were fragmented and breaking down into single cells.

After identifying a good working concentration, we had to determine an appropriate reaction time in order to get the most effective coverage. The previous studies reacted the islets with NHS-PEG$_{3500}$-NH2 for 1 hour at r.t., which we used as a starting point for testing. With our compounds, 1hr allowed for robust surface labelling, but bordered on toxic at doses greater than 25μM. Additionally, time-course experiments suggest that islets may be sufficiently labelled after just 10-30 minutes (Figure 3.9). It's unadvisable to go longer than 1 hour to reduce intracellular reactions with the NHS compound.

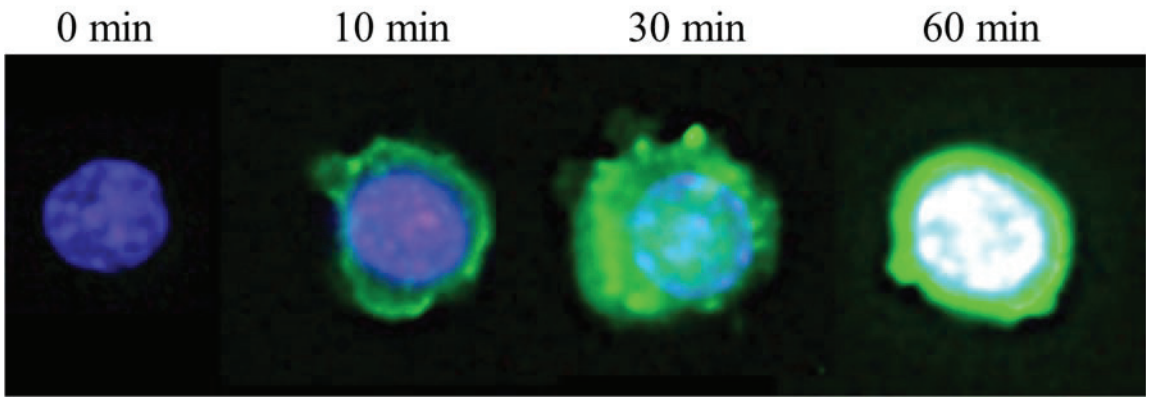

Figure 3.10. Time-dependent fluorescent labelling of cells. Single cells were reacted with NHS-DBCO for the indicated time points, then with N_3-488 for 30 minutes.

In conclusion, multiple compounds were tested in preliminary experiments for their efficacy against different aspects of IBMIR and sterile inflammation. IBET762 is a potent anti-inflammatory and remains of great interest in diseases with chronic inflammation such as cancer. CD47 has been demonstrated to be effective in reducing the activation of the innate immune system and is being studied for applications in nanoparticle delivery and medical device surface coatings. TAK-242 was found to have broad anti-inflammatory effects despite targeting a single cell-surface receptor. The chemical modification protocol, while presented here is a working protocol, could be further optimized to reduce the time necessary for sufficient labelling.

CHAPTER FOUR

Early TLR Blockade Attenuates Sterile Inflammation-Mediated Stress in Islets During Isolation and Promotes Successful Transplant Outcomes

Abstract

Background: During the isolation process, pancreatic islets are exposed to an environment of sterile inflammation resulting in an upregulated inflammatory state prior to transplantation. Toll-like receptor 4 (TLR4) has been identified as a major mediator of sterile inflammation. Therefore, we sought to determine whether early TLR4 blockade would be effective in reducing inflammatory burden in islets pre-transplant.

Methods: Pancreatic islets from C57BL/6 mice were treated with a TLR4 antagonist during the pancreatic ductal perfusion and digestion steps of the isolation process. Isolated islets were then analyzed for inflammatory markers by RT-PCR, western blot, immunofluorescent staining, and functionality in vitro. A syngeneic transplant model using a marginal mass of islets transplanted intraportally into mice with streptozotocin (STZ)-induced diabetes was performed to determine transplant outcomes after early TLR4 blockade.

Results: Diabetic mice receiving 150 islets treated with early TLR4 blockade achieved euglycemia at a higher rate than mice receiving untreated islets (75% vs 29%; $p < 0.05$). Serum markers for islet damage and inflammation were significantly reduced post-transplant. Both the expression of key inflammatory genes and the activation of mitogen-activated protein kinases (MAPKs) were reduced by early TLR4 blockade

Conclusions: Early TLR4 blockade protects islets from sterile inflammation-mediated stress sustained during isolation and promotes positive transplant outcomes. Our findings support the use of early TLR4 blockade during clinical islet isolation procedures to reduce pre-transplant inflammation and improve transplant outcomes.

Introduction

Pancreatic islet cell transplantation is an important treatment option for brittle type 1 diabetes and as an adjunct procedure after total pancreatectomy to prevent brittle type 3c diabetes [194,195]. Research into multiple facets of islet transplantation, such as anti-inflammatory regimens, has significantly improved islet transplant outcomes [6,196]. Yet, despite improvements made in the past two decades, early graft loss and sustained islet function remain a challenge [197].

Evidence suggests that islet transplant outcomes are compromised by inflammation sustained prior to transplantation [23]. The organ procurement process subjects tissues to sterile stressors and damage, which may promote acute rejection post-transplant, but also provides a potential window for therapeutic interventions prior to transplantation which remains largely unexplored [190,198]. The process of isolating pure islets generates an environment of sterile inflammation mediated by ischemia, hypoxia, enzymatic and mechanical digestion, oxidative stress, and the release of endogenous damage-associated molecular patterns (DAMPs) from stressed and damaged cells [199,200]. Therefore, reducing sterile inflammation pre-transplant may be an important step for improving transplant outcomes.

While the upregulation and activation of inflammatory markers in tissues is commonly observed after organ procurement and islet isolation, the mechanisms by which this inflammation occurs remain understudied. Previously, studies highlighted the roles of mitogen-activated protein kinases (MAPKs) and the NFkB pathway in upregulated stress responses in islets during and after isolation [201-203]. Targeting these pathways has demonstrated protection of islets against apoptosis post-transplant [204-206]. However, these treatments require sustained exposure to agents that inhibit vital signaling pathways and are potentially toxic to islet physiology or require systemic administration of compounds with broader off-target toxic side effects, making them inappropriate for clinical use [207-209]. Thus, selectively targeting a nonvital innate inflammatory pathway with a clinically safe compound is necessary to avoid these complications and provide a rapidly translatable methodology.

Recent evidence has elucidated the role of Toll-like receptors (TLRs) in poor transplant outcomes [148,210]. Of particular interest is TLR4, the canonical receptor for Gram-negative bacterial lipopolysaccharide. Activation of TLR4 triggers a robust inflammatory cascade that ultimately results in the production of inflammatory cytokines and possibly cell death [211]. Recently, TLR4 has been described as a promiscuous, noncanonical receptor for DAMPs and certain cytokines such as high mobility group box 1 (HMGB1) and C-X-C motif chemokine 10 (CXCL10) [212,213]. Moreover, TLR4 is observed to be highly upregulated in multiple organs by ischemia/reperfusion injury after transplant [21,154,214]. TLR4-deficient murine models and treatments targeting TLR4 and its endogenous ligands have shown positive results in preventing aberrant inflammation and acute graft rejection [20,160]. Therefore, we hypothesized that TLR4 was complicit in mediating islet inflammation

during the isolation process and that early blockade could reduce this inflammation and improve transplant outcomes.

In the present study, we investigated the therapeutic potential of early TLR4 blockade in the islet isolation process to inhibit sterile inflammation sustained during and after isolation. We used a syngeneic murine model to determine whether a single early treatment is sufficient to significantly improve transplant outcomes. Then we examined the expression of key proteins and cytokines known to compromise transplant outcomes, as well as elucidated the downstream signaling pathways differentially activated by early TLR4 blockade. For this study, TAK-242, a small molecular inhibitor of TLR4, was identified as an ideal candidate due to its immunity to enzymatic degradation and its safety as demonstrated by a phase II clinical trial [22,163].

Materials and Methods

Mice

Male wild-type C57BL/6 mice 6-7 weeks old were purchased from Envigo (Houston, TX) and housed at the Institute of Metabolic Disease at Baylor University Medical Center for at least 5 days prior to use. Animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor Scott & White Research Institute.

Islet Isolation

Islets were isolated from C57BL/6 mice using a previously described method with minor modifications [215]. Briefly, pancreases were perfused with collagenase type V (1 mg/mL; Sigma-Aldrich, St. Louis, MO) or Liberase TL (0.125 mg/mL; Roche Diagnostics, Indianapolis, IN, USA) containing either 0.01% DMSO or 3 μM TAK-242

(MedChemExpress, Monmouth Junction, NJ) via common bile duct injection, digested at 37°C for 18-20 minutes, and then purified via discontinuous density gradient. After isolation, islets were either used immediately or cultured in RPMI supplemented with 1% penicillin/streptomycin, 10 mM HEPES, and 1% BSA (Sigma-Aldrich) at 37°C and 5% CO2.

Islet Transplantation

Recipient mice were made diabetic by a single intra-peritoneal injection of streptozotocin (STZ; 200mg/kg; Sigma-Aldrich) 5-7 days prior to transplant. Diabetic mice are defined as having a nonfasting blood glucose >400 mg/dL for two consecutive days. Immediately after isolation, 150 Islets were transplanted intraportally via a 27 G winged infusion set into diabetic recipient mice under general anesthesia with isoflurane. After transplant, mice are considered cured on the first day of two consecutive blood glucose measurements <200 mg/dL. Blood glucose was measured with a Breeze 2 Blood Glucose Meter (Bayer HealthCare LLC, Mishawaka, IN, USA) by tail-vein prick. Mouse body weight was measured concurrently with blood glucose.

Intraperitoneal Glucose Tolerance Test

Mice were fasted for 6 hours by placing them in fresh cages with no food but access to water ad libitum. Then, a 2 mg/kg bolus of glucose was injected intraperitoneally into the mice as a 20% w/v glucose solution. Blood glucose was measure before injection (time 0) and then every 30 minutes for 150 minutes by tail-vein prick.

Serum Biomarker Analysis

Immediately after isolation with or without TAK-242, 200 islets were transplanted into the kidney subcapsular space of STZ-induced diabetic mice. Serum was isolated from ~200μL of blood taken from mice via the tail vein prior to transplant, 4 hours post-transplant, then days 1, 2, 3, and 7. Serum samples were frozen at -80°C after collection until analysis.

miRNA was isolated from 50μL serum using the miRCURY RNA Isolation Kit – Biofluids (Exiqon Inc, Woburn, MA, USA) according to manufacturer instructions with optional DNAse treatment, converted to cDNA with the miRCURY LNA Universal RT microRNA PCR kit with UniSp6 RNA for loading control according to manufacturer instructions (Exiqon). cDNA was diluted 1:40 in nuclease-free water and assayed by real-time PCR using ExiLENT SYBR Green master mix with miRCURY LNA primers for hsa-miR-375 and UniSp6 (Exiqon) on a Bio-rad CFX Connect (Hercules, California, USA) with the following program: 95°C 10 min, then 40 cycles of 95°C, 10 s; 60°C, 1 min. Relative expression was calculated using the 2^-ΔΔCT method normalized to UniSp6.

Serum cytokines for IL-6, CXCL1, and CXCL10 was measured by multiplex analysis with a Milliplex MCYTOMAG-70K kit (EMD Millipore Corporation, Billerica, MA, USA) according to manufacturer instructions with undiluted serum. Samples were incubated overnight at 4°C with shaking and analyzed on a Luminex 200 (Luminex Corporation, Austin, TX, USA).

Islet qRT-PCR Analysis

Total RNA was isolated from samples using TRIzol (Invivogen, San Diego, CA) and converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied

Biosystems, Waltham, MA) following manufacturer instructions. Quantitative expression of genes of interest was determined using SsoAdvanced Universal SYBR Green Supermix (Bio-rad) on a Bio-Rad CFX Connect with the following program: 95°C, 10 minutes; 40 cycles of 95°C, 15 seconds; and then 60°C, 1 minute. Primers for qRT-PCR analysis were purchased from commercially available stock (Integrated DNA Technologies, Coralville, IA). Relative gene expression was calculated using the 2^-ΔΔCT method normalized to 18S mRNA.

Western Blotting

At different time points, islets from the different groups were rinsed 2x with ice-cold DPBS supplemented 1:100 with Halt Protease and Phosphatase Inhibitor (Halt) Cocktail (ThermoScientific, Rockford, Il, USA) lysed in RIPA buffer (ThermoScientific) supplemented 1:50 with Halt by sonication with a Bioruptor (Diagenode, Denville, NJ, USA) for 4 rounds of: 15 s ON, 1 min OFF, on low power at 4°C. After lysis, 4x Laemmli sample buffer (Bio-Rad) supplemented with 0.25% 2-mercaptoethanol was added to the samples and immediately boiled at 95°C for 5 min or frozen at -20°C. Samples were then loaded into and resolved on 12% Tris-Glycine nUView gels (NuSep, Germantown, MD, USA) in a Mini-PROTEAN electrophoresis chamber (Bio-Rad) at 90V for 10 minutes, then 200V for 50 min. Proteins were transferred to 0.22µm PVDF membranes (azure biosystems, Dublin, CA, USA) via semi-dry transfer with a Trans-Blot SD (Bio-Rad) at 10V for 40 minutes using Schafer-Nielsen buffer with 10% methanol. After transfer, membranes are air-dried for at least 30 minutes prior to blocking with 5% BSA in TBST 0.1% for 1 hr at room temperature. Primary antibodies were incubated with the blot overnight at 4°C with gentle agitation, and secondary antibodies were incubated with the

blot for 1 hr at room temperature before ECL development with SuperSignal West Dura Extended Duration Substrate (ThermoScientific) according to manufacturer instructions with 3 x 5 min TBST washes between each step. After development, blots were stripped with Restore Western Blot Stripping Buffer (ThermoScientific) for 20 min at 37°C with gentle agitation, re-blocked with 5% BSA, then re-probed. Stripping efficacy was confirmed by developing the stripped blots with SuperSignal West Femto Maximum Sensitivity Substrate and imaging for 30-60s (ThermoScientific). All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at 1:1000 dilution unless otherwise stated. Antibodies used: β-actin (1:2000, #4970), phospho-ERK1/2 (1:2000, #4370), ERK1/2 (#9102), phospho-P38 (#4511), P38 (#9212), phospho-P65 (#3033), P65 (#8242), phospho-SAPK (#4668), SAPK (9252), α-Rabbit IgG HRP-linked (1:2000, #7074). Band density was measure using Fiji (http://fiji.sc/) [216]. Relative densities were calculated and compared using the ([phospho]:[actin])/([total]:[actin]) method to normalize relative activation across membranes.

Islet Viability Assessment

Whole islets are stained with 1μg/mL Hoechst 33342 and propidium iodide (PI) for 20 minutes at room temperature, rinsed 3 x 5 min with DPBS, then mounted on slides with cover slips before imaging on an Olympus FSX100. Viability was determined by counting the total number of both Hoechst 33342- and PI-positive cells using Fiji (http://fiji.sc/) and calculating the percentage of PI-positive cells compared to Hoechst 33342-positive cells.

Glucose-Stimulated Insulin Secretion

Islets are incubated with low glucose (2mM) for 1 hour to equilibrate the islets, low glucose again for 1 hr, then 1 hr in high (20mM) glucose solution in KRBH + 0.2% BSA at 37°C. Media samples were collected immediately after islet incubation and frozen at -80°C or analyzed immediately for insulin content measured using a mouse insulin ELISA kit (ALPCO Diagnostics, Salem, NH, USA) according to manufacturer instructions. Stimulation index is defined by the concentration of insulin in high glucose solution divided by insulin concentration in low glucose solution after equilibration.

Statistical Analysis

All data were presented as means ± SEM. Survival was compared by log-rank (Mantel-Cox) test. Single pairwise comparisons were performed using a two-tailed unpaired t test with Welch's correction. Multiple t test with Holm-Sidak's post hoc test was used for multiple comparisons. Statistical analysis was performed in GraphPad Prism 6 (San Diego, CA, USA). Graphs were made in either GraphPad Prism 6 or Microsoft Office 2016 (Redmond, WA, USA). Statistical significance was achieved when $p < 0.05$.

Results

Early TLR4 Blockade Promotes Successful Intraportal Islet Transplantation Outcomes

To determine whether early blockade of TLR4 affects islet transplant outcomes, we used a syngeneic transplant model. A marginal dose of 150 islets for intraportal transplant was chosen based on previous islet dose titrations [20]. Islets were isolated from male C57BL/6 mice with enzyme containing 0.01% DMSO (control group) or with enzyme containing 3 μM TAK-242 (TAK group). Immediately after isolation, islets were

transplanted intraportally into STZ-induced diabetic mice and followed for 8 weeks (Figures 4.1A, 4.2A,B). Mice receiving TAK islets had superior transplant outcomes, with 75% (6/8) achieving euglycemia, compared with 29% (2/7) of those receiving control islets (Figure 4.1B; $P < 0.05$). The time to euglycemia was also significantly reduced by early TLR4 blockade, with the TAK group having a mean time to euglycemia of 21.2 ± 3.7 days compared to the control group with 35.0 ± 1.0 days (Figure 4.1C; $P < 0.05$).

Long-term function of the islet grafts in vivo was assessed by IPGTT on day 45 post-transplant (Figure 4.1D). Area-under-the-curve analysis revealed superior islet function in mice from the TAK group compared to the control group (Figure 4.1E; $p < 0.05$). Lastly, since insulin deficiency hinders weight gain, the mean body weight of both groups over the monitoring period was compared, and mice in the TAK group gained significantly more weight than mice in the control group (Figure 4.1F; $P < 0.0001$). The results demonstrate that simple early TLR4 blockade during islet isolation is sufficient to significantly improve transplant outcomes.

Serum Markers of Islet Stress are Reduced Post-Transplant by Early TLR4 Blockade To examine islet damage post-transplant between the TAK and control groups, we analyzed serum for MiR-375 and proinflammatory cytokines. 200 islets isolated with (TAK, $n = 3$) or without (control, $n = 3$) early TLR4 blockade were transplanted into the kidney subcapsular space of diabetic mice. Serum was collected from recipient mice pre-transplant and at 0.15, 1, 2, 3, and 7 days post-transplant for analysis. We detected significantly higher levels of serum MiR-375 in control animals (20.6 ± 7.4-fold) compared to TAK animals (3.0 ± 0.6-fold) only at 0.15 days post-transplant ($P < 0.0001$), but not on days 1, 2, 3, or 7 (Figure 4.3A).

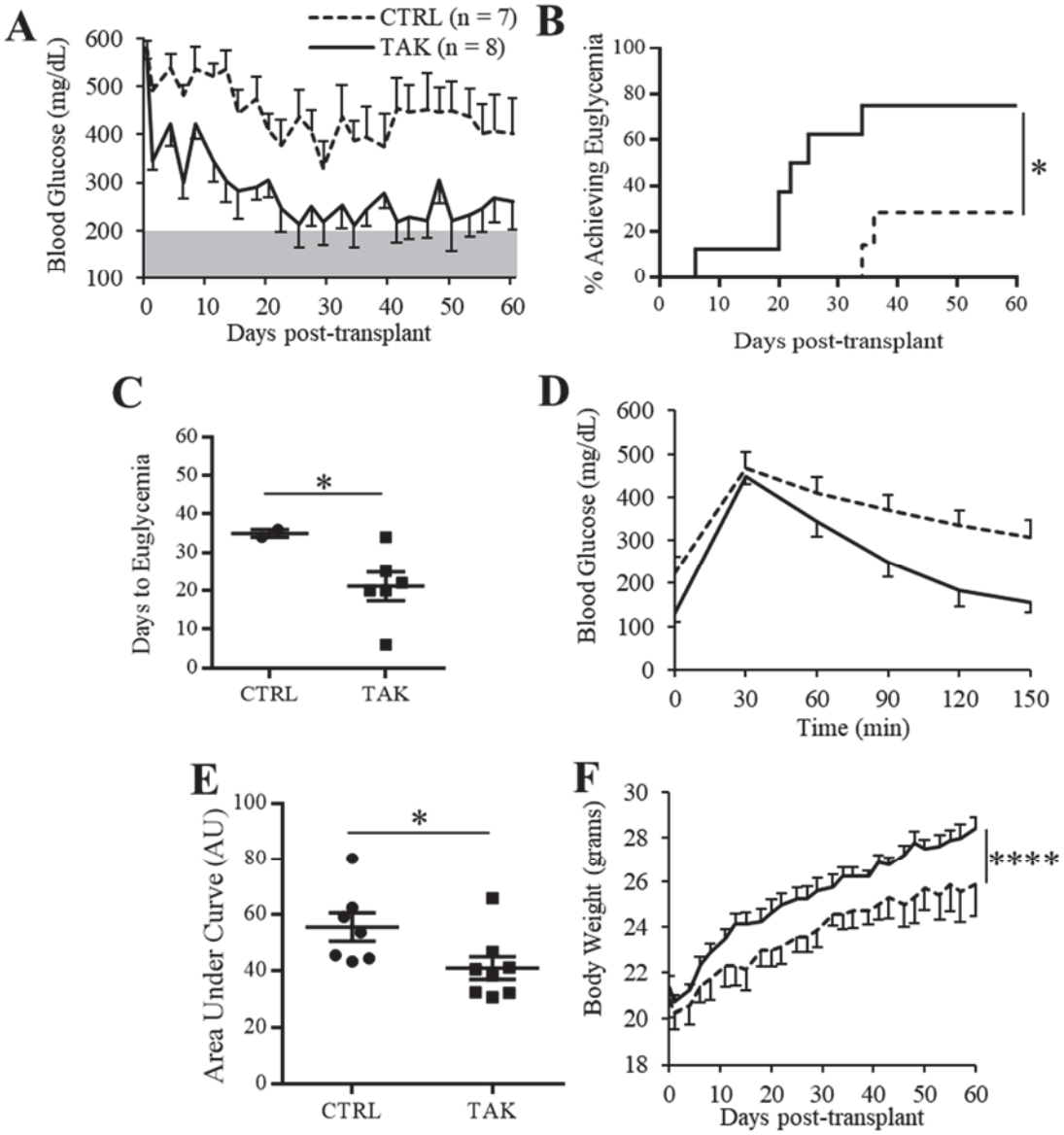

Figure 4.1**.** Islet transplant outcomes. (**A**) 150 islets isolated with early TLR4 blockade (TAK; solid line) transplanted intraportally into STZ-induced diabetic mice resulted in reduced non-fasting blood glucose compared to control islets treated with 0.01% DMSO (CTRL; dashed line)**. (B)** The TAK group achieved a euglycemia rate of 75% (6/8) compared to 29% (2/7) in the control group, with **(C)** significantly reduced time to euglycemia. **(D, E)** Additionally, the TAK group had superior long-term glycemic control determined by IPGTT on day 45 and **(F)** overall gained more weight than the control group. *P* values determined by log-rank (Mantel-Cox), unpaired *t* test, or two-way ANOVA. *$p < 0.05$, ****$p < 0.0001$.

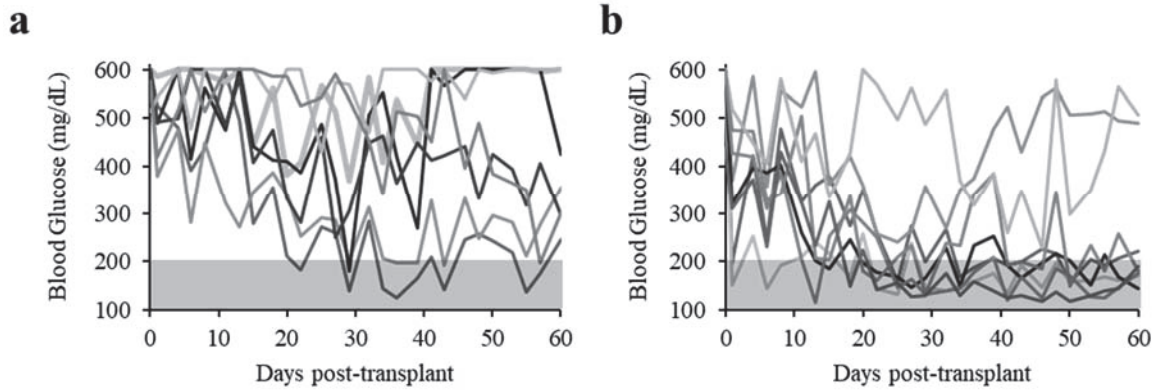

Figure 4.2. Blood glucose graphs of individual islet recipients. Non-fasting blood glucose from diabetic recipients receiving either islets isolated with untreated enzyme (CTRL; A) or early TLR4 blockade (TAK; B) was measured three times per week for 2 months. Grey box denotes the euglycemic threshold.

Serum Markers of Islet Stress are Reduced Post-Transplant by Early TLR4 Blockade

To examine islet damage post-transplant between the TAK and control groups, we analyzed serum for MiR-375 and proinflammatory cytokines. 200 islets isolated with (TAK, n = 3) or without (control, n = 3) early TLR4 blockade were transplanted into the kidney subcapsular space of diabetic mice. Serum was collected from recipient mice pre-transplant and at 0.15, 1, 2, 3, and 7 days post-transplant for analysis. We detected significantly higher levels of serum MiR-375 in control animals (20.6 ± 7.4-fold) compared to TAK animals (3.0 ± 0.6-fold) only at 0.15 days post-transplant ($P < 0.0001$), but not on days 1, 2, 3, or 7 (Figure 4.3A).

Next, we analyzed the sera for the pro-inflammatory cytokine IL-6, neutrophil chemoattractant CXCL1, and the chemokine CXCL10. Serum concentrations of both IL-6 and CXCL1 were significantly higher in control animals than in TAK animals at 1-day post-transplant (Figure 4.3B,C; both $P < 0.05$). We also saw a nonstatistically significant increase in the level of IL-6 ($P = 0.51$) and CXCL1 ($P = 0.21$) at day 3 post-transplant in control islets. Serum levels of these two cytokines remained largely unchanged in TAK

animals throughout the sampling period. Levels of serum CXCL10 were significantly higher in control animals at day 0.15 post-transplant ($P < 0.01$) than in TAK animals (Figure 4.3D). Graft-bearing kidneys were excised on day 7 for histological analysis. We observed reduced intragraft edema and necrosis in the TAK group, but no differences in macrophage infiltration between the groups (Figure 4.4). Overall, these data suggest that a significant amount of islet damage normally observed after transplantation is inhibited by early TLR4 blockade, and that treatment ameliorates systemic inflammatory cytokine reactions.

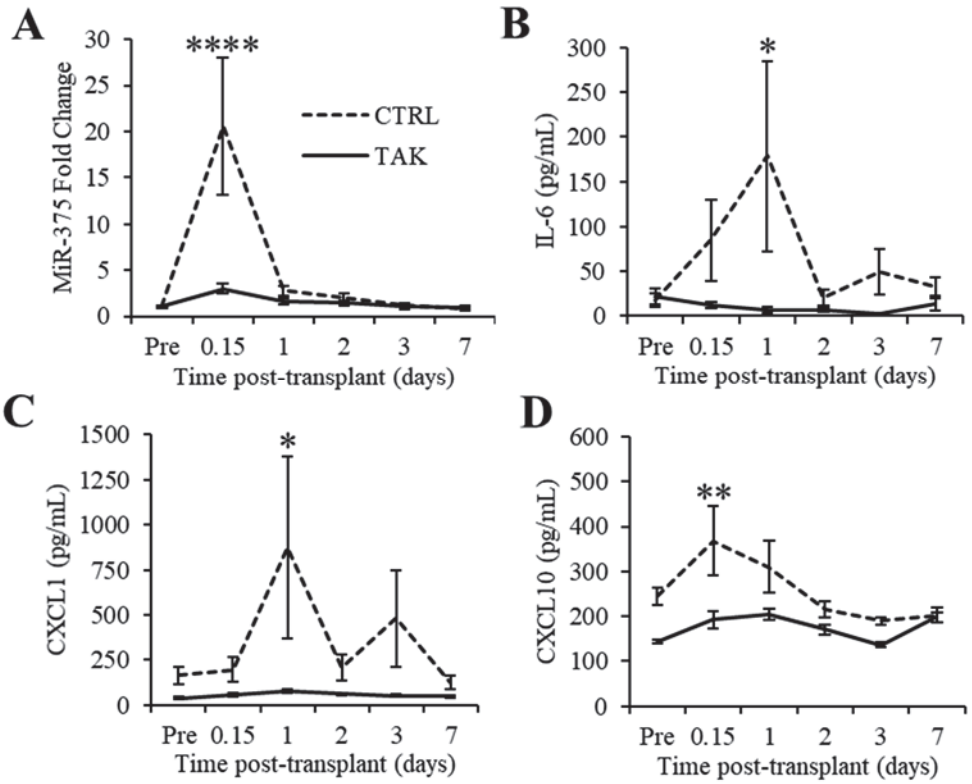

Figure 4.3. Serum markers of islet damage and inflammation post-transplant. **(A)** MiR-375 analysis of serum from islet recipients showed elevated islet damage 0.15 days post-transplant in the control group (dashed line), which was significantly reduced in the TAK group (solid line). Similarly, multiplex analysis was performed for serum **(B)** IL-6, **(C)** CXCL1, and **(D)** CXCL10 post-transplant, and these cytokines were significantly upregulated in control recipients immediately post-transplant but remained low in the TAK group. *P* values determined by multiple *t* test with Holm-Sidak's post hoc test. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$.

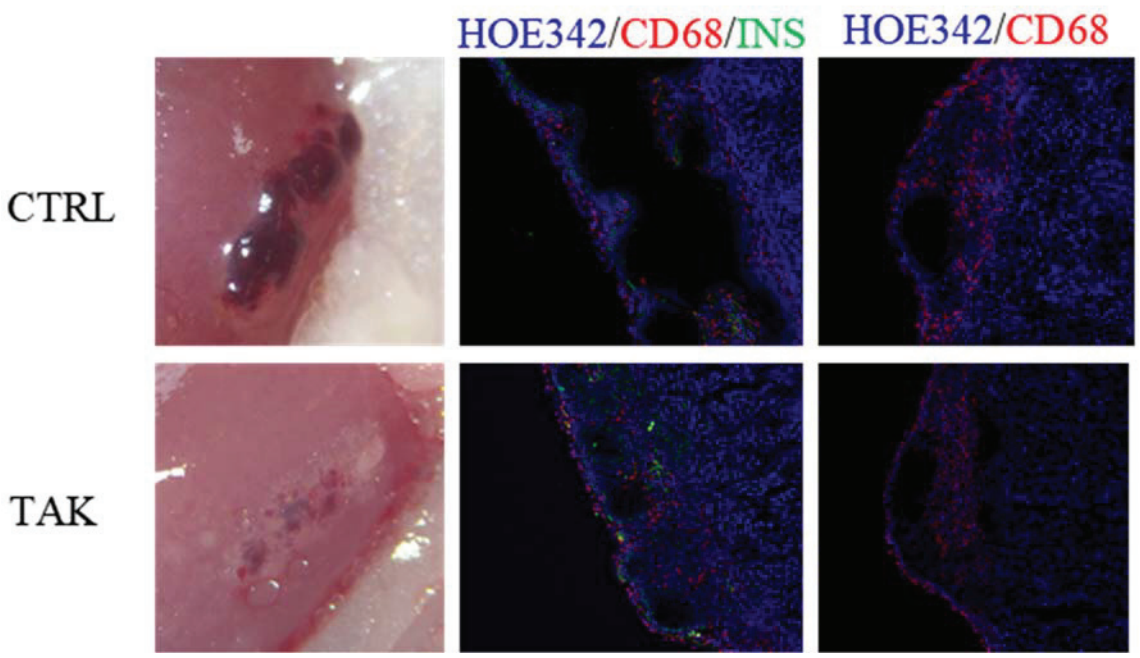

Figure 4.4. Islet kidney graft histological analysis. Islets isolated with untreated enzyme (CTRL) or TAK-242-treated enzyme (TAK) were transplanted into the subcapsular space of diabetic recipients and recovered on day 7 post-transplant. Grafts were frozen in OCT, sectioned into 8µm sections, and stained for insulin and/or CD68. Nuclei were stained with Hoechst 33342. The grafts from TAK mic displayed less edema than CTRL mice, but comparable levels of macrophage infiltration.

TLR4 Blockade During Islet Isolation Reduces Expression of Key Inflammatory Proteins

To examine the immediate post-isolation effects of early TLR4 blockade, we examined the expression of key pro-inflammatory genes known to compromise graft function and transplant outcomes (Figure 4.5A-F). Islets isolated with or without early TLR4 blockade were cultured for 4 hours post-isolation in serum-free media to provide sufficient time to upregulate mRNA expression. In TAK islets, tissue factor upregulation was reduced by ~52% (Figure 4.5A; $P < 0.05$), CXCL10 by ~65% (Figure 4.5B; $P < 0.01$), ICAM-1 by ~60% (Figure 4.5C; $P < 0.01$), CCL2 by ~79% (Figure 4.5D; $P < 0.01$), TLR4 by ~47% (Figure 4.5E; $P = 0.13$), and IL-6 by ~63% (Figure 4.5F; $P = 0.08$).

We also examined the expression of IL-1β, TNF-α, and IFN-γ at 4 and 24 hours post-isolation in control and TAK islets. Relative expression of islets that had been cultured for 48 hours were included for baseline reference. TAK islets had reduced IL-1β expression at 4 hours (~-75%, $P < 0.05$) and 24 hours (~-50%, $P = 0.15$) after isolation (Figure 4.5G). TNF-α expression was slightly increased at both timepoints in both groups compared to cultured islets, but there were no statistically significant differences among the groups (Figure 4.5H). IFN-γ expression was below the detection threshold in all groups. Genes encoding the inflammasome proteins PYCARD, NLRP3, and Caspase-3 were also examined, but observed no significant upregulation or differences (Figure 4.6A-C). We also observed that early TLR4 blockade indeed inhibited inflammation mediated exclusively during, not after, the isolation process as determined by a significant reduction of IL-6 expression in islets treated with TAK-242 during and after islet isolation compared to islets treated only after isolation (Figure 4.7). These data demonstrate that early TLR4 blockade effectively reduces the upregulation of many pro-inflammatory genes mediated by sterile inflammation which are detrimental to transplant outcomes.

The Protective Effects of Early TLR4 Blockade are Independent of Enzyme Blend

The enzyme primarily used in this study (collagenase type V) is commonly used in research to isolate islets from murine pancreases. However, its composition differs from clinically used enzyme blends. Therefore, to investigate if the reduction in inflammation post-isolation would be similarly observed using an enzyme blend related to clinical formulations, islets were isolated as described above except with Liberase enzyme from Roche instead of collagenase type V. After isolation, the islets were analyzed for expression of CCL2 and CXCL10. CCL2 upregulation was reduced by ~66% (Figure 4.8A;

P = 0.15), and CXCL10 was reduced by ~92% (Figure 4.8B; P < 0.05). These data demonstrate that the reduction of inflammation by early TLR4 blockade is independent of enzyme blend.

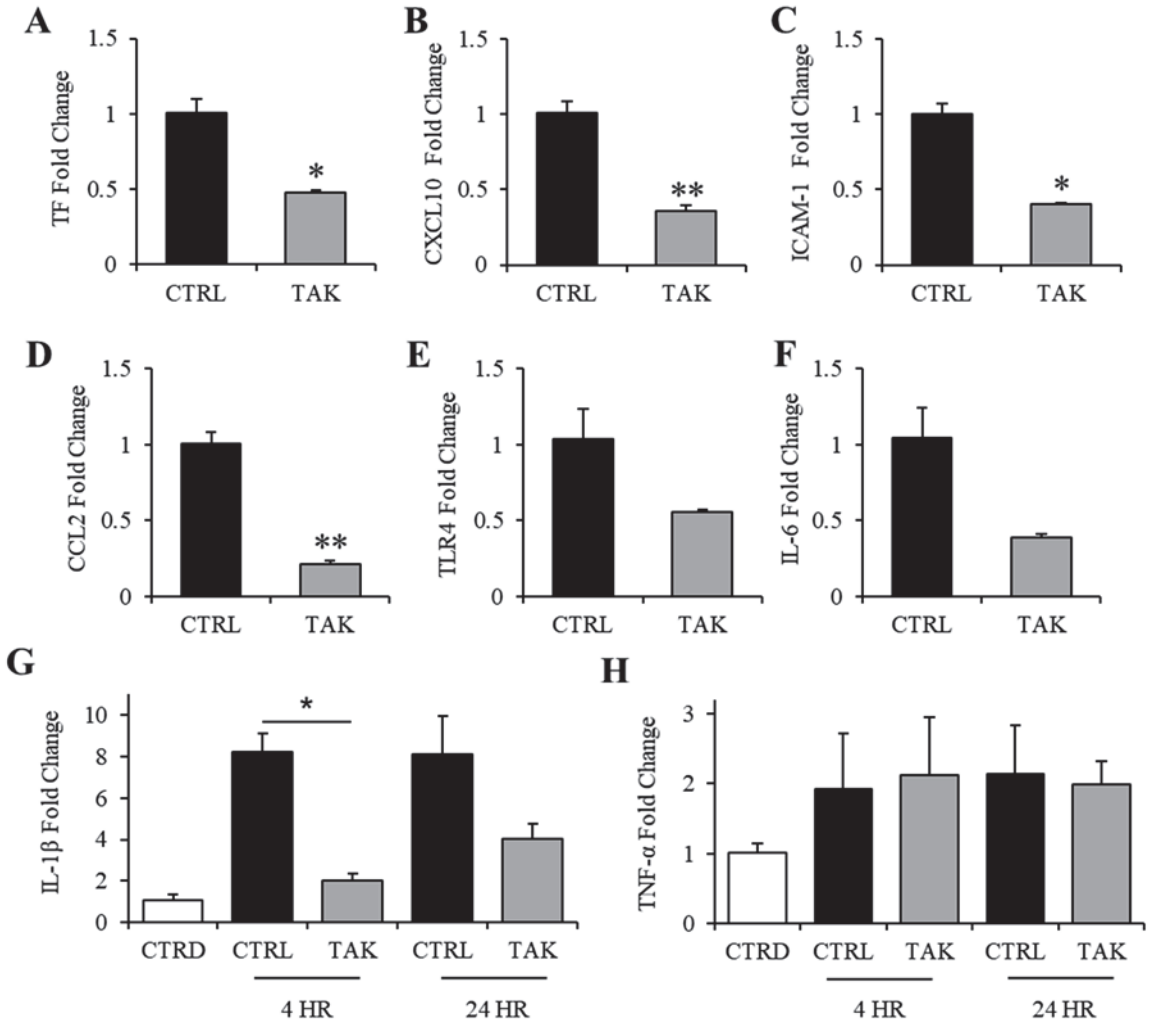

Figure 4.5. Inflammatory gene expression analysis after islet isolation. Analysis of (A) tissue factor, (B) CXCL10, (C) ICAM-1, and (D) CCL2 revealed significantly reduced upregulation with TLR4 inhibition during isolation. Numerical, but not statistically significant, reductions were observed in expression of (E) TLR4 (P = 0.13) and (F) IL-6 (P = 0.08). (G) IL-1β and (H) TNF-α were similarly analyzed at 4- and 24-hours post-isolation and compared with 48-hour cultured islets (CTRD). IL-1β upregulation was inhibited by TLR4 inhibition during isolation at 4 hours (P < 0.05) and 24 hours (P = 0.15). No statistically significant differences were detected in TNF-α expression. P values determined by unpaired t test. n = 3/group in 2 experiments. *P < 0.05; **P < 0.01.

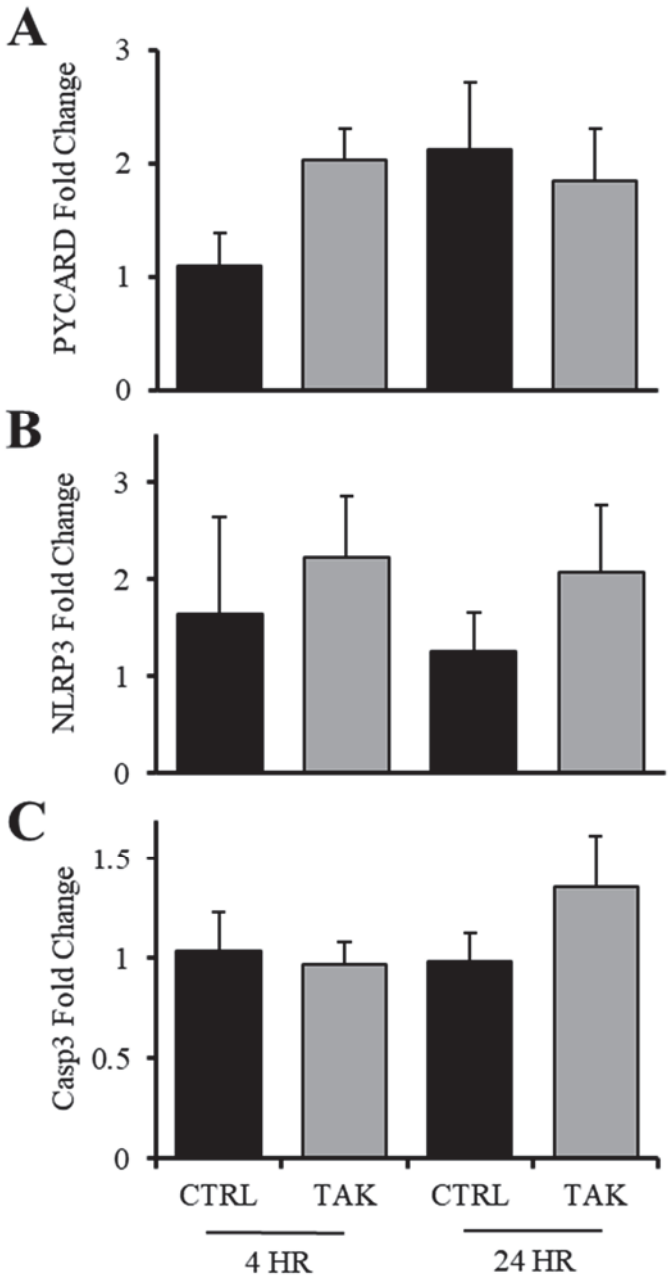

Figure 4.6. Gene expression levels of inflammasome proteins. 4- and 24-hours post-isolation, mRNA from islets isolated with untreated enzyme (CTRL) or TAK-242-treated enzyme (TAK) was recovered for analysis of the major proteins of the inflammasome pathway, including PYCARD (A), NLRP3 (B), and caspase-3 (C). No statistically significant differences were detected among the groups as determined by unpaired t test. n = 3/group.

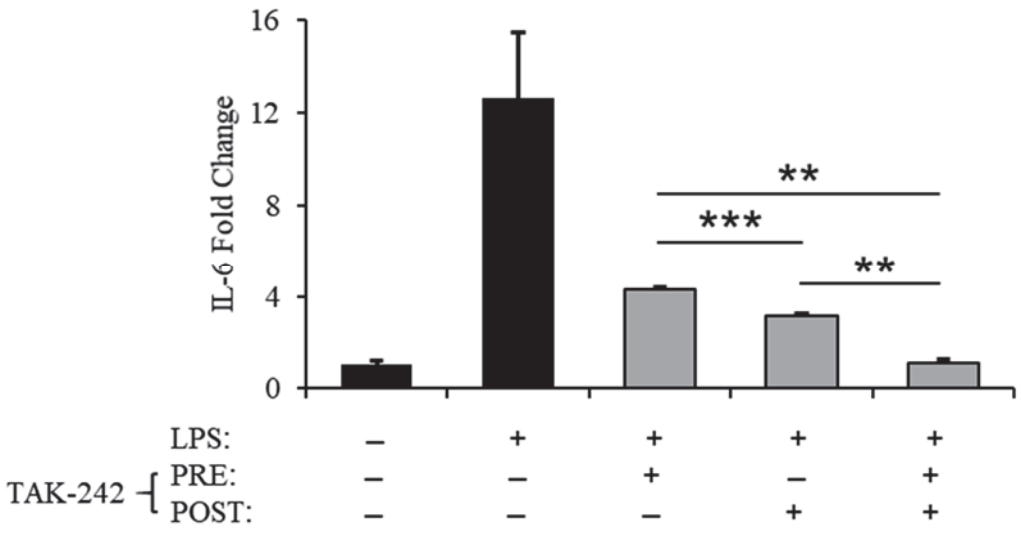

Figure 4.7. Proinflammatory gene expression in islets treated with TAK-242 before or after isolation. Islets were isolated with untreated enzyme or TAK-242-treated enzyme then further treated with or without TAK-242 before challenge with LPS. n = 3/group. **p < 0.01, ***P < 0.001, determined by unpaired t test.

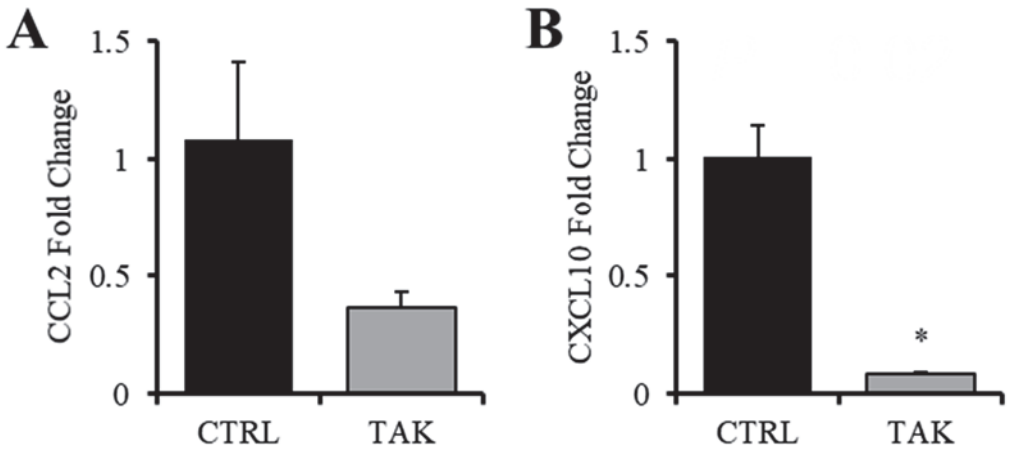

Figure 4.8. Proinflammatory chemokine expression in islets isolated with Liberase TL. Islets were isolated with Liberase TL with (TAK) or without (CTRL) TAK-242. mRNA was isolated 4 hours post-isolation and analyzed for the expression of major chemokines CCL2 (A) and CXCL10 (B). CCLs was numerically, but not statistically lower by TAK treatment (p = 0.15), however CXCL10 was significantly downregulated by TAK treatment (*p = 0.02). Statistical significance was determined by unpaired t test. n = 3/group.

Early TLR4 Blockade Reduces MAPK Activation in Islets

The TLR4/MyD88 signaling axis activates the MAPK and NFkB pathways [217]. To investigate if these pathways are upregulated following islet isolation, and to see if early TLR4 blockade differentially inhibits the activation of these pathways, we performed western blot analysis on islets isolated with or without early TLR4 blockade. Islets cultured for 2 days were used for baseline reference. Immediately after isolation, the activation of P65 and MAPK family proteins ERK1/2, P38, and SAPK were significantly upregulated compared to baseline (Figure 4.7). Activation of ERK1/2 was moderately inhibited in early TLR4 blockade islets compared to control islets at time 0 and remained below control activation levels for 24 hours. Both groups increased slightly at 6 hours, then rapidly became dephosphorylated by 24 hours (Figure 4.7A,B). Relatively equal levels of P38 activation were observed in both groups immediately after isolation; however, P38 activation in control islets increased at 6 hours post-isolation, but decreased in the early TLR4 blockade group (Figure 4.7C,D). SAPK activation began trending downward after isolation in both groups but was slightly reduced by early TLR4 blockade immediately post-isolation. By 6 hours, SAPK was near undetectable levels in both groups (Figure 4.7E,F). We saw no major differences in P65 activation between both groups at any time point (Figure 4.7G,H). These observations support the reduction of inflammatory gene expression seen in Figure 3 and suggest that this reduction is primarily due to reduced MAPK activation during and after isolation.

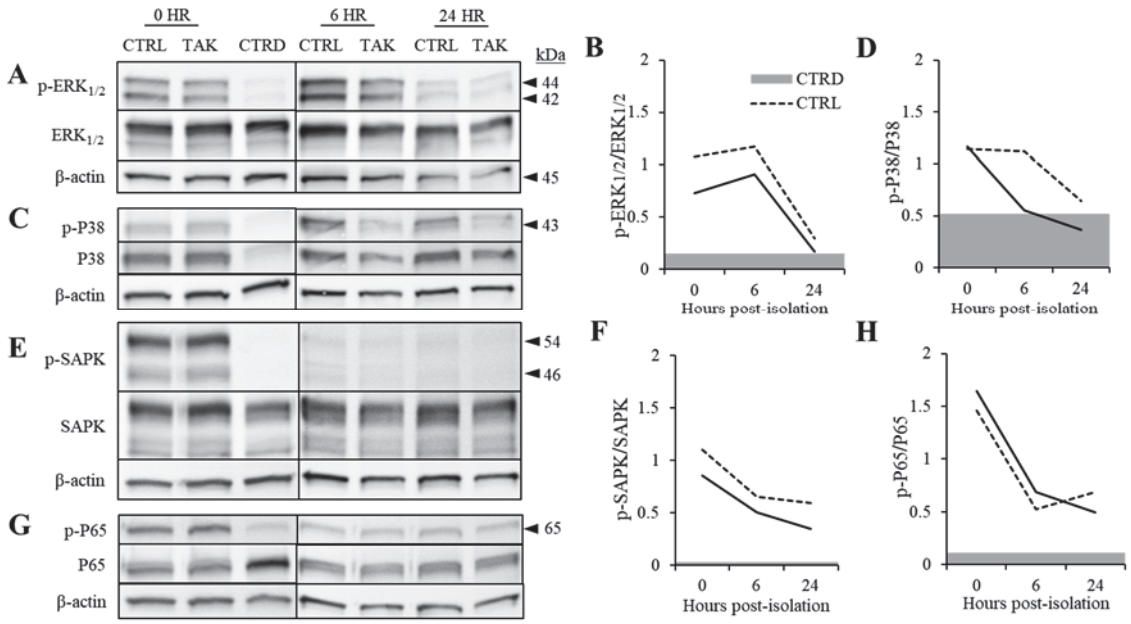

Figure 4.9. Semiquantitative western blot analysis of MAPKs and P65. Whole-islet lysates were prepared from islets with (TAK) or without (CTRL) early TLR4 blockade immediately after isolation (0 hours) and at 6- and 24-hours post-isolation. Lysates were immunoblotted for (A) ERK1/2, (C) P38, (E) SAPK, and (G) P65, and analyzed for relative activation (phospho:total) normalized to β-actin. (B, D, F, H) The relative activation in CTRL islets (dashed line), TAK islets (solid line), and cultured islets (grey area) is graphed.

TLR4 Blockade Rescues Islet Viability Immediately After Isolation

To examine if early TLR4 blockade has any effects on islet viability after isolation, we examined viability immediately and on days 1, 2, 3, and 7 post-isolation by Hoechst 33342/propidium iodide staining (Figure 4.10A). Pancreases were kept on ice for at least 30 minutes prior to digestion to increase cold ischemia time. Immediately after isolation, we observed a significant reduction in viability in the control islets compared to TAK islets ($P < 0.05$), but no significant differences on days 1, 2, 3, or 7 (Figure 4.10B). We saw no toxic effects of acute TAK-242 treatment, but to determine if long-term treatment impaired viability, we cultured islets in media containing 3 μM or 0.3 μM TAK-242 or 0.01% DMSO.

Finally, we examined if early TLR4 blockade had any impact on islet function. A glucose-stimulated insulin secretion assay was performed on islets isolated with or without early TLR4 blockade at 4 hours (Figure 4.10C,D) and 24 hours (Figure 4.10E,F) post-isolation. In low (2 mM) glucose at 4 hours, we saw higher basal insulin secretion in TAK islets than in control islets (7.87 ± 0.19 vs 5.86 ± 0.15 pg/mL, respectively; $P \leq 0.01$) but similar insulin levels in high (20 mM) glucose (27.32 ± 1.81 vs 28.66 ± 0.36 pg/mL, respectively; $P = 0.54$), resulting in a technically lower stimulation index (3.46 ± 0.16 vs 4.90 ± 0.17; $p < 0.01$). No significant differences were measured at 24 hours for insulin secretion or stimulation index (Figure 4.10E,F), but the stimulation index for TAK was slightly higher than that of control (4.64 ± 0.44 vs 3.74 ± 0.20, respectively; $P = 0.17$).

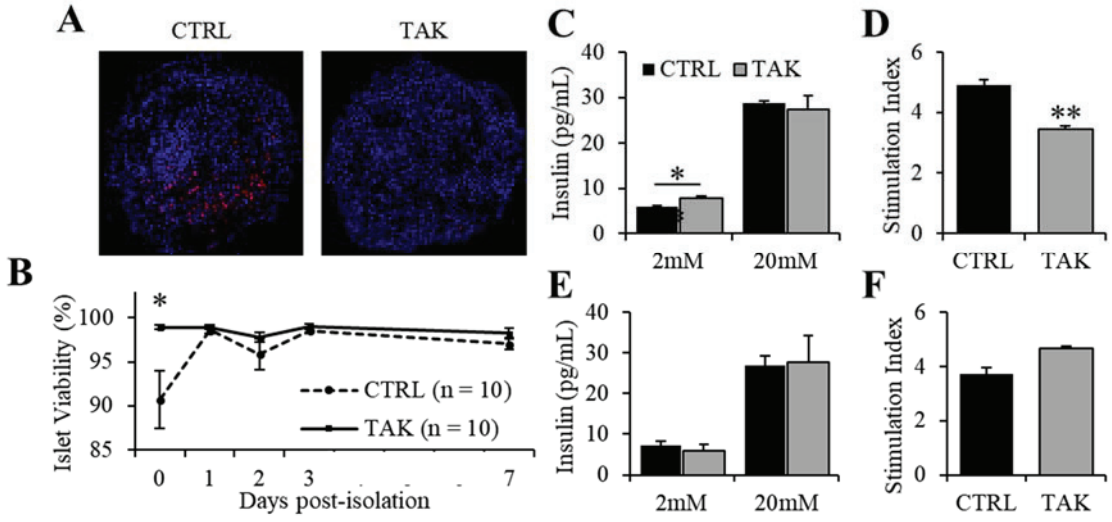

Figure 4.10. Assessment of islet viability and function. (A) Representative islet viability images of CTRL and TAK islets immediately post-isolation. (B) After isolation, CTRL (dashed line) and TAK (solid line) islets were cultured and stained for viability with Hoechst 33342/propidium iodide on the indicated days (n = 10 islets per group per timepoint). At time 0, CTRL islets had significantly reduced islet viability compared to TAK islets ($P < 0.05$). Islet functionality of CTRL islets (black bar) and TAK islets (grey bar) was determined with glucose-stimulated insulin secretion at (C, D) 4 hours and (E, F) 24 hours post-isolation (n = 10 islets per group per timepoint in triplicate). Statistical significance was determined by multiple t test with Holm-Sidak post hoc test (B) or unpaired t test (C-F). *$P < 0.05$; **$P < 0.01$.

Discussion

Evidence suggests, and we have shown here, that prior to transplant, islets are significantly stressed and inflamed due to the organ procurement and islet isolation procedure which may prime islets for dysfunction and apoptosis post-transplant [199,201,202,204]. Therefore, early intervention targeting nonvital innate inflammatory pathways, such as TLR4, is an attractive therapeutic option. Indeed, in our syngeneic intraportal transplant model, we found that early TLR4 blockade is sufficient to significantly improve transplant outcomes.

For our study, we added the TLR4 inhibitor TAK-242 directly to the collagenase used to isolate islets. A concentration of 3 μM for this study, based on prior experience with this compound [215], but optimizing the dosage should be a priority for future studies and clinical usage. This compound is attractive for our study since it is immune to degradation by proteases and has been clinically tested for safety [163]. We did not observe any inhibition of enzymatic activity or islet yield by the addition of TAK-242 during the isolation process. Including TAK-242 in the preservation solution and wash buffers may further increase the efficiency and protective effects of early TLR4 blockade. Additionally, incorporating this compound into perfusion solutions for solid-organ machine perfusion may also be a simple treatment option with profound effects.

Post-transplant, islets are damaged and inflamed, which can be detected and measured in patient serum. We used MiR-375, a beta-cell–specific microRNA, as an islet damage biomarker [218,219]. Early blockade of TLR4 significantly inhibited serum MiR-375 levels post-transplant. Additionally, key inflammatory proteins (IL-6, CXCL1, CXCL10)

were also inhibited. These findings suggest that more of the islet mass is preserved with treatment and that the post-transplant inflammatory response is ameliorated.

The instant blood-mediated inflammatory reaction is a well-studied phenomenon in intraportal cell transplantation and is posited to be influenced by TLR signaling [220,221]. Tissue factor is one of the key mediators of instant blood-mediated inflammatory reaction, triggering thrombin formation. In our analysis, the expression of tissue factor in islets was significantly reduced by early TLR4 blockade, as were other key mediators of inflammation and innate immune responses (CXCL10, ICAM-1, CCL2, TLR4, IL-6, IL-1β). Our group demonstrated that CXCL10 is one of the key mediators of islet graft failure [222]. CCL2 has also been investigated as a major chemokine implicit in early graft failure [223]. The reduction in TLR4 is welcome, since this receptor is commonly upregulated after organ transplant and may act in a positive feedback mechanism to induce further inflammation [150]. The significant reduction in IL-1β expression by early TLR4 blockade was not unexpected, since TLR4 activation is known to upregulate this cytokine [224]. We observed no reduction in TNF-α expression, which partially explains the lack of P65 inhibition observed in our blots, since this cytokine could be functioning in an autocrine/paracrine fashion to activate NFkB [225]. Expression of IFN-γ was not detected, suggesting the MyD88-independent IRF3 pathway does not participate in the early phases of sterile inflammation.

We detected relatively high levels of endotoxin (~220 EU/mL) in collagenase type V at working concentrations. However, we still achieved similar reductions of islet inflammation even with low-endotoxin Liberase enzyme. Since collagenase preparations used for islet isolation are produced in Gram-positive Clostridia bacteria, we believe the

endotoxin found in the enzyme preparation is lipoteichoic acid, a TLR2/6 agonist. Moreover, lipoteichoic acid from Clostridia histolyticum has been found to be minimally immunogenic [226]. Thus, we do not believe this had a major impact on our findings.

Our analysis of intracellular signaling pathways supports previous work showing that the MAP kinases, as well as P65, are upregulated during and after isolation [23,199,201]. MAPK phosphorylation, which activate AP-1 family proteins c-Fos and c-Jun, was reduced by early TLR4 blockade, either immediately after isolation or after short culture, compared to untreated islets [227]. Our observations support previous work correlating MAPK activation in isolated islets with impaired viability [228]. Unexpectedly, no significant differences in P65 activity were observed, suggesting that other receptors, such as TNF receptors, may be the primary regulators of NFkB activation during islet isolation.

In conclusion, our findings demonstrate that early therapeutic intervention is a viable method for reducing tissue inflammation pre-transplant, and that TLR4 is a major mediator of sterile inflammation during the islet isolation process and post-transplant. Incorporation of TAK-242 to perfusion solutions and wash buffers may provide further benefits. The results of our study support the use of TAK-242 during the islet isolation process to reduce TLR4-mediated sterile inflammation and to improve islet transplant outcomes.

CHAPTER FIVE

Ex-Vivo Generation of Drug-Eluting Islets Improves Transplant Outcomes by Inhibiting TLR4-Mediated NFkB Upregulation

This chapter published as: Chang CA, Akinbobuyi B, Quintana JM, Yoshimatsu G, Naziruddin B, Kane RR. Ex-vivo generation of drug-eluting islets improves transplant outcomes by inhibiting TLR4-Mediated NFkB upregulation. Biomaterials. 2018;159:13-24.

Abstract

The systemic administration of immunosuppressive and anti-inflammatory drugs is routinely employed in organ transplantation to minimize graft rejection and improve graft survival. Localized drug delivery has the potential to improve transplant outcomes by providing sustained exposure to efficacious drug concentrations while avoiding systemic immunosuppression and off-target effects. Here, we describe the synthesis of a novel prodrug and its direct covalent conjugation to pancreatic islets via a cleavable linker. Post-transplant, linker hydrolysis results in the release of a potent anti-inflammatory antagonist TLR4, localized to the site of implantation. This covalent islet modification significantly reduces the time and the minimal effective dose of islets necessary to achieve normoglycemia in a murine transplantation model. In streptozotocin-induced diabetic C57BL/6 mice a syngeneic transplant of ~100 modified islets achieved a 100% cure rate by the end of a 4-week monitoring period, compared to a 0% cure rate for untreated control islets. Overall, this direct prodrug conjugation to islets is well tolerated and preserves their functionality while affording significantly superior transplant outcomes. The development

of drug-eluting tissues that deliver sustained and localized doses of small-molecule therapeutics represents a novel pathway for enhancing success in transplantation.

Introduction

Organ and cellular transplantation is often a necessary life-saving treatment option for patients suffering from a wide range of diseases and disorders resulting in impaired organ function and failure. The systemic administration of immunosuppression and anti-inflammatory drugs is used routinely to prevent acute graft loss and promote successful engraftment. However, the efficacy of these drugs is compromised due to the sub-optimal drug trough concentrations in order to minimize the risk of opportunistic infections [229], nephrotoxicity [230], and other undesired off-target effects [231,232]. Thus, a localized drug delivery system is desirable to ameliorate off-target side effects and optimize drug delivery.

An archetypal model for transplantation is the transplantation of pancreatic islets of Langerhans for the treatment of brittle diabetes as a result of type 1 or type 3c diabetes mellitus. Brittle diabetics are characterized by having significantly impaired or nonexistent insulin production, problematic hypoglycemic unawareness, severe hypoglycemic events, and glycemic lability [60,233]. While most diabetics successfully manage their diabetes with exogenous insulin with a multiple daily injection regimen or insulin pumps, a sub-population fail to respond to existing insulin therapies and account for a significant proportion of diabetes-related morbidity and mortality [234,235], but have been shown to respond well to islet transplantation [236].

In islet transplantation, acute inflammation during the peri-transplant period was found to result in the loss of as much as 50-70% of transplanted islets [237] and contribute to the need for multiple donor pancreases to achieve insulin independence [6]. This

inflammation is mediated by sterile inflammation mechanisms such as innate immunity [14], ischemia/reperfusion injury (IRI), and hypoxia. Acute graft injury is not a problem isolated to islet transplantation, but manifests itself as primary graft dysfunction in lung, liver, heart, kidney, and pancreas transplantation [238-243], which occurs in a significant proportion (~20 to 30%) of organ transplants [244,245]. Several methods to protect islet grafts from inflammatory damage have been proposed, including treatment with anti-inflammatory agents [196,246,247], encapsulation [248], and islet surface modification [249,250]. There is a growing revelation to the importance of Toll-like Receptors (TLRs) in transplantation outcomes [148]. Specifically, TLR4 is identified as a major mediator or graft inflammation and dysfunction after organ transplantation [251], making it a putative therapeutic target for alleviating graft injury post-transplant.

Here, we report a chemical modification strategy and the resulting drug-eluting islets which release a potent anti-inflammatory TLR4-antagonist. This chemical modification utilizes bioorthogonal "click" chemistry, providing rapid reaction times and modularity, and is performed in physiologically compatible conditions. Our covalent modification significantly reduces the islet mass and time needed to achieve euglycemia in a streptozotocin (STZ)-induced diabetic mouse model. We use C57BL/6 mice due to their robust inflammatory foreign body response [252]. A syngeneic model was used to study the anti-inflammatory effects of our pro-drug on the innate inflammatory response independent of adaptive immunity [253]. Our results suggest that the chemical modification of islets with drug-eluting compounds has the potential for superior localized drug efficacy and improved transplant outcomes with minimal handling and invasiveness.

Materials and Methods

Study Design

The objective of this project was to examine the feasibility of modifying living tissue into carriers for drugs in order to increase the local dose of the drug to a therapeutic concentration while localizing the drug to the site of transplantation, thereby minimizing any undesirable off-target effects of systemic drug administration. Initially, we performed in vitro assays to test the effects of blocking TLR4 with TAK-242 in pancreatic islet inflammation using standard biological assays. Next, we tested the hypothesis that the chemistry used to create drug-eluting macromolecules could be successfully translated to living tissue with minimal invasiveness and toxicity. Finally, we used an in vivo transplantation model to see whether the application of this chemistry to transplanted living tissue would provide superior outcomes. Animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor Scott & White Research Institute.

MTT Viability Assay

Impact of TAK-242 on beta cell viability was assessed by co-culturing with MIN6 cells. MIN6 cells were seeded in a 96-well plate at 15,000 cells per well in RPMI. After overnight culture, culture media was replaced with media containing the indicated TAK-242 concentrations. The control (0 nM TAK-242) contained 0.1% DMSO. On the indicated days, media was removed from the plates and a 0.5 mg/mL solution of MTT (Sigma-Aldrich) in DPBS was added to the wells and incubated at 37°C for 4 h. After incubation, the MTT solution was removed and 100 µL of DMSO was added to the wells to dissolve the resulting

formazan crystals and absorbance read at 570 nm on a Cytation 5 (BioTek). Media was changed on day 3.

NFkB Reporter Cell Assay

HEK Blue hTLR4 NFkB SEAP reporter cells were purchased from Invitrogen and maintained in DMEM + 10% FBS with selections antibiotics. For experiments, cells were seeded at 5000 cells per well in a 96-well plate and cultured for 48 h prior to usage. Cells were pre-treated with the indicated compounds at the indicated concentrations for 10-30 minutes prior to LPS challenge (10 ng/mL) in HEK-Blue Detection media (Invitrogen). At the indicated times, the plates were read on a Cytation 5 plate reader for absorbance at 635 nm.

Synthesis of Compounds

Synthesis of TAK-PhSO2-Linker: Pyridine (27.3 μL, 0.34 mmol) was added dropwise to a stirred solution of 7-azido-1-(phenylsulfonyl)heptan-2-ol (45.5 mg, 0.15 mmol) and triphosgene (79 mg, 0.27 mmol) in 2.1 mL of anhydrous tetrahydrofuran. The resulting suspension was stirred for 20 min and filtered and concentrated to give the crude chloroformate as an oil. To the solution of the crude chloroformate in tetrahydrofuran (2.5 mL) was added ethyl 6-(N-(2-chloro-4-fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate (55 mg, 0.15 mmol) and triethylamine (38.4 μL, 0.28 mmol). The solution was stirred for 2 h at room temperature and diluted with ethyl acetate and washed with 1 M HCl, water, saturated sodium bicarbonate, and brine (5 mL each). The organic phase was dried over magnesium sulfate, concentrated, and subjected to flash chromatography using 25% ethyl acetate/hexanes. ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.3, 166.1, 163.5, 163.5, 161.5, 161.5, 151.4, 151.3, 148.1, 146.8, 139.1, 139.0, 137.0, 136.9, 135.8, 135.7, 134.2,

134.2, 133.8, 133.0, 133.0, 132.2, 132.1, 130.6, 130.6, 129.5, 129.5, 129.5, 129.4, 128.4, 128.3, 127.5, 124.0, 123.6, 118.1, 117.9, 117.6, 117.4, 115.3, 115.1, 114.9, 114.8, 71.8, 71.8, 61.3, 61.2, 59.4, 58.7, 58.6, 58.5, 51.2, 44.6, 33.9, 33.6, 28.6, 26.2, 26.1, 25.2, 24.8, 24.0, 23.9, 23.8, 23.4, 16.8, 16.1, 14.4, 14.3; HRMS (+ESI) calculated for $C_{29}H_{34}ClFN_4NaO_8S_2$ ($M+Na^+$) 707.1383 found 707.1383 (Δ 0.0 ppm).

Synthesis of BODIPY-PhSO$_2$-Linker: Pyridine (5.5 µL, 0.068 mmol) was added dropwise to a stirred solution of 7-azido-1-(phenylsulfonyl)heptan-2-ol (8.9 mg, 0.03 mmol) and triphosgene (17.4 mg, 0.06 mmol) in 0.4 mL of anhydrous tetrahydrofuran. The resulting suspension was stirred for 20 min and filtered and concentrated to give the crude chloroformate as an oil. To the solution of the crude chloroformate in tetrahydrofuran (2.5 mL) was added BODIPY TR Cadaverine hydrochloride (4.2 mg, 0.008 mmol) and triethylamine (2.5 µL, 0.018 mmol). The reaction was monitored by TLC (10% methanol/dichloromethane) and stirred for 2.5 h at room temperature. The solution was then diluted with ethyl acetate (10 mL) and washed with water (2 × 5 mL) and concentrated by the rotary evaporator. The crude material was purified by flash chromatography using 40% ethyl acetate/hexanes and then 4% to 5% methanol/dichloromethane to provide 2.2 mg of the product (34% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.13–8.08 (m, 1H), 7.99–7.95 (m, 2H), 7.94–7.89 (m, 2H), 7.66–7.62 (m, 1H), 7.57–7.52 (m, 2H), 7.47 (d, *J* = 6.0 Hz, 1H), 7.19 (s, 1H), 7.16–7.13 (m, 1H), 7.08 (dd, *J* = 12.2, 4.3 Hz, 2H), 7.04–6.99 (m, 2H), 6.82 (d, *J* = 4.3 Hz, 1H), 6.66 (d, *J* = 4.4 Hz, 1H), 5.12–5.05 (m, 1H), 4.56 (s, 2H), 4.44 (t, *J* = 6.0 Hz, 1H), 3.45–3.34 (m, 3H), 3.22 (t, *J* = 6.8 Hz, 2H), 3.05 (q, *J* = 6.8 Hz, 2H), 1.41–1.57 (m, 8H), 1.23–1.37 (m, 8H); HRMS (+ESI) calculated for $C_{40}H_{44}BF_2N_7NaO_6S_2^+$ ($M+Na^+$) 854.2748 found 854.2737.

Mouse Islet Isolation

Islets were isolated from 7- to 8-week-old male C57BL/6 mice (Envigo) by common-bile duct cannulation, using a 27 G needle, and pancreatic perfusion with Collagenase Type V (1 mg/mL; Sigma-Aldrich) in Hank's balanced salt solution (HBSS; Mediatech) followed by pancreatectomy and digestion in a water bath at 37°C for 20 min with periodic agitation by hand. Digested pancreatic tissue was washed twice with HBSS supplemented with 5 mM glucose, 20 mM HEPES, 0.5% bovine serum albumin, and 1% penicillin/streptomycin (Sigma-Aldrich) and filtered through a 600-micron mesh strainer. Islets were purified using a discontinuous Biocoll Separating Solution gradient (1.077 and 1.100 g/mL; Biochrom GmbH). Islets were hand-picked to >95% purity before culturing in Roswell Park Memorial Institute medium (RPMI) 1640 supplemented with 10% etal bovine serum and 1% penicillin/streptomycin solution (Sigma-Aldrich) at 37°C and 5% CO2. Islets were cultured overnight (12-18 h) prior to use in experiments.

TAK-242 Inhibition of Inflammation in vitro

After overnight culture, islets are washed in DPBS (Caisson Labs) before treatment with TAK-242 (3 μM, 30 min; MedchemExpress) and 2 μg/ml LPS-EB (Ultrapure from *Escherichia coli* O111:B4; Invivogen) for 24 h at 37°C and 5% CO_2. Islets were washed once with cold DPBS immediately after treatment and total RNA was isolated from samples using TRIzol (Invivogen) and converted to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems) following manufacturer protocols. Quantitative expression of genes of interest were determined using RT^2 SYBR Green qPCR master mix (Qiagen) on a Bio-Rad CFX Connect (Bio-Rad) with the following program: 95°C, 10 min; 40 cycles of (95°C, 15 s, 60°C, 1 min). Primers for qRT-PCR

analysis were purchased from commercially available stock from Integrated DNA Technologies. Relative gene expression was calculated using the 2-ΔΔCT method normalized to 18S mRNA.

Cytokines from assay culture media were analyzed using Milliiplex MCYTOMAG-70K Assay (EMD Millipore). Samples were prepared following manufacturer guidelines. Analysis was performed after overnight incubation at 4°C on a Luminex 200 (Luminex). Samples were assayed in triplicate and analyzed in triplicate for cDNA or duplicate for multiplex.

Islet Modification

Isolated islets from culture are rinsed once with Krebs-Ringer Bicarbonate Buffer (KRBH; pH 7.4) (Sigma-Aldrich) then incubated with 25 μM NHS-PEG4-DBCO (Sigma-Aldrich) in KRBH (pH 7.7) for 1 h at room temperature. Islets were rinsed once with KRBH (pH 7.4) to remove unreacted NHS-PEG4-DBCO then subsequently reacted with TAK-PhO_2-Linker (10-25 μM), TAK-Linker (10-25μM), or BODIPY-Linker (10 μM) for 1 h at room temperature. Islets are then washed twice with KRBH (pH 7.4) to generate TAK-PhO_2-Linker, TAK-Linker, or BODIPY-Linker modified islets. Modified islets are then cultured or used immediately for experiments.

Fluorescent Staining and Imaging

For fluorescent islet modification experiments, islets were surface modified as described above with 10μM carboxyrhodamine-110 azide (N_3-488) and nuclei stained with 1 μg/mL Hoechst 33342 (Sigma-Aldrich). For viability assays, islets were stained with 1 μg/mL Hoechst 33342 and 1 μg/mL propidium iodide (Sigma-Aldrich) in DPBS for 30

min at 37°C. Islets were washed twice with DPBS for 5 min before mounting on slides. Images were acquired with a fluorescent microscope (FSX100; Olympus) with exposure set to auto. ImageJ software (http://rsb.info.nih.giv/ij/) was used to count stained cells and measure relative fluorescent intensity (RFI). Fluorescent modification was measured as the ratio of the RFI of N_3-488 to Hoechst 33342 staining. Viability was calculated as the percentage of propidium iodide–positive cells out of total Hoechst 33342-positive cells per image.

Modified Islet Function Assays

Glucose-stimulated insulin secretion was assessed on days 1 and 2 postmodification. TAK-PhSO2-Linker modified and unmodified control islets (n = 10 per sample) were placed in a 8 μm cell strainer and then incubated with low glucose (1.67 mM) for 1 h to equilibrate the islets, low glucose again for 1 h, and then high (16.7 mM) glucose solution in Krebs-Ringer bicarbonate HEPES buffer (KRBH) + 0.2% bovine serum albumin at 37°C. Media samples were collected immediately after islet incubation and frozen at –30°C until analysis. Insulin content was measured with a mouse insulin enzyme-linked immunosorbent assay kit (ALPCO) in duplicate. The stimulation index was calculated as the concentration of insulin in high-glucose solution divided by the insulin concentration in low-glucose solution after equilibration.

To measure intracellular Ca2+ signaling, TAK-PhSO2-Linker modified and control islets were dissociated with Accutase (Innovative Cell Technologies) postmodification and seeded in a 96-well optical-bottom plate (Nunc). After overnight culture, islet cells were incubated with 2μM fura-2AM (Thermo Fisher) in low glucose (2 mM) KRBH for 1 h at 37°C. After fura-2AM incubation, well contents were dumped out and rinsed 2 x 5 min

with fresh low glucose KRBH. After the final wash, 20 µL of low glucose buffer was added to the cells. A basal 340 nm/380–508 nm excitation-emission ratio was measured in the cells for 2 min every 5 sec with a Cytation 5 (Biotek). At 2 min, a high-glucose (40 mM) solution was injected into the wells to bring the final glucose concentration to 20 mM. The 340 nm/380–508 nm excitation-emission ratio was continuously monitored for another 6 min. The 340/380 emission ratio was normalized to the basal mean ratio.

Islet Transplantation

Recipient male C57BL/6 mice 6-7 weeks old were made diabetic by a single intra-peritoneal injection of STZ (200 mg/kg; Sigma-Aldrich). Diabetic mice are defined as having a non-fasting blood glucose >400 mg/dL for two consecutive days. Prior to transplantation, islets were untreated, pre-treated with 3 µM TAK-242, or modified with TAK-PhSO_2-Linker as described above. Diabetic C57BL/6 mice are anesthetized under isofluorane and a field between the rib cage and pelvis on the left side was disinfected by 1% providone-iodine scrub and 70% ethanol. A minimal incision (~8 to 10 mm) into the peritoneum is made to expose the kidney and a small cut (~2 mm) is made in the kidney capsule using a 30 G needle. Then a flame-blunted glass pipette tip is inserted into the kidney capsule through the cut to create a pocket between the renal cortex and capsule. Islets are then injected into the pocket via polyethylene tubing (PE-50) connected to a 1mL syringed filled with RPMI 1640 driven by a NE-300 syringe pump (New Era Pump Systems). At the completion of islet infusion, the incision was closed with 4-0 violet monofilament (Ethicon) via continuous nonlocking sutures. After closing, mice receive a 0.1mg/kg bolus of buprenorphine subcutaneously near the incision. After transplant, blood glucose from mice were measured 5 times per week with a Breeze 2 Glucose Monitor

(Bayer). mice are considered cured on the first day of 2 consecutive glucose measurements <200 mg/dL.

Intraperitoneal Glucose Tolerance Test

IPGTT was conducted on day 30 post–islet transplant to assess graft function. Mice were placed in fresh cages with access to water ad libitum but no food and fasted for 6 h. Then, 2 g/kg of glucose in a 20% solution was administered to the mice intraperitoneally, mimicking a postprandial glucose bolus. Blood glucose was checked prior to glucose administration at time 0 and then at 30, 60, 90, 120, and 150 min following administration. AUC response in blood glucose was then calculated between each group.

Statistical Analysis

All data are represented as the means ± SEM. Single pairwise comparisons were performed using the two-tailed Student's *t* test in Microsoft Office Excel 2016. One-way ANOVA with Tukey's test or Newman-Keuls test was performed for multiple comparisons and Kaplan-Meier survival function curves were compared using the Mantel-Cox log rank method with Graphpad Prism 7.0 for Windows. Statistical significance is defined as $P < 0.05$. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Results

Identification of a Safe and Potent Small Molecule TLR4 Antagonist

Several potent TLR4 antagonists have been described in the literature, including Lipid A mimetics CRX-526 and E5564 [254,255], and the arylsulfonamide TAK-242 [256] (Figure 5.1A). We chose to work with TAK-242 because of the sulfonamide motif, which

is a safe and common motif if medicinal and prodrug chemistry [257,258], the potent TLR4 inhibition (IC_{50} values of 1 to 30 nM) in both human and murine models [22], and its clinical safety profile as demonstrated in human clinical trials (NCT00633477) [163]. Critical for our purposes, the sulfonamide motif in TAK-242 offers a potential reactive group for chemical modification into a covalently-linked prodrug [258]. In our preliminary testing, TAK-242 showed negligible toxicity to beta cells at effective concentrations. MIN6 cells cultured with increasing concentrations of TAK-242 displayed no major reductions in viability at 1, 3, or 7 d (Figure 5.1B).

TAK-242 functions by covalently binding to Cys747 in the intracellular TIR domain of TLR4 and inhibiting the adapter proteins TIRAP and TRAM from associating with the receptor [259]. Blockade of this pathway inhibits the inflammatory TLR4-MyD88-NFkB axis. A NFkB reporter cell line expressing human TLR4 was used to demonstrate that TAK-242 inhibited TLR4-mediated inflammation in a dose-dependent manner by inhibiting NFkB upregulation. A brief 30-min pre-treatment with increasing concentrations of TAK-242 (0 to 6000 nM) significantly reduced or completely inhibited NFkB upregulation in response to LPS challenge at 8 h (Figure 5.1C), while after 22 h, only TAK-242 concentrations greater than 1000 nM were effective (Figure 5.1D).

To investigate whether TAK-242 protects islets from TLR4-mediated inflammation, islets from C57BL/6 mice were challenged with the canonical TLR4 ligand lipopolysaccharide (LPS). Islets were treated with TAK-242 before a 24-h challenge with a high dose of LPS (2µg/ml). After 24 h, culture media was analyzed by multiplex assay and real-time PCR analysis was performed on the islets. LPS-treated islets had significantly increased expression of both CXCL10 and CCL2, which was completely blocked by the

TAK-242 pre-treatment ($P < 0.05$) (Figures 5.2A and B). Similar results were observed when islets were challenged with high-mobility group box 1 (HMGB1; Figure 5.3), an endogenous damage-associated molecular pattern (DAMP) known to elicit acute inflammation through TLR4 [260]. Culture media was examined by multiplex analysis for IL-6, CXCL10, CXCL1, CCL2, and TNF-α. Islets treated with LPS alone showed significantly higher concentrations of these proinflammatory cytokines (Figures 5.2C-G). This is in contrast to the cytokine levels from islets that were pre-treated with TAK-242 before LPS challenge which showed significantly lower levels of these cytokines. The data here demonstrates both the sensitivity of islets to TLR-mediated inflammation, and the potency of TAK-242 as a TLR4-antagonist.

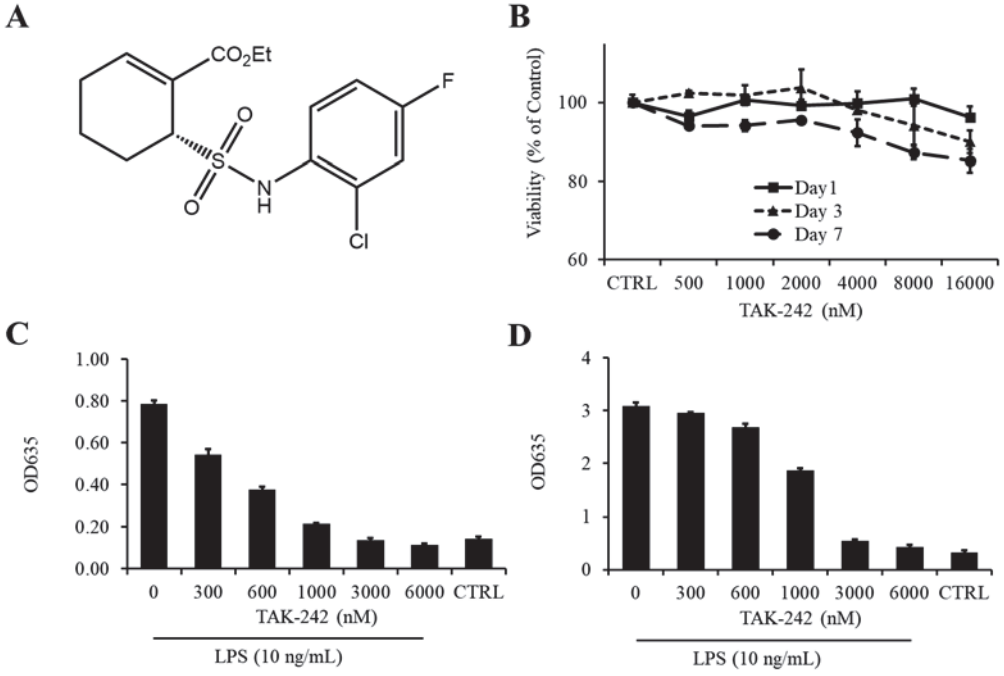

Figure 5.1. TAK-242 is non-toxic to beta cells and dose-dependently inhibits TLR4-mediated NFkB upregulation. (A) Chemical structure of the arylsulfonamide TAK-242. (B) TAK-242 showed negligible toxicity to the beta cells at effective concentrations. Data are represented as means ± SEM. $P > 0.05$ for all samples (one-way ANOVA with Tukey's multiple-comparisons test). (C) The efficacy of TAK-242 was assessed by pre-treating HEK Blue hTLR4 colorimetric reporter cells with increasing concentrations for 30 min before LPS challenge (10 ng/mL) for 8 h (C) and 22 h (D).

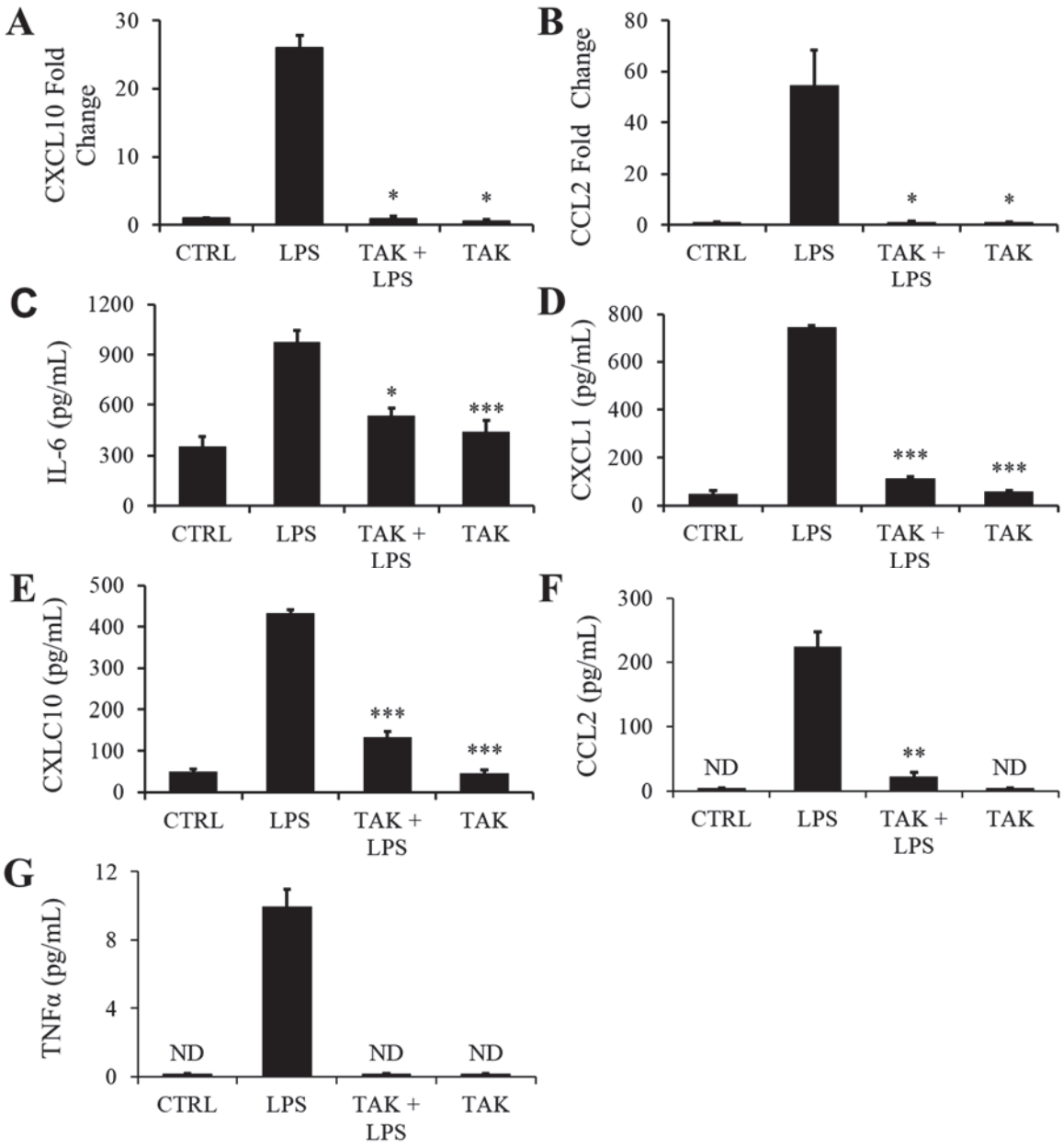

Figure 5.2. TAK-242 inhibits TLR4-mediated inflammation in murine pancreatic islets. (A and B) mRNA analysis of proinflammatory cytokines CXCL10 and CCL2 in mouse islets 24 h after 2 μg/mL LPS challenge with or without 3 μM TAK-242. (C-G) Luminex analysis of proinflammatory cytokines IL-6, CXCL1, CXCL10, CCL2, and TNF-α 24 h after LPS challenge with or without TAK-242. n = 3 per condition. (*P < 0.05, **P < 0.01, ***P < 0.001, two-tailed unpaired t-test). ND, not detected.

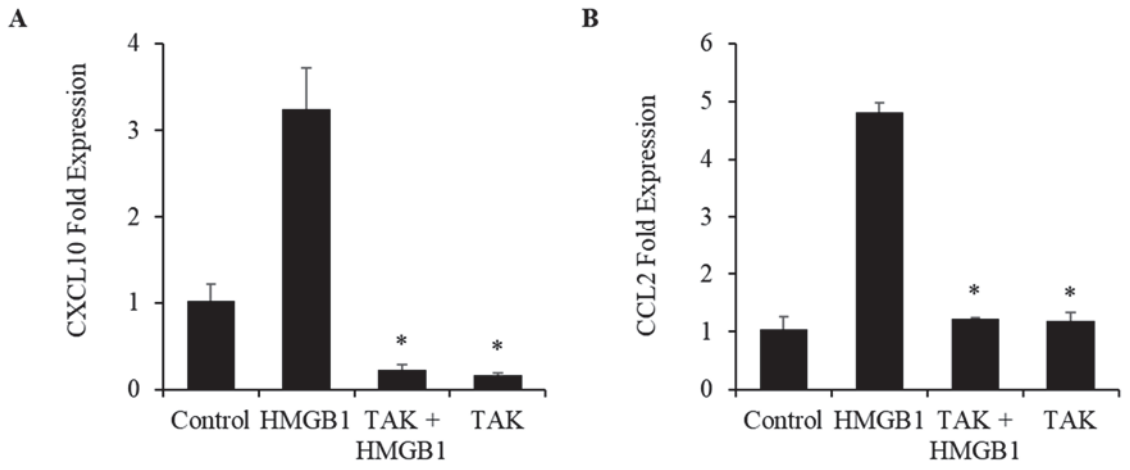

Figure 5.3. Protection of mouse islets against rHMGB1-mediated TLR4 inflammation with TAK-242. Islets from C57BL/6 mice were challenged with 1 μg/mL rHMGB1 with or without TAK-242 pretreatment. After 4 h, total RNA was isolated from the islets for gene expression analysis. The proinflammatory genes (A) CXCL10 and (B) CCL2 were examined in the samples. Data represented as means ± SEM. *P < 0.05 (n = 3 for all samples, unpaired two-tailed t test).

Design and Synthesis of TAK-242 Prodrug for Conjugation

Our approach for covalently attaching a TAK-242 prodrug to tissue surfaces for sustained release utilizes a recently reported azide-functionalized linker [184,261] and an azide/alkyne bioconjugation reaction [262,263]. Briefly, a linker alcohol was converted to its chlorocarbonate, which was directly reacted with the TAK-242 sulfonamide amine to afford the TAK-PhSO2-Linker prodrug in moderate yield (Figure 5.4A). A fluorescent BODIPY-PhSO2-Linker compound was similarly synthesized for use in quantification studies (Figure 5.4B). This linker design has been shown to hydrolytically release simple amines via a β-elimination reaction that is only modestly dependent on the basicity of the drug, independent of enzymatic cleavage mechanisms, and amenable to modulation by the selection of substituents (modulators) to adjust the pKa. We selected a phenyl sulphonyl substituted linker, with a reported $t_{1/2}$ of approximately 75 h for carbamates derived from simple aliphatic amines (pH 7.4, 37°), for these initial studies. While the nitrogen in TAK-

242 is an electron poor aryl sulfonamide, N-acyl sulfonamides have been described as enzyme-stable prodrug candidates [264], and it was anticipated that TAK-242 would be likely released from the conjugated TAK-PhSO2-Linker by β-elimination (Figure 5.4C).

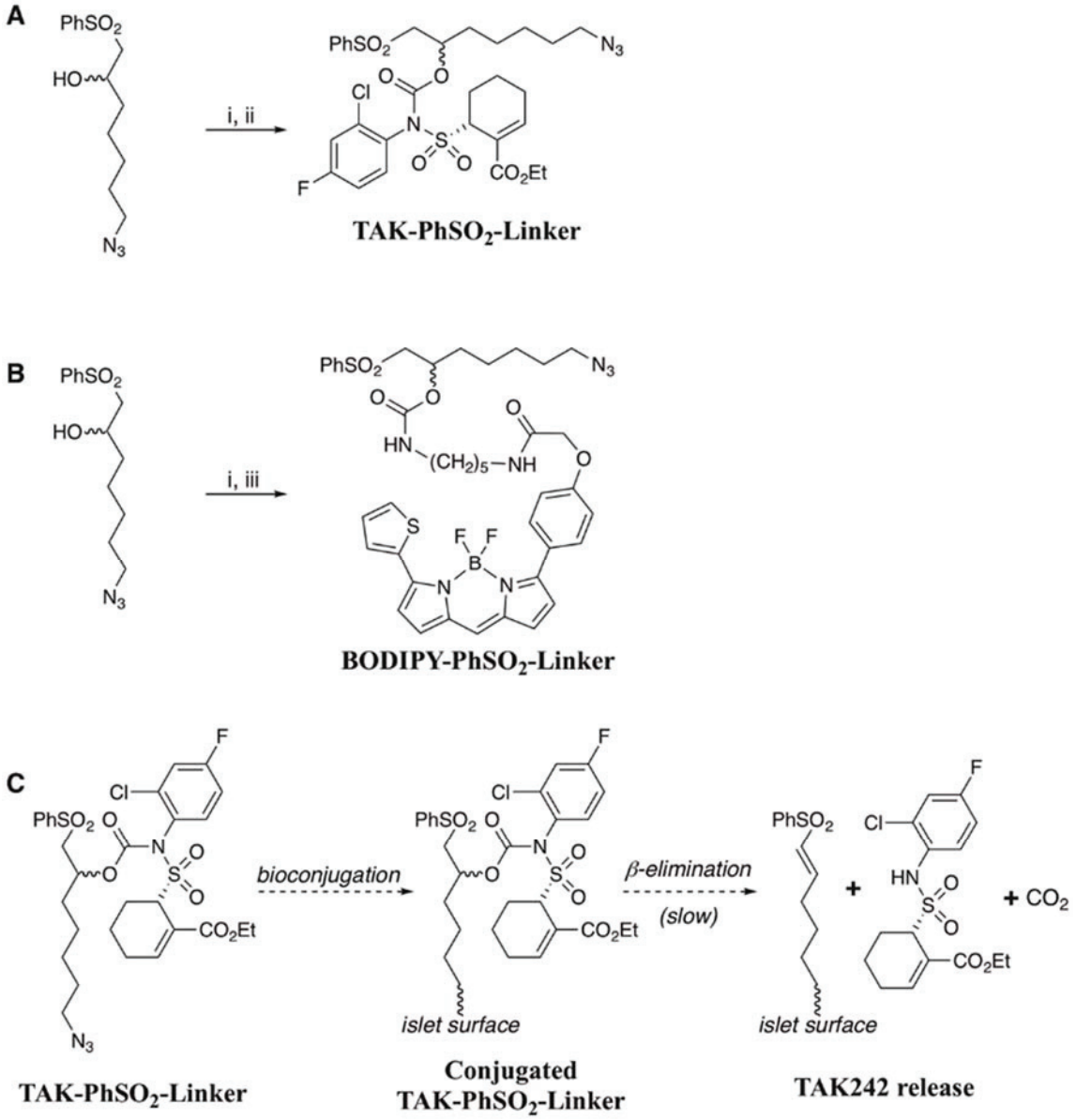

Figure 5.4. Cleavable prodrug chemistry. (A) Synthesis of TAK-PhSO2-Linker prodrug: (i) Triphosgene, pyridine, THF (ii) TAK-242, Et3N, CH2Cl2 (69% overall). (B) Synthesis of BODIPY-PhSO2-Linker: (i) Triphosgene, pyridine, THF (iii) BODIPY-TR-cadaverine, Et3N, THF (34% overall). (C) Proposed TAK-PhSO2-Linker conjugation to tissue surface and release of free TAK-242 by β-elimination.

Islet Conjugation Chemistry

Our group previously demonstrated that murine, porcine, and human islets are amenable to surface modifications using bioorthogonal conjugation chemistries [193]. The chemistry used in the present study to functionalize tissue surfaces, depicted in Figure 5.5A, is a two-step process that harnesses the bio-compatibility and modularity of copper-free "click" chemistry [265]. A commercially available bifunctional linker, the dibenzocyclooctyne-PEG4-N-hydroxysuccinimidiyl ester (NHS-PEG4-DBCO), is used to label reactive amines on surface of the islets with a strained alkyne, which we then react with our linker compounds to create a covalent linkage via strain-promoted azide-alkyne cycloaddition (SPAAC). To confirm the initial acylation reaction, we treated islets with NHS-PEG4-DBCO followed by carboxyrhodamine-110 azide (N_3-488) and observed significant surface labeling, while islets treated with N3-488 alone showed no labeling (Fig 5.5B). Confocal microscopy was used to confirm that the reactions occurred on the islet surface (Figure 5.5C).

Next, we used a competitive binding model to demonstrate that our compound, TAK-PhSO_2-Linker, reacts with alkyne-functionalized islet surfaces. In this experiment, we observed that incubating surface-functionalized islets with the TAK-PhSO_2-Linker prior to treatment with N_3-488 significantly reduced the fluorescent labeling ($P < 0.0001$; Figures 5.5D and E), demonstrating that our linker compounds successfully reacted with islet surface alkynes. We have also demonstrated that murine kidneys are also amenable to this conjugation chemistry (Figure 5.6), suggesting that the drug-eluting live tissue concept is readily translatable to different organs and tissue types.

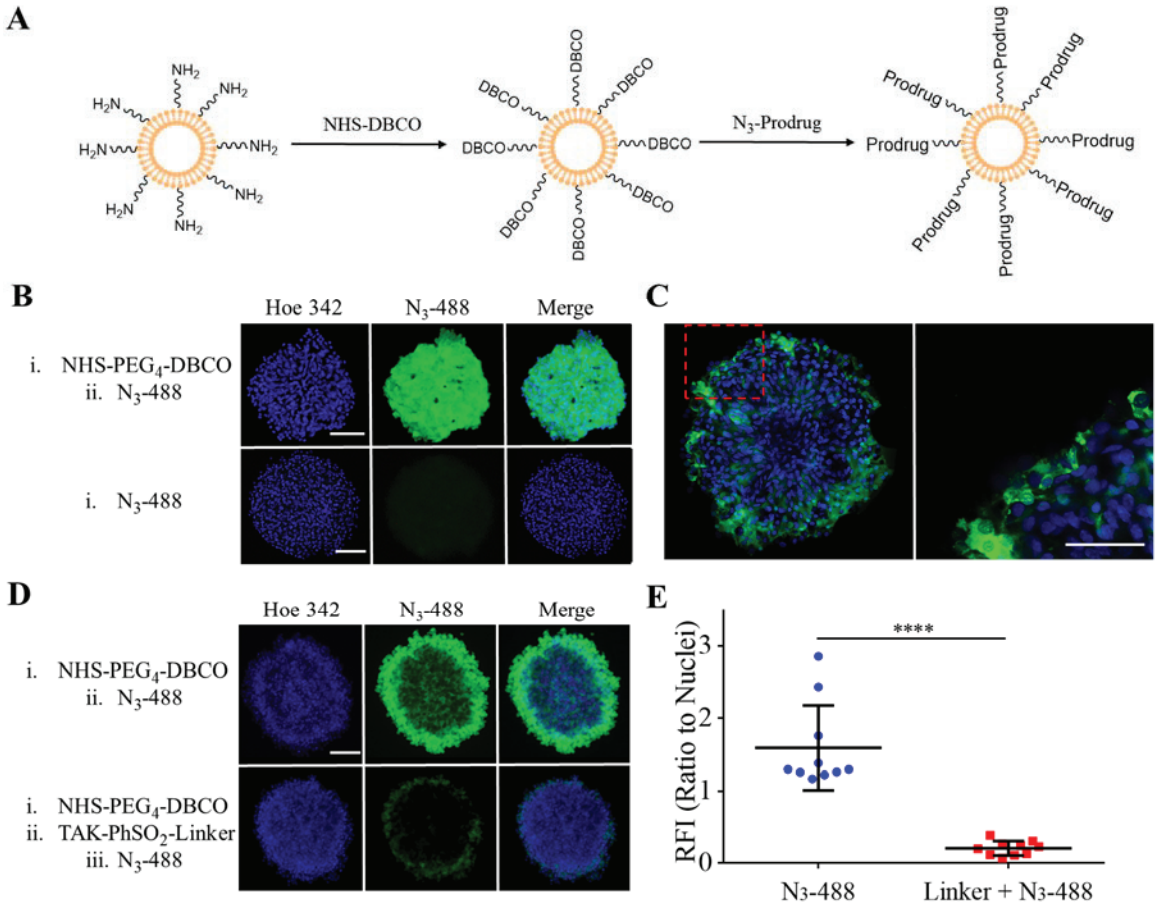

Figure 5.5. Verification of linker chemistry. (A) Scheme of functionalizing tissue surfaces using bioorthogonal "click" chemistry. NHS-PEG4-DBCO can be used to attach reactive alkynes (DBCO) by the reaction of N-hydroxysuccinimide (NHS) esters with primary amines on the tissue surface. This allows an azide-substituted prodrug to be covalently linked to the tissue surface via azide-alkyne cycloaddition reaction. (B) Representative fluorescent images of a surface-functionalized islet successfully labeled with a fluorescent dye via SPAAC compared to an islet that was not surface functionalized with NHS-PEG4-DBCO before staining with N3-488. Nuclei are stained with Hoechst 33342. (C) Confocal image of fluorescently modified islets showing the modification on the surface of the islets. (D) Representative fluorescent images of alkyne-functionalized islets reacting with N3-488 alone or with TAK-PhSO2-Linker followed with N3-488. (E) Quantification of fluorescent modification from both groups normalized to nuclei staining (n = 10 per group, ****P < 0.0001 two-tailed unpaired t-test).

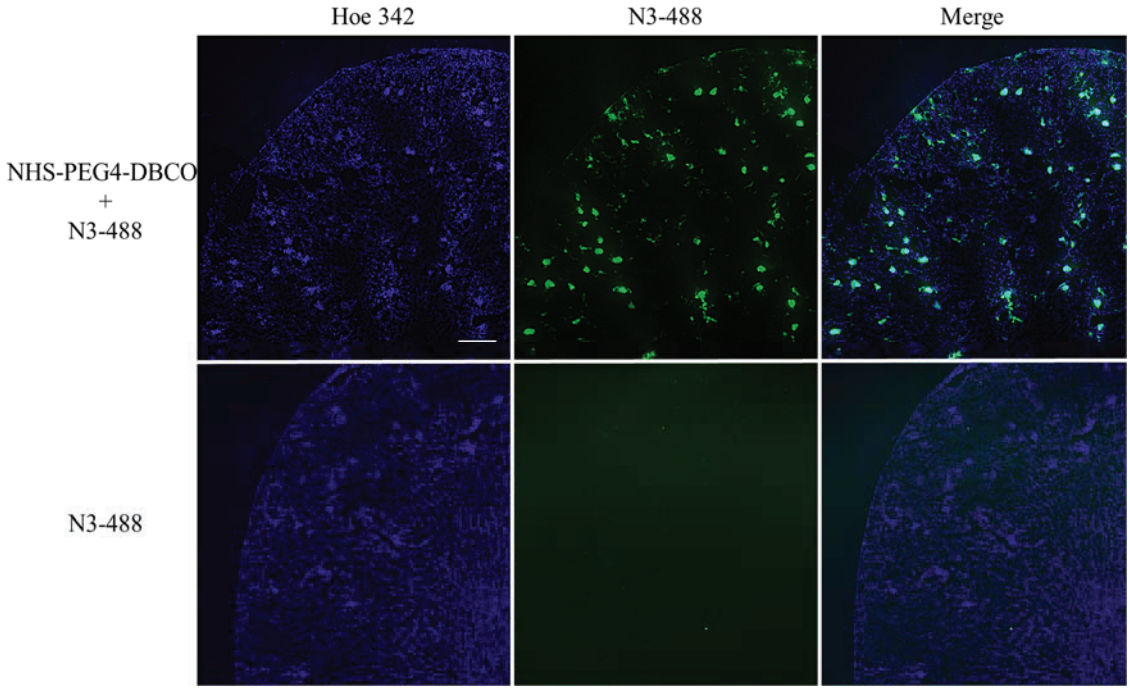

Figure 5.6. Chemically modified kidneys. Kidneys from nude mice were cannulated through the renal artery and perfused with Hoechst 33342 and N_3-488 alone or NHS-PEG4-DBCO plus N_3-488. After perfusion, kidneys were resected and embedded in optimal cutting temperature compound. Frozen sections were prepared and mounted on slides for fluorescent microscope imaging. While the kidney perfused with N_3-488 alone showed only background fluorescence, the kidney functionalized with NHS-PEG4-DBCO displayed significant labeling with N_3-488. Scale bar = 100µm.

Drug Capacity and Release Kinetics

The stability and release kinetics of our linker compounds were explored next. TAK-PhSO2-Linker was incubated in phosphate-buffered saline (PBS; pH 7.4) at 37°C, and the solution sampled over the course of several days. Liquid chromatography-mass spectrometry (LC-MS) was used to identify and quantitate the compounds released from this prodrug. This experiment revealed that the half-life of TAK-PhSO2-Linker was ~25 h, and that a second compound resulting from sulfonamide hydrolysis was also formed under these conditions (Figures 5.7A and B). The observed half-life of TAK-$PhSO_2$-Linker

was shorter than what has been previously reported for this linker attached to primary amines, but may be partially accounted for by the higher pKa of the sulfonamide amine.

The potential drug dose delivered is another important parameter, and so we measured the effective islet drug-loading capacity of this DBCO/azide-linker chemistry. This was accomplished by modifying islets with NHS-PEG4-DBCO followed by our fluorophore/linker molecule (BODIPY-PhSO2-Linker) (Figure 5.7C). The hydrolysable linkage on the resulting islets was then rapidly cleaved in a cleavage buffer, and the released dye was quantified by plotting the relative fluorescent intensity of the cleavage buffer against a standard curve. From 300 modified islets, we measured a BODIPY concentration of 3101.57 ± 690.73 nM in 400 μL of cleavage buffer (Figures 5.7D and E), or approximately 1.24 ± 0.276 nmol of compound released. This equates to 4.13 ± 0.92 pmol of compound per islet. Since the effective concentrations (IC50) of TAK-242 as a TLR4 antagonist are in the low nM range [22], this suggests the capacity for the release of biologically-relevant amounts of the active drug. The theoretical cumulative compound release over time is depicted in Figure 5.7F.

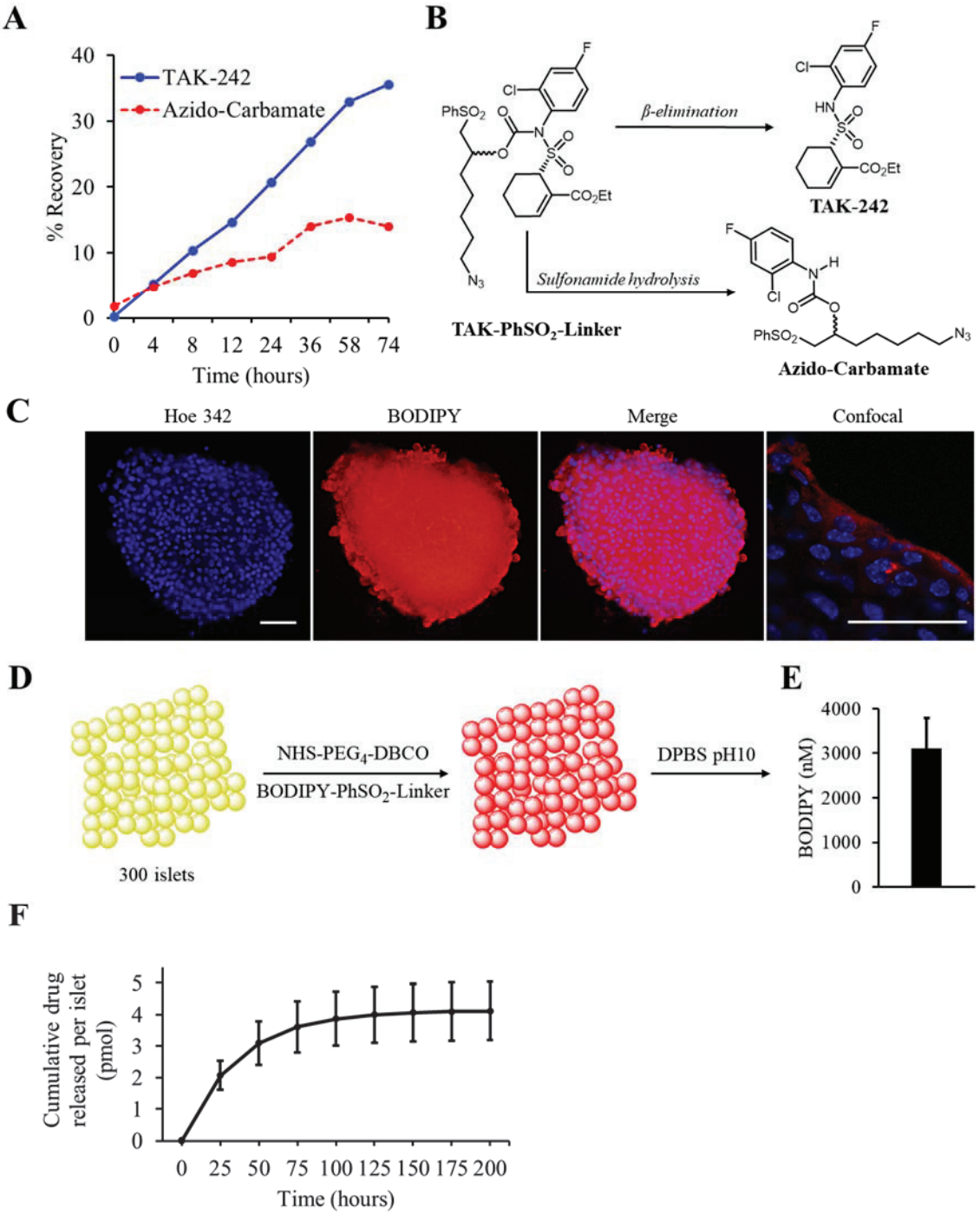

Figure 5.7. Assessment of linker kinetics. (A) Conversion of TAK-PhSO2-Linker into TAK-242 or the azido-carbamate side product. (B) TAK-PhSO2-Linker hydrolysis products. (C) Fluorescent images of a representative islet modified with BODIPY-PhSO2-Linker. (D and E) Experimental design and quantification of islet drug-loading capacity. (F) Theoretical cumulative release of TAK-242 over time.

Viability and Functionality of Modified Islets

We assessed the viability of islets after covalent modification by staining islets with Hoechst 33342 and propidium iodide at various time points to ensure that the islet manipulations, reactive compounds, and modification reactions did not injure or were otherwise toxic to the islets. Islets isolated from C57BL/6 mice were modified with NHS-PEG4-DBCO followed by TAK-PhSO2-Linker and cultured for up to 5 days. The viability of unmodified and modified islets (n = 10 per group) was evaluated on days 1, 2, and 5 post-modification by calculating the ratio of PI-positive cells to Hoechst 33342–positive cells (Fig 5.8A). We observed no significant differences in mean viability between unmodified and modified islets at any time points (P = 0.13), which is in-line with published safety data on SPAAC modifications in living systems [262].

Preserving islet function and insulin secretion is an important consideration in proposed islet treatment or modification. Glucose-stimulated insulin secretion (GSIS) assays were performed on unmodified and modified islets at 24 h (Figure 5.8B) and 72 h (Figure 5.8C) post modification to assess islet function. No differences were detected in the stimulation index between unmodified and modified islets at day 1 (P = 0.22) or at day 3 (P = 0.44). We also examined Ca^{2+} signaling in modified and unmodified islet cells, since intracellular Ca^{2+} signaling is a key factor in insulin secretion [266]. Unsurprisingly, given the results of the GSIS assay, intracellular Ca^{2+} flux in response to increased glucose remained identical in modified islets (Figure 5.8D). Overall, the modification of islets with TAK-$PhSO_2$-Linker presents no significant effects on islet viability or function, demonstrating the tolerance of islets to this chemical surface modification with a TLR4 antagonist.

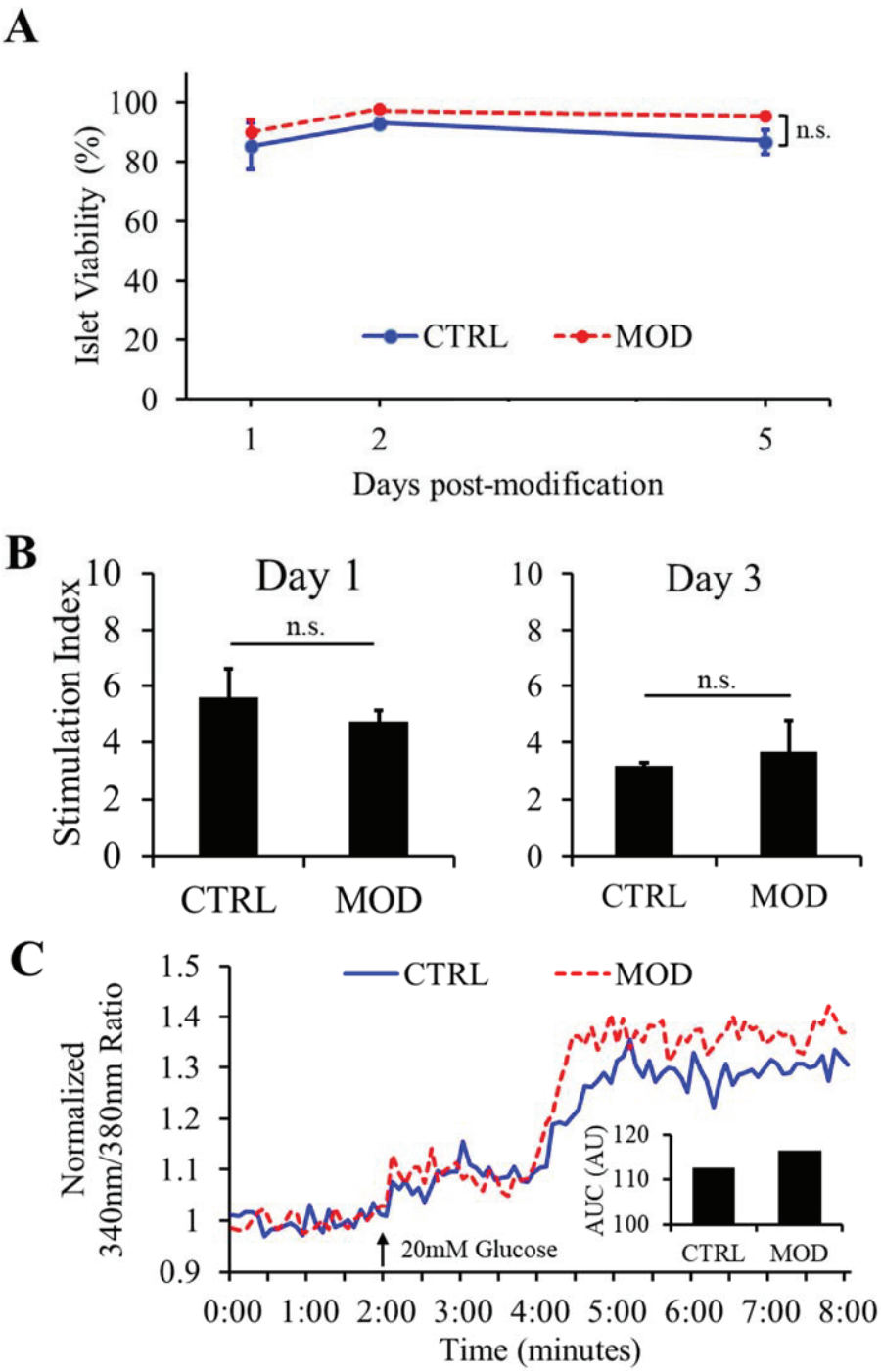

Figure 5.8. Modified islet viability and functionality assays. (A) Viability of control and modified islets assessed by Hoechst 33342 and PI staining on days 1, 2, and 5 post-modification (n = 10 per group, P = 0.13). (B and C) Stimulation index of control and modified islets (B) 24 h and (C) 72 h post-modification (n = 2 per group; P = 0.22 and 0.44, respectively; two-tailed unpaired t-test). (D) Representative intracellular Ca2+ flux assessment by fura-2AM staining. Arrow indicates an injection of 40 mM glucose. AUC is quantified in the inset graph. n.s. not significant.

Protection of Drug-Eluting Islets in Vitro

In order to study the function and efficacy of our cleavable pro-drug, we performed *in vitro* experiments challenging mouse islets with LPS at different times post-modification. First, we show that, although TAK-242 is a potent TLR4 antagonist, the intact prodrug construct (TAK-PhSO$_2$-Linker) has negligible TLR4-antagonist activity. HEK Blue hTLR4 reporter cells were treated with either free TAK-242 or the TAK-PhSO2-Linker prodrug, and TAK-Linker, which lacks the pKa modulator, before LPS challenge. We see that while TAK-242 completely inhibits the upregulation of NFkB after LPS challenge, there was no significant protection provided by the unconjugated prodrugs TAK-PhSO$_2$-Linker and TAK-Linker (Figure 5.9A).

When conjugated to islets, TAK-PhSO2-Linker significantly protects against LPS-mediated inflammation 24 h after modification ($P < 0.001$) while TAK-Linker provides moderate, but not statistically significant protection ($P = 0.14$; Figure 5.9B). We also observed that modified islets provide potent protection, comparable to a TAK-242 treatment 30 min prior, and superior to TAK-242 24 h prior ($P < 0.05$), for at least 48 h post-modification (Figure 5.9C and D). Not surprisingly, simple covalent surface modification lacking the prodrug functionality did not reduce the LPS-mediated IL-6 upregulation ($P = 0.60$; Figure 5.9E). These results demonstrate that our cleavable TLR4-antagonist prodrug functions as anticipated and provides potent and durable protection against TLR4-mediated inflammation when covalently attached to islet surfaces *in vitro*.

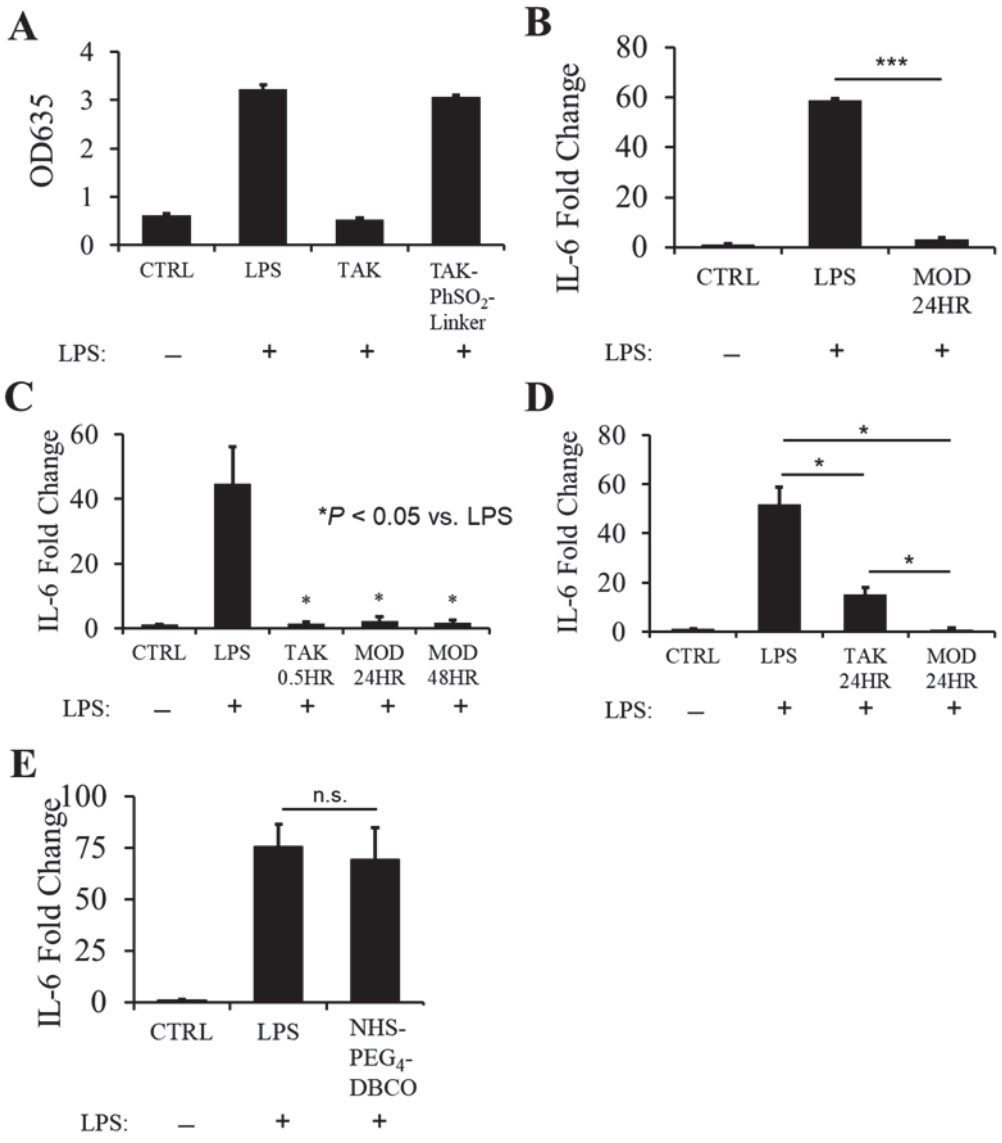

Figure 5.9. Assessment of drug-eluting islet protection in vitro. (A) Inhibition of TLR4-mediated NFkB upregulation was assessed for free TAK-242 and the TAK-PhSO2-Linker prodrug using HEK Blue hTLR4 colorimetric assay. TAK-242 completely blocked LPS while the intact prodrug provided no protection. (B) Islets covalently modified with TAK-PhSO2-Linker were significantly protected from LPS challenge (***P < 0.001) as determined by IL-6 expression. (C) Protection of modified islets was explored out to 48 h post-modification. At both 24 and 48 h after modification, covalently modified islets were protected against LPS as well as islets treated with TAK-242 30 minutes prior to challenge. (D) Both free TAK-242 and the covalent TAK-PhSO2-Linker modification provided protection from TLR4-mediated inflammation 24 hours post treatment, but the protection provided by the surface modification was significantly better than that of free TAK-242 alone. (E) Islet surfaces functionalized with NHS-PEG4-DBCO demonstrated no protection against LPS-mediated inflammation (P = 0.60). n = 3 (A) or n = 2 (B – E). Statistical significance determined by two-tailed unpaired t-test. n.s. not significant.

Efficacy of Drug-Eluting Islets in an Islet Transplant Model

After the promising *in vitro* results described above, we performed islet transplantation experiments in a syngeneic wild-type C57BL/6 streptozotocin-induced diabetes model. Engraftment success was measured by nonfasting blood glucose measurements 5 times a week. Mice were considered cured on the first day of two consecutive glucose measurements below 200 mg/dL. Diabetic mice were separated into untreated (n = 4), TAK-242 (n = 6), and TAK-PhO2-Linker (n = 6) groups. A marginal dose of ~100 islets, determined by titrating doses of TAK-242–treated islets (Figure 5.10), was transplanted into the kidney subcapsular space in each group and blood glucose was monitored over a 4-week period (Figure 5.11A). 100% of mice receiving modified islets became euglycemic with a mean time-to-cure of 17.2 ± 2.6 days (Figure 5.11B), and 67% of mice receiving TAK-242 treated islets became euglycemic at 21.0 ± 2.4 days (Figure 5.11B). None of the mice in the control group achieved euglycemia during the monitoring period (Figure 5.11B). The cure rate of the modified islets was significantly superior to the TAK-242 treated islets (P = 0.0037 and 0.0521 respectively, log-rank). Visual analysis of the modified islet grafts showed preservation of islet mass and significant vascularization in the euglycemic mice (Figure 5.11C), demonstrating the treatment does not impair revascularization of islets post-transplant.

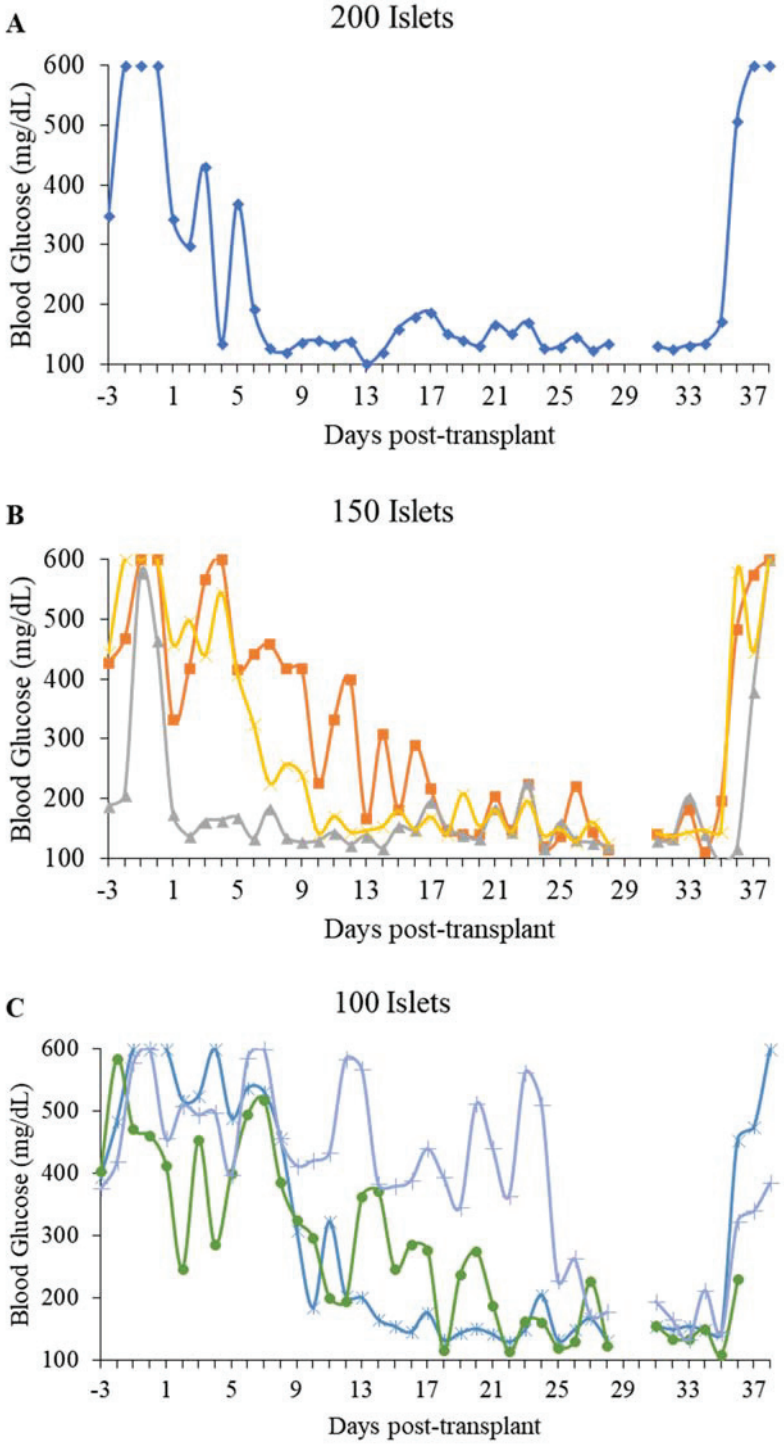

Figure 5.10. Islet dose titration with TAK-242 pretreated islets. Diabetic C57BL/6 mice were transplanted with (A) 200, (B) 150, or (C) 100 TAK-242–treated islets. Blood glucose was monitored for 4 weeks before IPGTT on day 30 and at nephrectomy on day 35. The quantity of 200 and 150 islets showed quick cure times and good glycemic control; the quantity of 100 islets had delayed cure times and exhibited significant glycemic lability.

An intraperitoneal glucose tolerance test (IPGTT) was performed at day 30 posttransplant to assess graft function. IPGTT area under the curve (AUC) analysis showed that the mice receiving the modified islets had the most robust glucose clearance response (Figures 5.11D and E). Islet grafts were removed by nephrectomy of the transplanted kidney to demonstrate that the mice became cured due to the grafts and not because of regeneration of endogenous insulin, and we saw prompt return to a diabetic state in previously cured animals (Figure 5.12). These data demonstrate the superior outcomes of drug-eluting modified islets by significantly reducing the time and islet dose required for achieving euglycemic in diabetic mice

Discussion

In this study, we have demonstrated the ex vivo modification of live tissue surface with a cleavable TLR4-antagonist prodrug, the ability of the locally released drug to provide sustained protection to islets against TLR4-mediated inflammation, and the use of these 'drug-eluting transplants' to provide significantly improved outcomes in a murine model of islet transplantation.

The role of TLRs in innate antipathogenic immunity is widely known [267]. Recently, TLR4 has been demonstrated to play a major role in sterile inflammation [268], acute graft dysfunction and allograft rejection [148], and autoimmune disease [269]. Studies involving anti-TLR4 antibodies, siRNA, and TLR4-/- animals have shown significant therapeutic benefits against acute injury mediated by inflammatory DAMPs [20,270]. TAK-242 is a selective TLR4-antagonist that was initially developed and clinically tested for the treatment of sepsis [163]. The protective effects of TAK-242 against TLR4-mediated inflammation has

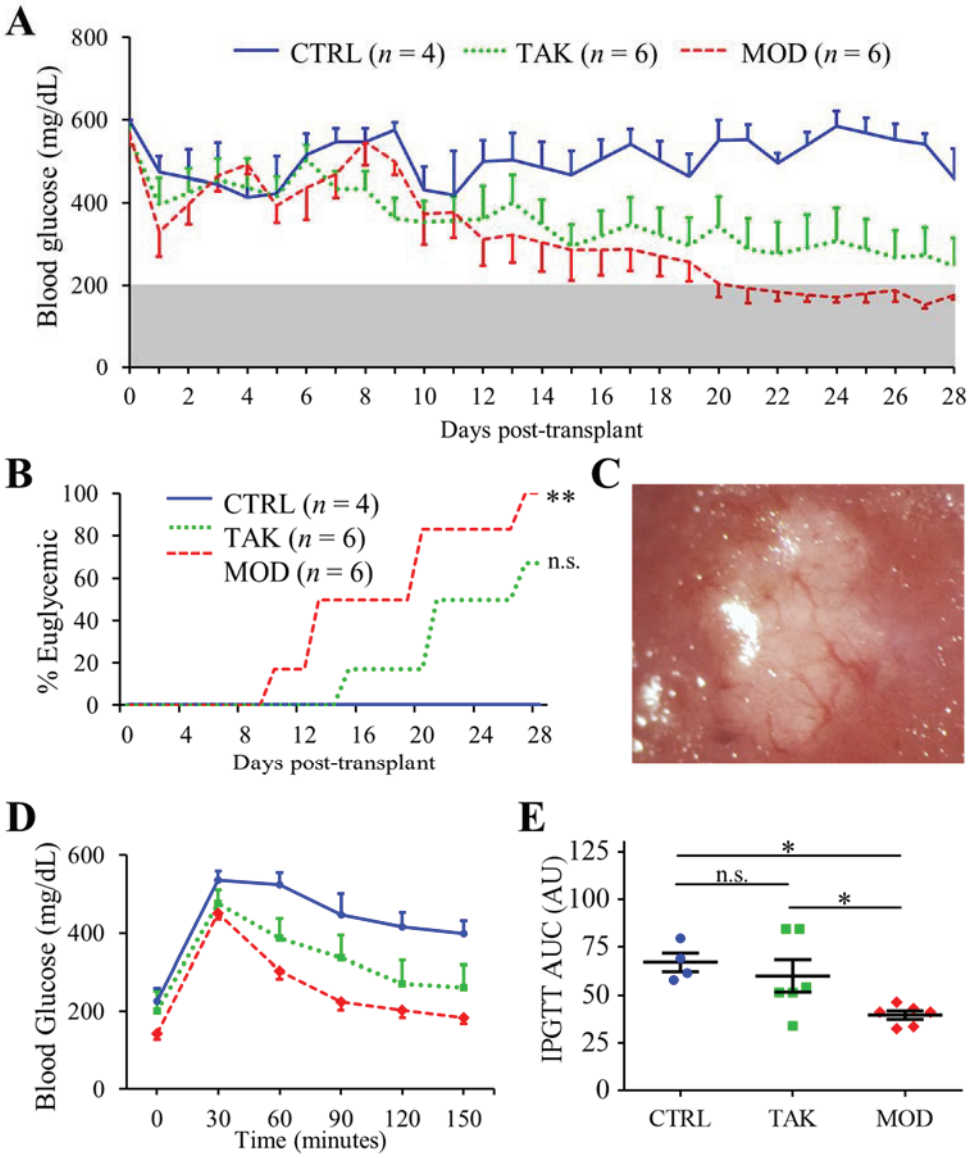

Figure 5.11. Islet transplant outcomes and function in a syngeneic model. (A) Mean nonfasting blood glucose in control (n = 4), TAK-242 (n = 6), and modified (n = 6) groups. Mice receiving 100 untreated islets (control) did not demonstrate improving blood glucose control, while mice receiving 100 TAK-242–treated and modified islets demonstrated some improvement. Only the group receiving modified islets achieved a mean blood glucose concentration in the normoglycemic range (<200 mg/dL). (B) All mice receiving 100 modified islets (6/6) achieved euglycemia (P = 0.004) compared to 67% (4/6) of mice receiving 100 TAK-242–treated islets (**P = 0.052). No mice in the control group achieved euglycemia. (C) Dissecting light microscopic image of a neovascularized islet graft from a mouse that received 100 modified islets. (D and E) IPGTT data from mice on day 30 posttransplant. AUC analysis showed no difference between the control and TAK-242 group (P > 0.05), but a significant difference between the modified group and both the control and TAK-242 group (*P < 0.05, one-way ANOVA with Newman-Keuls multiple comparisons test). n.s. not significant.

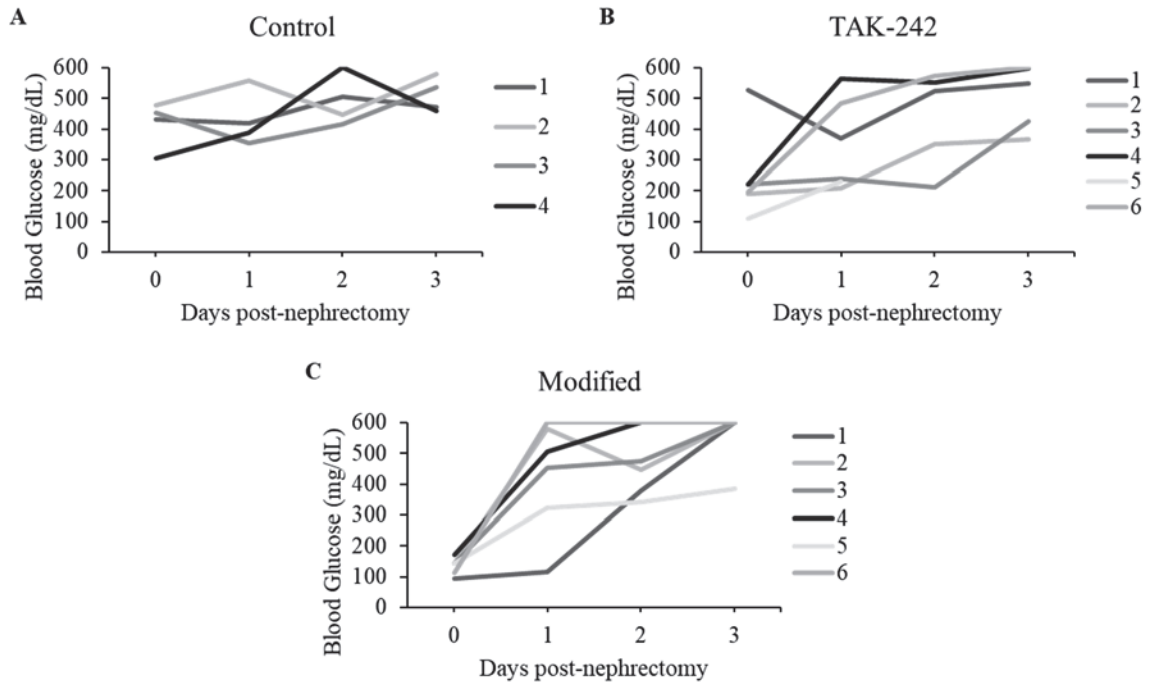

Figure 5.12. Blood glucose of mice after islet graft removal by nephrectomy. Nephrectomy of the islet-transplanted kidney was performed on day 35 posttransplant. (A) Control mice remained diabetic. Cured mice in the (B) TAK-242 group and (C) modified group quickly returned to a diabetic state.

been previously demonstrated in models of neural stress [271], liver IRI [272], and even autoimmune disease [166].

We evaluated TAK-242 as a candidate for blocking TLR4-mediated inflammation in islets *in vitro*. TAK-242 completely blocked LPS-mediated inflammation in islets as demonstrated by gene and cytokine analysis. TLR4 stimulation in islets significantly upregulates both CXCL10 and CCL2, both known to have a significant negative effect on islet transplantation outcomes [273,274], but this inflammation largely inhibited by TAK-242 treatment. Cytokine profile analysis from LPS-challenged islets reveals that in addition to CXCL10 and CCL2, the acute inflammatory cytokines IL-6 and TNF-α are high upregulated, as is the myeloid cell chemoattractant CXCL10.

Methods for the ex vivo covalent modification of islet cell clusters have been previously investigated as a strategy to protect transplanted islets. While simple steric barriers have been most often linked to islet surfaces [110,275], functional molecules aimed at reducing thrombosis have also been covalently tethered to the islet surface [276,277]. Cleavable linkers for drug delivery from macromolecules and biocompatible polymers have been previously utilized for their ability to significantly prolong the half-life of compounds [184]. Additionally, the use of 'Click' chemistry for modifying the surface of live cells has been widely explored and can be accomplished with no apparent effects on cell viability [262]. The combination of these two tools provides colocalization of drug treatment with the grafted tissues, resulting in an extended duration of elevated local concentrations with minimal systemic exposure. Based on the promising findings from our in vitro assays, we selected TAK-242 as a candidate to evaluate the advantages of tissue surface modification using cleavable prodrug linker chemistry. To this end, we synthesized TAK-PhSO2-Linker, a conjugatable, slow-release prodrug form of TAK-242, which can be readily covalently linked to surface-functionalized tissues, and which can elute TAK-242 via β-elimination at a tunable rate. Analysis of cleavage kinetics revealed that free TAK-242 is released from the prodrug with a half-life of approximately 25 h.

In preliminary islet modification experiments, we demonstrated the surface labeling of murine islets with a fluorophore using a "click" chemistry approach. A competitive binding assay revealed that we could also successfully conjugate the TAK-PhSO2-Linker prodrug to islets. The conjugation of a BODIPY dye using the same linker chemistry allowed for an estimation of islet drug-loading capacity, which we calculated to be approximately 4.13 ± 0.92 pmol per islet. Importantly, this conjugation chemistry can be

applied to other tissues and organs, as demonstrated by our preliminary modification of murine kidney via renal artery perfusion (Figure 4.6), suggesting that this 'drug-eluting transplant' strategy may have broad applicability. Modification of the kidney by vascular perfusion appears to be concentrated in the glomeruli, which may have important implications in protecting renal grafts from glomerulitis and other acute injuries post-transplant. Coincidentally, the ex vivo machine perfusion of organs for transplantation is gaining popularity to reduce IRI and expand the donor pool [278], presenting a convenient opportunity for the chemical modification of organs prior to transplantation.

Viability and functionality assays in vitro revealed the safety profile and tolerability of islets to our conjugation chemistry. We observed no differences in islet viability at days 1, 2, and 5 post-modification with TAK-PhSO2-Linker as compared to unmodified control islets. The stimulation index and intracellular Ca^{2+} flux of islets was also preserved in the modified islet group. Our results reinforce findings from other groups who report the safety and tolerability of islets to surface modification [109,250]. The modified islets exhibited lasting protection against TLR4-mediated inflammation in vitro. At 24 and 48 h post-modification, modified islets still demonstrated essentially complete inhibition of LPS-mediated inflammation. On the other hand, TAK-242 was able to completely inhibit LPS-mediated inflammation immediately after treatment, but began to lose its effectiveness by 24 h.

Data from our in vivo transplant model clearly demonstrate the advantage of our chemically modified drug-eluting islets. We initially transplanted 200 modified syngeneic islets into diabetic C57BL/6 mice to assess their function and saw that all mice rapidly returned to euglycemia, however this was also obtainable with 200 TAK-242 treated islets (unpublished data). Therefore, we titrated a lower islet dose in order to stratify the two

treatment groups (Figure 5.10). With 100 islets, 67% of subjects in the TAK-242 group were able to achieve euglycemia, albeit with delayed cure times (21. ± 2.4 days) and with significant glycemic lability. On the other hand, 100 modified islets abled to achieve a 100% cure rate (17.2 ± 2.6 days) and also demonstrated superior glucose clearance in response to a IPGTT. Human islet transplantation is often complicated by the requirement of high doses of islets (>10,000 IEQ/kg) and multiple donors [279]. The transplant data here suggest that the translation of our chemistry into the clinic may substantially reduce the dose of islets needed to achieve insulin independence and facilitate single-donor transplant success.

In conclusion, results from our study demonstrate the feasibility and benefits of *ex vivo* modification of living tissue with potent anti-inflammatory prodrugs to provide drug-eluting transplants. The *ex vivo* modification of tissues can be easily implemented during organ procurement or prior to transplant by normothermic machine perfusion in vascularized organs, which is increasingly common [280,281], or by directly washing tissues and cells in modification buffers. Clinical implementation of our chemistry in the field of transplantation may prove to provide a significant advantage in reducing primary graft dysfunction and improving long-term outcomes.

CHAPTER SIX

Conclusion

Islet transplantation is an important treatment option for brittle T1Ds with problematic hypoglycemia unawareness and severe hypoglycemic events who do not respond well to intense insulin therapy [236]. As a byproduct of advances in and facilities for allogeneic islet transplant, autologous islet transplant after total pancreatectomy was developed as a procedure to prevent the development of brittle diabetes after pancreatectomy for intractable pain [282]. The aims of this project were to elucidate the protective effects of TLR4 inhibition on the outcomes of islet transplantation using a clinically-tested compound, and to develop the concept of drug-eluting live tissue for transplantation. TLR4 was chosen as a therapeutic target due to its reported role in compromising graft function and survival during the peri-transplant period. Indeed, through our experiments we observed that inhibiting TLR4 in islets prior to and post-transplant significantly reduced inflammation and islet damage, and improved transplant outcomes.

In one study, the impact of using the potent TLR4 antagonist TAK242 during islet isolation and culturing was studied. While the role of TLR4 has been studied in islets and other organs post-transplant. The state of the graft prior to transplant is critical since this will affect the amount of inflammation triggered post-transplant. Although there is minimal LPS during the organ procurement and islet isolation process, sterile inflammation still occurs and TLR4 is a major mediator. Indeed, in our experiments we saw significant reductions in major mediators of islet graft dysfunction by the early blockade of TLR4.

The ability to perfuse an organ with this anti-inflammatory drug has the benefits of prophylactic inhibition of sterile inflammation and avoiding the direct administration of a potentially immunosuppressive drug to the patient. The aim of treating the islets directly instead of the patient systemically is an important distinction since islets are known to produce significant amounts of self-reactive inflammatory isletokines [222]. Although in this study, TAK-242 was only added to the collagenase solution, it could also be added to the storage solution, wash buffers, and transplant media to ensure complete TLR4 inhibition prior to transplant. It could be imagined that the addition of TAK-242 to normothermic machine perfusion of solid organs could also limit sterile inflammation, and potentially improve transplant outcomes.

Weaknesses in the early TLR4 blockade study are that this data needs to be replicated in human islet isolation, which was not tested here due to the lack of research pancreases available. Transplant outcomes, however, may be different from the animal model due to differences in both the procurement, isolation, and transplant. Additionally, the western blotting data for MAPKs and NFkB could be repeated to address the observation that there was no reduction in p-P65, as this is thought to be upregulated by TLR4, and therefore TLR4 antagonism was expected to have reduced P65 activation. Future experimentation could include repeating the western blots and performing these analyses with human islet research isolations.

In the second study, we aimed to inhibit TLR4 in islets for several days post-transplant. After transplant, islets become inflamed due to IBMIR and other sterile inflammatory processes. This inflammation lasts for up to a week post-transplant and is correlated with poor transplant outcomes and the need for multiple islet infusions. Current

protocols include the systemic administration of anakinra (IL-1R blocker) and etanercept (TNF-α inhibitor) to reduce inflammation, however sub-optimal doses are used to reduce off-target side effects and the chances of opportunistic infections. To address these drawbacks, we aimed to develop a method for localizing anti-inflammatory treatments to the graft while maintaining therapeutic concentrations and prolonging treatment for some days. Due to the demonstrated role of TLR4 in graft dysfunction and TAK-242 as a potent anti-inflammatory, this compound was used for this proof-of-concept study. To develop a cleavable pro-drug which we could conjugate to the surface of islets, we adopted the concept of drug-eluting particles [283], which are already used for drug delivery of some pharmaceutical compounds such as exenatide [284]. By linking the pro-drug directly to the islet prior to transplant, the graft is enhanced with the ability to elute its own drug locally, eliminating the need for system administration of the drug.

Weaknesses in this study are that we could have in vitro data showing protection of modified islet from LPS beyond 48 hours compared to a single TAK-242 treatment. This was somewhat limited by the absolute number of donor mice and islets required to perform such a robust study. Additionally, the number of recipients in the transplant model could be increased to increase statistical power. Although the current data shows an advantage over untreated controls, it is debatable whether or not it has a statistically significant advantage over TAK-242 pre-treatment alone. Increasing the sample sizes should provide more definitive data. Future directions this would could take include: 1. Test in an allogeneic model of islet transplantation, and 2. Collaborate with another group to test the effects of self-medicating drug-eluting tissues for transplant with solid organ transplant,

taking advantage of the rise in importance of ex-vivo machine perfusion of organs to reduce ischemia and expand the donor pools.

In conclusion, I have presented evidence that targeting TLR4 on islets before and after isolation represents an attractive therapeutic option for improving the outcomes of islet transplantation. The addition of TAK-242, a clinically-tested TLR4 antagonist, to the collagenase solution or other buffers represents a rapidly clinically-translatable modification of current protocols that may serve to reduce islet injury due to sterile inflammation prior to transplantation. Additionally, the development for a rapid islet surface modification method with a pro-drug is a novel concept represents a promising development that has potential in future transplantation studies.

REFERENCES

1. Barker CF, Markmann JF. Historical overview of transplantation. *Cold Spring Harb Perspect Med.* 2013;3(4): a014977.

2. Linden PK. History of solid organ transplantation and organ donation. *Crit Care Clin.* 2009;25(1): 165-184, ix.

3. Starzl TE. The mystique of organ transplantation. *J Am Coll Surg.* 2005;201(2): 160-170.

4. Morrissey PE, Monaco AP. Donation after circulatory death: current practices, ongoing challenges, and potential improvements. *Transplantation.* 2014;97(3): 258-264.

5. Matsumoto S. Clinical allogeneic and autologous islet cell transplantation: update. *Diabetes Metab J.* 2011;35(3): 199-206.

6. Bellin MD, Barton FB, Heitman A, et al. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *Am J Transplant.* 2012;12(6): 1576-1583.

7. Molnar AO, Fergusson D, Tsampalieros AK, et al. Generic immunosuppression in solid organ transplantation: systematic review and meta-analysis. *BMJ.* 2015;350: h3163.

8. Chapman JR, Webster AC, Wong G. Cancer in the transplant recipient. *Cold Spring Harb Perspect Med.* 2013;3(7).

9. Patel MJ, Liegeois NJ. Skin cancer and the solid organ transplant recipient. *Curr Treat Options Oncol.* 2008;9(4-6): 251-258.

10. Gupta A, Kumer S, Kaplan B. Novel immunosuppressive strategies for composite tissue allografts. *Curr Opin Organ Transplant.* 2014;19(6): 552-557.

11. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. *N Engl J Med.* 2007;357(25): 2562-2575.

12. Gracia-Sancho J, Villarreal G, Jr., Zhang Y, et al. Flow cessation triggers endothelial dysfunction during organ cold storage conditions: strategies for pharmacologic intervention. *Transplantation.* 2010;90(2): 142-149.

13. Murphy SP, Porrett PM, Turka LA. Innate immunity in transplant tolerance and rejection. *Immunol Rev.* 2011;241(1): 39-48.

14. Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O. Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups J Med Sci.* 2000;105(2): 125-133.

15. Tsujimura T, Kuroda Y, Kin T, et al. Human islet transplantation from pancreases with prolonged cold ischemia using additional preservation by the two-layer (UW solution/perfluorochemical) cold-storage method. *Transplantation.* 2002;74(12): 1687-1691.

16. Eich T, Eriksson O, Sundin A, et al. Positron emission tomography: a real-time tool to quantify early islet engraftment in a preclinical large animal model. *Transplantation.* 2007;84(7): 893-898.

17. Turgeon NA, Avila JG, Cano JA, et al. Experience with a novel efalizumab-based immunosuppressive regimen to facilitate single donor islet cell transplantation. *Am J Transplant.* 2010;10(9): 2082-2091.

18. Murdock JL, Nunez G. TLR4: The Winding Road to the Discovery of the LPS Receptor. *J Immunol.* 2016;197(7): 2561-2562.

19. Peri F, Calabrese V. Toll-like receptor 4 (TLR4) modulation by synthetic and natural compounds: an update. *J Med Chem.* 2014;57(9): 3612-3622.

20. Gao Q, Ma LL, Gao X, Yan W, Williams P, Yin DP. TLR4 mediates early graft failure after intraportal islet transplantation. *Am J Transplant.* 2010;10(7): 1588-1596.

21. Kruger B, Yin N, Zhang N, et al. Islet-expressed TLR2 and TLR4 sense injury and mediate early graft failure after transplantation. *Eur J Immunol.* 2010;40(10): 2914-2924.

22. Ii M, Matsunaga N, Hazeki K, et al. A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. *Mol Pharmacol.* 2006;69(4): 1288-1295.

23. Cowley MJ, Weinberg A, Zammit NW, et al. Human islets express a marked proinflammatory molecular signature prior to transplantation. *Cell Transplant.* 2012;21(9): 2063-2078.

24. Rindler MJ. Isolation of zymogen granules from rat pancreas. *Curr Protoc Cell Biol.* 2006;Chapter 3: Unit 3 18.

25. Ferrucci JT, Jr., Eaton SB. Radiology of the pancreas. *N Engl J Med.* 1973;288(10): 506-510.

26. Malaisse WJ. Physiology and pathology of islet metabolism. *Diabete Metab.* 1994;20(2): 95-98.

27. Habegger KM, Heppner KM, Geary N, Bartness TJ, DiMarchi R, Tschop MH. The metabolic actions of glucagon revisited. *Nat Rev Endocrinol.* 2010;6(12): 689-697.

28. Lacy PE. The pancreatic beta cell. Structure and function. *N Engl J Med.* 1967;276(4): 187-195.

29. Briant LJB, Reinbothe TM, Spiliotis I, Miranda C, Rodriguez B, Rorsman P. delta-cells and beta-cells are electrically coupled and regulate alpha-cell activity via somatostatin. *J Physiol.* 2018;596(2): 197-215.

30. Hauge-Evans AC, King AJ, Carmignac D, et al. Somatostatin secreted by islet delta-cells fulfills multiple roles as a paracrine regulator of islet function. *Diabetes.* 2009;58(2): 403-411.

31. Andralojc KM, Mercalli A, Nowak KW, et al. Ghrelin-producing epsilon cells in the developing and adult human pancreas. *Diabetologia.* 2009;52(3): 486-493.

32. Dezaki K, Sone H, Koizumi M, et al. Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. *Diabetes.* 2006;55(12): 3486-3493.

33. Poher AL, Tschop MH, Muller TD. Ghrelin regulation of glucose metabolism. *Peptides.* 2018;100: 236-242.

34. Nakazato M, Miyazato M, Asai J, et al. Islet amyloid polypeptide, a novel pancreatic peptide, is a circulating hormone secreted under glucose stimulation. *Biochem Biophys Res Commun.* 1990;169(2): 713-718.

35. Johnson KH, O'Brien TD, Westermark P. Newly identified pancreatic protein islet amyloid polypeptide. What is its relationship to diabetes? *Diabetes.* 1991;40(3): 310-314.

36. Zhang Y, Song W. Islet amyloid polypeptide: Another key molecule in Alzheimer's pathogenesis? *Prog Neurobiol.* 2017;153: 100-120.

37. In't Veld P, Marichal M. Microscopic anatomy of the human islet of Langerhans. *Adv Exp Med Biol.* 2010;654: 1-19.

38. Alam U, Asghar O, Azmi S, Malik RA. General aspects of diabetes mellitus. *Handb Clin Neurol.* 2014;126: 211-222.

39. Hogan P, Dall T, Nikolov P, American Diabetes A. Economic costs of diabetes in the US in 2002. *Diabetes Care.* 2003;26(3): 917-932.

40. Dall TM, Yang W, Halder P, et al. The economic burden of elevated blood glucose levels in 2012: diagnosed and undiagnosed diabetes, gestational diabetes mellitus, and prediabetes. *Diabetes Care.* 2014;37(12): 3172-3179.

41. Gill GV. The spectrum of brittle diabetes. *J R Soc Med.* 1992;85(5): 259-261.

42. American Diabetes A. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. *Diabetes Care.* 2018;41(Suppl 1): S13-S27.

43. Pugliese A. Insulitis in the pathogenesis of type 1 diabetes. *Pediatr Diabetes.* 2016;17 Suppl 22: 31-36.

44. Campbell-Thompson M, Fu A, Kaddis JS, et al. Insulitis and beta-Cell Mass in the Natural History of Type 1 Diabetes. *Diabetes.* 2016;65(3): 719-731.

45. Bolande RP. The pathology and pathogenesis of juvenile diabetes mellitus. *Perspect Pediatr Pathol.* 1979;5: 269-291.

46. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. *Lancet.* 2014;383(9911): 69-82.

47. Khanpour Ardestani S, Karkhaneh M, Yu HC, Hydrie MZI, Vohra S. Primary outcomes reporting in trials of paediatric type 1 diabetes mellitus: a systematic review. *BMJ Open.* 2017;7(12): e014610.

48. Soltesz G, Patterson CC, Dahlquist G, Group ES. Worldwide childhood type 1 diabetes incidence--what can we learn from epidemiology? *Pediatr Diabetes.* 2007;8 Suppl 6: 6-14.

49. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. *Endocrinol Metab Clin North Am.* 2010;39(3): 481-497.

50. Golden SH, Sapir T. Methods for insulin delivery and glucose monitoring in diabetes: summary of a comparative effectiveness review. *J Manag Care Pharm.* 2012;18(6 Suppl): S1-17.

51. Rodbard D. Continuous Glucose Monitoring: A Review of Recent Studies Demonstrating Improved Glycemic Outcomes. *Diabetes Technol Ther.* 2017;19(S3): S25-S37.

52. Demmer RT, Zuk AM, Rosenbaum M, Desvarieux M. Prevalence of diagnosed and undiagnosed type 2 diabetes mellitus among US adolescents: results from the continuous NHANES, 1999-2010. *Am J Epidemiol.* 2013;178(7): 1106-1113.

53. Magliano DJ, Zimmet P, Shaw J. US Trends for Diabetes Prevalence Among Adults. *JAMA.* 2016;315(7): 705.

54. American Diabetes A. 2. Classification and Diagnosis of Diabetes. *Diabetes Care.* 2017;40(Suppl 1): S11-S24.

55. Courcoulas AP, Goodpaster BH, Eagleton JK, et al. Surgical vs medical treatments for type 2 diabetes mellitus: a randomized clinical trial. *JAMA Surg.* 2014;149(7): 707-715.

56. Liu J, Li L, Deng K, et al. Incretin based treatments and mortality in patients with type 2 diabetes: systematic review and meta-analysis. *BMJ.* 2017;357: j2499.

57. Group TS, Zeitler P, Hirst K, et al. A clinical trial to maintain glycemic control in youth with type 2 diabetes. *N Engl J Med.* 2012;366(24): 2247-2256.

58. Roeyen G, De Block C. A plea for more practical and clinically applicable criteria defining type 3c diabetes. *Pancreatology.* 2017;17(6): 875.

59. Hardt PD, Brendel MD, Kloer HU, Bretzel RG. Is pancreatic diabetes (type 3c diabetes) underdiagnosed and misdiagnosed? *Diabetes Care.* 2008;31 Suppl 2: S165-169.

60. Hart PA, Bellin MD, Andersen DK, et al. Type 3c (pancreatogenic) diabetes mellitus secondary to chronic pancreatitis and pancreatic cancer. *Lancet Gastroenterol Hepatol.* 2016;1(3): 226-237.

61. Ewald N, Kaufmann C, Raspe A, Kloer HU, Bretzel RG, Hardt PD. Prevalence of diabetes mellitus secondary to pancreatic diseases (type 3c). *Diabetes Metab Res Rev.* 2012;28(4): 338-342.

62. Lew D, Afghani E, Pandol S. Chronic Pancreatitis: Current Status and Challenges for Prevention and Treatment. *Dig Dis Sci.* 2017;62(7): 1702-1712.

63. Andersen DK. The practical importance of recognizing pancreatogenic or type 3c diabetes. *Diabetes Metab Res Rev.* 2012;28(4): 326-328.

64. Majumder S, Chari ST. Chronic pancreatitis. *Lancet.* 2016;387(10031): 1957-1966.

65. Albisser AM, Leibel BS, Ewart TG, Davidovac Z, Botz CK, Zingg W. An artificial endocrine pancreas. *Diabetes.* 1974;23(5): 389-396.

66. Mirouze J, Selam JL, Pham TC, Cavadore D. Evaluation of exogenous insulin homoeostasis by the artificial pancreas in insulin-dependent diabetes. *Diabetologia.* 1977;13(3): 273-278.

67. Yamasaki Y, Shichiri M, Kawamori R, et al. Counterregulatory system in an artificial endocrine pancreas. Glucose infusion algorithm. *Artif Organs.* 1979;3(3): 265-270.

68. Yagi T, Yamasaki Y, Bando K, Kawamori R, Kamada T, Shichiri M. The evaluation of glucagon infusion algorithms for a counterregulatory system in artificial endocrine pancreas. *Med Prog Technol.* 1988;13(4): 195-201.

69. Bequette BW. Fault detection and safety in closed-loop artificial pancreas systems. *J Diabetes Sci Technol.* 2014;8(6): 1204-1214.

70. Farrington C. The artificial pancreas: challenges and opportunities. *Lancet Diabetes Endocrinol.* 2015;3(12): 937.

71. Thabit H, Tauschmann M, Allen JM, et al. Home Use of an Artificial Beta Cell in Type 1 Diabetes. *N Engl J Med.* 2015;373(22): 2129-2140.

72. Anderson SM, Raghinaru D, Pinsker JE, et al. Multinational Home Use of Closed-Loop Control Is Safe and Effective. *Diabetes Care.* 2016;39(7): 1143-1150.

73. Messer LH, Forlenza GP, Sherr JL, et al. Optimizing Hybrid Closed-Loop Therapy in Adolescents and Emerging Adults Using the MiniMed 670G System. *Diabetes Care.* 2018;41(4): 789-796.

74. Foerster V, Severn M. A Hybrid Closed-Loop Insulin Delivery System for the Treatment of Type 1 Diabetes. *CADTH Issues in Emerging Health Technologies*. Ottawa (ON)2016:1-11.

75. Stone JY, Haviland N, Bailey TS. Review of a commercially available hybrid closed-loop insulin-delivery system in the treatment of Type 1 diabetes. *Ther Deliv.* 2018;9(2): 77-87.

76. Gingras V, Smaoui MR, Cameli C, et al. Impact of erroneous meal insulin bolus with dual-hormone artificial pancreas using a simplified bolus strategy - A randomized controlled trial. *Sci Rep.* 2018;8(1): 2621.

77. Thabit H, Hovorka R. Coming of age: the artificial pancreas for type 1 diabetes. *Diabetologia.* 2016;59(9): 1795-1805.

78. Gruessner AC. 2011 update on pancreas transplantation: comprehensive trend analysis of 25,000 cases followed up over the course of twenty-four years at the International Pancreas Transplant Registry (IPTR). *Rev Diabet Stud.* 2011;8(1): 6-16.

79. Dholakia S, Mittal S, Quiroga I, et al. Pancreas Transplantation: Past, Present, Future. *Am J Med.* 2016;129(7): 667-673.

80. Gruessner AC, Gruessner RW. Long-term outcome after pancreas transplantation: a registry analysis. *Curr Opin Organ Transplant.* 2016;21(4): 377-385.

81. Cornejo-Carrasco CE, Fernandez-Cruz L. Re-operation in pancreas transplantation. *Transplant Proc.* 2014;46(9): 3050-3053.

82. Rudolph EN, Finger EB, Chandolias N, Kandaswamy R, Sutherland DE, Dunn TB. Outcomes of pancreas retransplantation. *Transplantation.* 2015;99(2): 367-374.

83. Gruessner AC, Gruessner RW. Declining numbers of pancreas transplantations but significant improvements in outcome. *Transplant Proc.* 2014;46(6): 1936-1937.

84. Niclauss N, Morel P, Berney T. Has the gap between pancreas and islet transplantation closed? *Transplantation.* 2014;98(6): 593-599.

85. Alejandro R, Mintz DH, Noel J, et al. Islet cell transplantation in type I diabetes mellitus. *Transplant Proc.* 1987;19(1 Pt 3): 2359-2361.

86. Sutherland DE, Matas AJ, Goetz FC, Najarian JS. Transplantation of dispersed pancreatic islet tissue in humans: autografts and allografts. *Diabetes.* 1980;29 Suppl 1: 31-44.

87. Kendall DM, Robertson RP. Pancreas and islet transplantation. Challenges for the twenty-first century. *Endocrinol Metab Clin North Am.* 1997;26(3): 611-630.

88. Robertson RP. Pancreatic islet transplantation for diabetes: successes, limitations, and challenges for the future. *Mol Genet Metab.* 2001;74(1-2): 200-205.

89. Berney T, Johnson PR. Donor pancreata: evolving approaches to organ allocation for whole pancreas versus islet transplantation. *Transplantation.* 2010;90(3): 238-243.

90. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med.* 2000;343(4): 230-238.

91. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes.* 2005;54(7): 2060-2069.

92. Kin T, Johnson PR, Shapiro AM, Lakey JR. Factors influencing the collagenase digestion phase of human islet isolation. *Transplantation.* 2007;83(1): 7-12.

93. Rheinheimer J, Ziegelmann PK, Carlessi R, et al. Different digestion enzymes used for human pancreatic islet isolation: a mixed treatment comparison (MTC) meta-analysis. *Islets.* 2014;6(4): e977118.

94. Matsumoto S, Takita M, Chaussabel D, et al. Improving efficacy of clinical islet transplantation with iodixanol-based islet purification, thymoglobulin induction, and blockage of IL-1beta and TNF-alpha. *Cell Transplant.* 2011;20(10): 1641-1647.

95. Manzoli V, Villa C, Bayer AL, et al. Immunoisolation of murine islet allografts in vascularized sites through conformal coating with polyethylene glycol. *Am J Transplant.* 2018;18(3): 590-603.

96. Basta G, Calafiore R. Immunoisolation of pancreatic islet grafts with no recipient's immunosuppression: actual and future perspectives. *Curr Diab Rep.* 2011;11(5): 384-391.

97. Korsgren O. Islet Encapsulation: Physiological Possibilities and Limitations. *Diabetes.* 2017;66(7): 1748-1754.

98. Dhanasekaran M, George JJ, Loganathan G, et al. Pig islet xenotransplantation. *Curr Opin Organ Transplant.* 2017;22(5): 452-462.

99. Matsumoto S, Tan P, Baker J, et al. Clinical porcine islet xenotransplantation under comprehensive regulation. *Transplant Proc.* 2014;46(6): 1992-1995.

100. Rezania A, Bruin JE, Riedel MJ, et al. Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. *Diabetes.* 2012;61(8): 2016-2029.

101. Liu C, Zhang W, Peradze N, et al. Mesenchymal stem cell (MSC)-mediated survival of insulin producing pancreatic beta-cells during cellular stress involves signalling via Akt and ERK1/2. *Mol Cell Endocrinol.* 2018.

102. Goland R, Egli D. Stem Cell-Derived Beta Cells for Treatment of Type 1 Diabetes? *EBioMedicine.* 2014;1(2-3): 93-94.

103. Pagliuca FW, Millman JR, Gurtler M, et al. Generation of functional human pancreatic beta cells in vitro. *Cell.* 2014;159(2): 428-439.

104. Lilly MA, Davis MF, Fabie JE, Terhune EB, Gallicano GI. Current stem cell based therapies in diabetes. *Am J Stem Cells.* 2016;5(3): 87-98.

105. Sordi V, Pellegrini S, Piemonti L. Immunological Issues After Stem Cell-Based beta Cell Replacement. *Curr Diab Rep.* 2017;17(9): 68.

106. Krishnan R, Ko D, Foster CE, 3rd, et al. Immunological Challenges Facing Translation of Alginate Encapsulated Porcine Islet Xenotransplantation to Human Clinical Trials. *Methods Mol Biol.* 2017;1479: 305-333.

107. Kumagai-Braesch M, Jacobson S, Mori H, et al. The TheraCyte device protects against islet allograft rejection in immunized hosts. *Cell Transplant.* 2013;22(7): 1137-1146.

108. Omami M, McGarrigle JJ, Reedy M, et al. Islet Microencapsulation: Strategies and Clinical Status in Diabetes. *Curr Diab Rep.* 2017;17(7): 47.

109. Cabric S, Sanchez J, Lundgren T, et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. *Diabetes.* 2007;56(8): 2008-2015.

110. Giraldo JA, Molano RD, Rengifo HR, et al. The impact of cell surface PEGylation and short-course immunotherapy on islet graft survival in an allogeneic murine model. *Acta Biomater.* 2017;49: 272-283.

111. Papas KK, Avgoustiniatos ES, Suszynski TM. Effect of oxygen supply on the size of implantable islet-containing encapsulation devices. *Panminerva Med.* 2016;58(1): 72-77.

112. Ryan AJ, O'Neill HS, Duffy GP, O'Brien FJ. Advances in polymeric islet cell encapsulation technologies to limit the foreign body response and provide immunoisolation. *Curr Opin Pharmacol.* 2017;36: 66-71.

113. Robles L, Storrs R, Lamb M, Alexander M, Lakey JR. Current status of islet encapsulation. *Cell Transplant.* 2014;23(11): 1321-1348.

114. Strand BL, Coron AE, Skjak-Braek G. Current and Future Perspectives on Alginate Encapsulated Pancreatic Islet. *Stem Cells Transl Med.* 2017;6(4): 1053-1058.

115. Wilson JT, Cui W, Chaikof EL. Layer-by-layer assembly of a conformal nanothin PEG coating for intraportal islet transplantation. *Nano Lett.* 2008;8(7): 1940-1948.

116. Hirshberg B, Montgomery S, Wysoki MG, et al. Pancreatic islet transplantation using the nonhuman primate (rhesus) model predicts that the portal vein is superior to the celiac artery as the islet infusion site. *Diabetes.* 2002;51(7): 2135-2140.

117. Suszynski TM, Avgoustiniatos ES, Papas KK. Intraportal islet oxygenation. *J Diabetes Sci Technol.* 2014;8(3): 575-580.

118. Cantarelli E, Piemonti L. Alternative transplantation sites for pancreatic islet grafts. *Curr Diab Rep.* 2011;11(5): 364-374.

119. Bellin MD, Parazzoli S, Oseid E, et al. Defective glucagon secretion during hypoglycemia after intrahepatic but not nonhepatic islet autotransplantation. *Am J Transplant.* 2014;14(8): 1880-1886.

120. Rajab A. Islet transplantation: alternative sites. *Curr Diab Rep.* 2010;10(5): 332-337.

121. Barshes NR, Wyllie S, Goss JA. Inflammation-mediated dysfunction and apoptosis in pancreatic islet transplantation: implications for intrahepatic grafts. *J Leukoc Biol.* 2005;77(5): 587-597.

122. Montazeri L, Hojjati-Emami S, Bonakdar S, et al. Improvement of islet engrafts by enhanced angiogenesis and microparticle-mediated oxygenation. *Biomaterials.* 2016;89: 157-165.

123. Citro A, Cantarelli E, Pellegrini S, Dugnani E, Piemonti L. Anti-Inflammatory Strategies in Intrahepatic Islet Transplantation: A Comparative Study in Preclinical Models. *Transplantation.* 2018;102(2): 240-248.

124. Kim MJ, Lee Y, Jon S, Lee DY. PEGylated bilirubin nanoparticle as an anti-oxidative and anti-inflammatory demulcent in pancreatic islet xenotransplantation. *Biomaterials.* 2017;133: 242-252.

125. Citro A, Cantarelli E, Piemonti L. Anti-inflammatory strategies to enhance islet engraftment and survival. *Curr Diab Rep.* 2013;13(5): 733-744.

126. Farrar CA, Kupiec-Weglinski JW, Sacks SH. The innate immune system and transplantation. *Cold Spring Harb Perspect Med.* 2013;3(10): a015479.

127. Georgel P. Innate immune receptors in solid organ transplantation. *Hum Immunol.* 2016;77(11): 1071-1075.

128. Obhrai J, Goldstein DR. The role of toll-like receptors in solid organ transplantation. *Transplantation.* 2006;81(4): 497-502.

129. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell.* 2006;124(4): 783-801.

130. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A.* 1998;95(2): 588-593.

131. Gay NJ, Gangloff M. Structure and function of Toll receptors and their ligands. *Annu Rev Biochem.* 2007;76: 141-165.

132. Fekonja O, Avbelj M, Jerala R. Suppression of TLR signaling by targeting TIR domain-containing proteins. *Curr Protein Pept Sci.* 2012;13(8): 776-788.

133. Arancibia SA, Beltran CJ, Aguirre IM, et al. Toll-like receptors are key participants in innate immune responses. *Biol Res.* 2007;40(2): 97-112.

134. Oosting M, Cheng SC, Bolscher JM, et al. Human TLR10 is an anti-inflammatory pattern-recognition receptor. *Proc Natl Acad Sci U S A.* 2014;111(42): E4478-4484.

135. Brikos C, O'Neill LA. Signalling of toll-like receptors. *Handb Exp Pharmacol.* 2008(183): 21-50.

136. De Nardo D. Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine.* 2015;74(2): 181-189.

137. Beg AA. Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. *Trends Immunol.* 2002;23(11): 509-512.

138. Tsan MF, Gao B. Endogenous ligands of Toll-like receptors. *J Leukoc Biol.* 2004;76(3): 514-519.

139. Jozefowski S. The danger model: questioning an unconvincing theory. *Immunol Cell Biol.* 2016;94(2): 164-168.

140. Reynolds JM, Pappu BP, Peng J, et al. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity.* 2010;32(5): 692-702.

141. Abdollahi-Roodsaz S, Joosten LA, Roelofs MF, et al. Inhibition of Toll-like receptor 4 breaks the inflammatory loop in autoimmune destructive arthritis. *Arthritis Rheum.* 2007;56(9): 2957-2967.

142. Kim KW, Cho ML, Oh HJ, et al. TLR-3 enhances osteoclastogenesis through upregulation of RANKL expression from fibroblast-like synoviocytes in patients with rheumatoid arthritis. *Immunol Lett.* 2009;124(1): 9-17.

143. Guiducci C, Gong M, Xu Z, et al. TLR recognition of self nucleic acids hampers glucocorticoid activity in lupus. *Nature.* 2010;465(7300): 937-941.

144. Li M, Zhou Y, Feng G, Su SB. The critical role of Toll-like receptor signaling pathways in the induction and progression of autoimmune diseases. *Curr Mol Med.* 2009;9(3): 365-374.

145. Green NM, Marshak-Rothstein A. Toll-like receptor driven B cell activation in the induction of systemic autoimmunity. *Semin Immunol.* 2011;23(2): 106-112.

146. Jansen MAA, Spiering R, Broere F, et al. Targeting of tolerogenic dendritic cells towards heat-shock proteins: a novel therapeutic strategy for autoimmune diseases? *Immunology.* 2018;153(1): 51-59.

147. Wang S, Schmaderer C, Kiss E, et al. Recipient Toll-like receptors contribute to chronic graft dysfunction by both MyD88- and TRIF-dependent signaling. *Dis Model Mech.* 2010;3(1-2): 92-103.

148. Braza F, Brouard S, Chadban S, Goldstein DR. Role of TLRs and DAMPs in allograft inflammation and transplant outcomes. *Nat Rev Nephrol.* 2016;12(5): 281-290.

149. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature.* 1997;388(6640): 394-397.

150. Wu H, Chen G, Wyburn KR, et al. TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest.* 2007;117(10): 2847-2859.

151. Chen J, John R, Richardson JA, et al. Toll-like receptor 4 regulates early endothelial activation during ischemic acute kidney injury. *Kidney Int.* 2011;79(3): 288-299.

152. Hsieh JL, Shen PC, Wu PT, et al. Knockdown of toll-like receptor 4 signaling pathways ameliorate bone graft rejection in a mouse model of allograft transplantation. *Sci Rep.* 2017;7: 46050.

153. Oetting WS, Guan W, Schladt DP, et al. Donor polymorphisms of toll-like receptor 4 associated with graft failure in liver transplant recipients. *Liver Transpl.* 2012;18(12): 1399-1405.

154. Zhao H, Perez JS, Lu K, George AJ, Ma D. Role of Toll-like receptor-4 in renal graft ischemia-reperfusion injury. *Am J Physiol Renal Physiol.* 2014;306(8): F801-811.

155. Wu H, Ma J, Wang P, et al. HMGB1 contributes to kidney ischemia reperfusion injury. *J Am Soc Nephrol.* 2010;21(11): 1878-1890.

156. Samstein B, Johnson GB, Platt JL. Toll-like receptor-4 and allograft responses. *Transplantation.* 2004;77(3): 475-477.

157. Goldstein DR, Tesar BM, Akira S, Lakkis FG. Critical role of the Toll-like receptor signal adaptor protein MyD88 in acute allograft rejection. *J Clin Invest.* 2003;111(10): 1571-1578.

158. Helming L. Inflammation: cell recruitment versus local proliferation. *Curr Biol.* 2011;21(14): R548-550.

159. Semeraro F, Ammollo CT, Morrissey JH, et al. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood.* 2011;118(7): 1952-1961.

160. Shao Z, Jiao B, Liu T, Cheng Y, Liu H, Liu Y. TAK-242 treatment ameliorates liver ischemia/reperfusion injury by inhibiting TLR4 signaling pathway in a swine model of Maastricht-category-III cardiac death. *Biomed Pharmacother.* 2016;84: 495-501.

161. Frelin C, Imbert V, Bottero V, et al. Inhibition of the NF-kappaB survival pathway via caspase-dependent cleavage of the IKK complex scaffold protein and NF-kappaB essential modulator NEMO. *Cell Death Differ.* 2008;15(1): 152-160.

162. Harper SJ, LoGrasso P. Signalling for survival and death in neurones: the role of stress-activated kinases, JNK and p38. *Cell Signal.* 2001;13(5): 299-310.

163. Rice TW, Wheeler AP, Bernard GR, et al. A randomized, double-blind, placebo-controlled trial of TAK-242 for the treatment of severe sepsis. *Crit Care Med.* 2010;38(8): 1685-1694.

164. Matsunaga N, Tsuchimori N, Matsumoto T, Ii M. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. *Mol Pharmacol.* 2011;79(1): 34-41.

165. Wu C, Ding X, Zhou C, et al. Inhibition of intimal hyperplasia in murine aortic allografts by administration of a small-molecule TLR4 inhibitor TAK-242. *Sci Rep.* 2017;7(1): 15799.

166. Shibata A, Sugiura K, Furuta Y, Mukumoto Y, Kaminuma O, Akiyama M. Toll-like receptor 4 antagonist TAK-242 inhibits autoinflammatory symptoms in DITRA. *J Autoimmun.* 2017;80: 28-38.

167. Pan LF, Yu L, Wang LM, et al. The Toll-like receptor 4 antagonist TAK-242 protects against chronic pancreatitis in rats. *Mol Med Rep.* 2017;16(4): 3863-3868.

168. Zhang D, Li H, Li T, et al. TLR4 inhibitor resatorvid provides neuroprotection in experimental traumatic brain injury: implication in the treatment of human brain injury. *Neurochem Int.* 2014;75: 11-18.

169. Tramullas M, Finger BC, Moloney RD, et al. Toll-like receptor 4 regulates chronic stress-induced visceral pain in mice. *Biol Psychiatry.* 2014;76(4): 340-348.

170. Joo SH. Lipid A as a Drug Target and Therapeutic Molecule. *Biomol Ther (Seoul).* 2015;23(6): 510-516.

171. Tidswell M, Tillis W, Larosa SP, et al. Phase 2 trial of eritoran tetrasodium (E5564), a toll-like receptor 4 antagonist, in patients with severe sepsis. *Crit Care Med.* 2010;38(1): 72-83.

172. Zou Y, Zhang L, Yang L, et al. "Click" chemistry in polymeric scaffolds: Bioactive materials for tissue engineering. *J Control Release.* 2018;273: 160-179.

173. Baskin JM, Prescher JA, Laughlin ST, et al. Copper-free click chemistry for dynamic in vivo imaging. *Proc Natl Acad Sci U S A.* 2007;104(43): 16793-16797.

174. Bertozzi CR. A decade of bioorthogonal chemistry. *Acc Chem Res.* 2011;44(9): 651-653.

175. Prescher JA, Bertozzi CR. Chemistry in living systems. *Nat Chem Biol.* 2005;1(1): 13-21.

176. Saxon E, Bertozzi CR. Cell surface engineering by a modified Staudinger reaction. *Science.* 2000;287(5460): 2007-2010.

177. Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proc Natl Acad Sci U S A.* 2008;105(7): 2415-2420.

178. Song CX, Szulwach KE, Fu Y, et al. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. *Nat Biotechnol.* 2011;29(1): 68-72.

179. Wee YM, Lim DG, Kim YH, et al. Cell surface modification by activated polyethylene glycol prevents allosensitization after islet transplantation. *Cell Transplant.* 2008;17(10-11): 1257-1269.

180. Cabric S, Sanchez J, Johansson U, et al. Anchoring of vascular endothelial growth factor to surface-immobilized heparin on pancreatic islets: implications for stimulating islet angiogenesis. *Tissue Eng Part A.* 2010;16(3): 961-970.

181. Lou S, Zhang X, Zhang J, Deng J, Kong D, Li C. Pancreatic islet surface bioengineering with a heparin-incorporated starPEG nanofilm. *Mater Sci Eng C Mater Biol Appl.* 2017;78: 24-31.

182. Fishburn CS. The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. *J Pharm Sci.* 2008;97(10): 4167-4183.

183. Alconcel SNS, Baas AS, Maynard HD. FDA-approved poly(ethylene glycol)-protein conjugate drugs. *Polymer Chemistry.* 2011;2(7): 1442-1448.

184. Santi DV, Schneider EL, Reid R, Robinson L, Ashley GW. Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates. *Proc Natl Acad Sci U S A.* 2012;109(16): 6211-6216.

185. Schneider EL, Henise J, Reid R, Ashley GW, Santi DV. Hydrogel Drug Delivery System Using Self-Cleaving Covalent Linkers for Once-a-Week Administration of Exenatide. *Bioconjug Chem.* 2016;27(5): 1210-1215.

186. Ramnath RD, Maillard E, Jones K, et al. In Vitro Assessment of Human Islet Vulnerability to Instant Blood-Mediated Inflammatory Reaction (IBMIR) and Its Use to Demonstrate a Beneficial Effect of Tissue Culture. *Cell Transplant.* 2015;24(12): 2505-2512.

187. Leal AS, Williams CR, Royce DB, Pioli PA, Sporn MB, Liby KT. Bromodomain inhibitors, JQ1 and I-BET 762, as potential therapies for pancreatic cancer. *Cancer Lett.* 2017;394: 76-87.

188. Liu R, Wei H, Gao P, et al. CD47 promotes ovarian cancer progression by inhibiting macrophage phagocytosis. *Oncotarget.* 2017;8(24): 39021-39032.

189. Ashrafi F, Rezaie N, Mousavi S. New Indications for Dabigatran: A Suggestion from a Drug Use Evaluation Study. *J Res Pharm Pract.* 2017;6(4): 211-216.

190. Shen H, Kreisel D, Goldstein DR. Processes of sterile inflammation. *J Immunol.* 2013;191(6): 2857-2863.

191. Bandukwala HS, Gagnon J, Togher S, et al. Selective inhibition of CD4+ T-cell cytokine production and autoimmunity by BET protein and c-Myc inhibitors. *Proc Natl Acad Sci U S A.* 2012;109(36): 14532-14537.

192. Rodriguez PL, Harada T, Christian DA, Pantano DA, Tsai RK, Discher DE. Minimal "Self" peptides that inhibit phagocytic clearance and enhance delivery of nanoparticles. *Science.* 2013;339(6122): 971-975.

193. SoRelle JA, Kanak MA, Itoh T, Horton JM, Naziruddin B, Kane RR. Comparison of surface modification chemistries in mouse, porcine, and human islets. *J Biomed Mater Res A.* 2015;103(3): 869-877.

194. Hering BJ, Clarke WR, Bridges ND, et al. Phase 3 Trial of Transplantation of Human Islets in Type 1 Diabetes Complicated by Severe Hypoglycemia. *Diabetes Care.* 2016;39(7): 1230-1240.

195. Bellin MD, Abu-El-Haija M, Morgan K, et al. A multicenter study of total pancreatectomy with islet autotransplantation (TPIAT): POST (Prospective Observational Study of TPIAT). *Pancreatology.* 2018;18(3): 286-290.

196. Takita M, Matsumoto S, Shimoda M, et al. Safety and tolerability of the T-cell depletion protocol coupled with anakinra and etanercept for clinical islet cell transplantation. *Clin Transplant.* 2012;26(5): E471-484.

197. Khosravi-Maharlooei M, Hajizadeh-Saffar E, Tahamtani Y, et al. THERAPY OF ENDOCRINE DISEASE: Islet transplantation for type 1 diabetes: so close and yet so far away. *Eur J Endocrinol.* 2015;173(5): R165-183.

198. Ploeg RJ, Friend PJ. New strategies in organ preservation: current and future role of machine perfusion in organ transplantation. *Transpl Int.* 2015;28(6): 633.

199. Abdelli S, Ansite J, Roduit R, et al. Intracellular stress signaling pathways activated during human islet preparation and following acute cytokine exposure. *Diabetes.* 2004;53(11): 2815-2823.

200. Itoh T, Iwahashi S, Shimoda M, et al. High-mobility group box 1 expressions in hypoxia-induced damaged mouse islets. *Transplant Proc.* 2011;43(9): 3156-3160.

201. Abdelli S, Papas KK, Mueller KR, Murtaugh MP, Hering BJ, Bonny C. Regulation of the JNK3 signaling pathway during islet isolation: JNK3 and c-fos as new markers of islet quality for transplantation. *PLoS One.* 2014;9(7): e99796.

202. Noguchi H, Nakai Y, Ueda M, et al. Activation of c-Jun NH2-terminal kinase (JNK) pathway during islet transplantation and prevention of islet graft loss by intraportal injection of JNK inhibitor. *Diabetologia.* 2007;50(3): 612-619.

203. Bottino R, Balamurugan AN, Tse H, et al. Response of human islets to isolation stress and the effect of antioxidant treatment. *Diabetes.* 2004;53(10): 2559-2568.

204. Noguchi H, Nakai Y, Matsumoto S, et al. Cell permeable peptide of JNK inhibitor prevents islet apoptosis immediately after isolation and improves islet graft function. *Am J Transplant.* 2005;5(8): 1848-1855.

205. Omori K, Valiente L, Orr C, et al. Improvement of human islet cryopreservation by a p38 MAPK inhibitor. *Am J Transplant.* 2007;7(5): 1224-1232.

206. Peng H, Olsen G, Tamura Y, et al. Inhibition of inflammatory cytokine-induced response in human islet cells by withaferin A. *Transplant Proc.* 2010;42(6): 2058-2061.

207. Chang HW, Li RN, Wang HR, et al. Withaferin A Induces Oxidative Stress-Mediated Apoptosis and DNA Damage in Oral Cancer Cells. *Front Physiol.* 2017;8: 634.

208. He Y, Cai C, Sun S, Wang X, Li W, Li H. Effect of JNK inhibitor SP600125 on hair cell regeneration in zebrafish (Danio rerio) larvae. *Oncotarget.* 2016;7(32): 51640-51650.

209. Papademetrio DL, Lompardia SL, Simunovich T, et al. Inhibition of Survival Pathways MAPK and NF-kB Triggers Apoptosis in Pancreatic Ductal Adenocarcinoma Cells via Suppression of Autophagy. *Target Oncol.* 2016;11(2): 183-195.

210. Alegre ML, Leemans J, Le Moine A, et al. The multiple facets of toll-like receptors in transplantation biology. *Transplantation.* 2008;86(1): 1-9.

211. Rosadini CV, Kagan JC. Early innate immune responses to bacterial LPS. *Curr Opin Immunol.* 2017;44: 14-19.

212. Goligorsky MS. TLR4 and HMGB1: partners in crime? *Kidney Int.* 2011;80(5): 450-452.

213. Schulthess FT, Paroni F, Sauter NS, et al. CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. *Cell Metab.* 2009;9(2): 125-139.

214. Howell J, Gow P, Angus P, Visvanathan K. Role of toll-like receptors in liver transplantation. *Liver Transpl.* 2014;20(3): 270-280.

215. Chang CA, Akinbobuyi B, Quintana JM, Yoshimatsu G, Naziruddin B, Kane RR. Ex-vivo generation of drug-eluting islets improves transplant outcomes by inhibiting TLR4-Mediated NFkB upregulation. *Biomaterials.* 2018;159: 13-24.

216. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 2012;9(7): 676-682.

217. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol.* 2001;2(8): 675-680.

218. Saravanan PB, Kanak MA, Chang CA, et al. Islet damage during isolation as assessed by miRNAs and the correlation of miRNA levels with posttransplantation outcome in islet autotransplantation. *Am J Transplant.* 2017.

219. Kanak MA, Takita M, Shahbazov R, et al. Evaluation of MicroRNA375 as a Novel Biomarker for Graft Damage in Clinical Islet Transplantation. *Transplantation.* 2015;99(8): 1568-1573.

220. Nilsson B, Ekdahl KN, Korsgren O. Control of instant blood-mediated inflammatory reaction to improve islets of Langerhans engraftment. *Curr Opin Organ Transplant.* 2011;16(6): 620-626.

221. Vivot K, Langlois A, Jeandidier N, et al. Instant blood-mediated inflammatory reaction during islet transplantation: the role of Toll-like receptors signaling pathways. *Transplant Proc.* 2011;43(9): 3192-3194.

222. Yoshimatsu G, Kunnathodi F, Saravanan PB, et al. Pancreatic beta-Cell-Derived IP-10/CXCL10 Isletokine Mediates Early Loss of Graft Function in Islet Cell Transplantation. *Diabetes.* 2017;66(11): 2857-2867.

223. Melzi R, Mercalli A, Sordi V, et al. Role of CCL2/MCP-1 in islet transplantation. *Cell Transplant.* 2010;19(8): 1031-1046.

224. Nackiewicz D, Dan M, He W, et al. TLR2/6 and TLR4-activated macrophages contribute to islet inflammation and impair beta cell insulin gene expression via IL-1 and IL-6. *Diabetologia.* 2014;57(8): 1645-1654.

225. Pekalski J, Zuk PJ, Kochanczyk M, et al. Spontaneous NF-kappaB activation by autocrine TNFalpha signaling: a computational analysis. *PLoS One.* 2013;8(11): e78887.

226. Tuovinen E, Keto J, Nikkila J, Matto J, Lahteenmaki K. Cytokine response of human mononuclear cells induced by intestinal Clostridium species. *Anaerobe.* 2013;19: 70-76.

227. Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem.* 1995;270(28): 16483-16486.

228. Paraskevas S, Aikin R, Maysinger D, et al. Modulation of JNK and p38 stress activated protein kinases in isolated islets of Langerhans: insulin as an autocrine survival signal. *Ann Surg.* 2001;233(1): 124-133.

229. Fishman JA. Infection in Organ Transplantation. *Am J Transplant.* 2017;17(4): 856-879.

230. Lim WH, Eris J, Kanellis J, et al. A systematic review of conversion from calcineurin inhibitor to mammalian target of rapamycin inhibitors for maintenance immunosuppression in kidney transplant recipients. *Am J Transplant.* 2014;14(9): 2106-2119.

231. Ojo AO, Held PJ, Port FK, et al. Chronic renal failure after transplantation of a nonrenal organ. *N Engl J Med.* 2003;349(10): 931-940.

232. Kuschal C, Thoms KM, Schubert S, et al. Skin cancer in organ transplant recipients: effects of immunosuppressive medications on DNA repair. *Exp Dermatol.* 2012;21(1): 2-6.

233. Ryan EA, Shandro T, Green K, et al. Assessment of the severity of hypoglycemia and glycemic lability in type 1 diabetic subjects undergoing islet transplantation. *Diabetes.* 2004;53(4): 955-962.

234. Pedersen-Bjergaard U, Pramming S, Heller SR, et al. Severe hypoglycaemia in 1076 adult patients with type 1 diabetes: influence of risk markers and selection. *Diabetes Metab Res Rev.* 2004;20(6): 479-486.

235. Choudhary P, Rickels MR, Senior PA, et al. Evidence-informed clinical practice recommendations for treatment of type 1 diabetes complicated by problematic hypoglycemia. *Diabetes Care.* 2015;38(6): 1016-1029.

236. Holmes-Walker DJ, Gunton JE, Hawthorne W, et al. Islet Transplantation Provides Superior Glycemic Control With Less Hypoglycemia Compared With Continuous Subcutaneous Insulin Infusion or Multiple Daily Insulin Injections. *Transplantation.* 2017;101(6): 1268-1275.

237. Delaune V, Berney T, Lacotte S, Toso C. Intraportal islet transplantation: the impact of the liver microenvironment. *Transpl Int.* 2017;30(3): 227-238.

238. Porteous MK, Diamond JM, Christie JD. Primary graft dysfunction: lessons learned about the first 72 h after lung transplantation. *Curr Opin Organ Transplant.* 2015;20(5): 506-514.

239. Lu L, Zhou H, Ni M, et al. Innate Immune Regulations and Liver Ischemia-Reperfusion Injury. *Transplantation.* 2016;100(12): 2601-2610.

240. Bzeizi KI, Jalan R, Plevris JN, Hayes PC. Primary graft dysfunction after liver transplantation: from pathogenesis to prevention. *Liver Transpl Surg.* 1997;3(2): 137-148.

241. Nankivell BJ, Kuypers DR. Diagnosis and prevention of chronic kidney allograft loss. *Lancet.* 2011;378(9800): 1428-1437.

242. Iyer A, Kumarasinghe G, Hicks M, et al. Primary graft failure after heart transplantation. *J Transplant.* 2011;2011: 175768.

243. Troppmann C. Complications after pancreas transplantation. *Curr Opin Organ Transplant.* 2010;15(1): 112-118.

244. Oto T, Excell L, Griffiths AP, et al. Association between primary graft dysfunction among lung, kidney and heart recipients from the same multiorgan donor. *Am J Transplant.* 2008;8(10): 2132-2139.

245. Lee DD, Croome KP, Shalev JA, et al. Early allograft dysfunction after liver transplantation: an intermediate outcome measure for targeted improvements. *Ann Hepatol.* 2016;15(1): 53-60.

246. Kanak MA, Takita M, Itoh T, et al. Alleviation of instant blood-mediated inflammatory reaction in autologous conditions through treatment of human islets with NF-kappaB inhibitors. *Transplantation.* 2014;98(5): 578-584.

247. Wang J, Sun Z, Gou W, et al. alpha-1 Antitrypsin Enhances Islet Engraftment by Suppression of Instant Blood-Mediated Inflammatory Reaction. *Diabetes.* 2017;66(4): 970-980.

248. Desai T, Shea LD. Advances in islet encapsulation technologies. *Nat Rev Drug Discov.* 2017;16(5): 338-350.

249. Teramura Y, Asif S, Ekdahl KN, Nilsson B. Cell Surface Engineering for Regulation of Immune Reactions in Cell Therapy. *Adv Exp Med Biol.* 2015;865: 189-209.

250. Teramura Y, Iwata H. Surface modification of islets with PEG-lipid for improvement of graft survival in intraportal transplantation. *Transplantation.* 2009;88(5): 624-630.

251. Alegre ML, Goldstein DR, Chong AS. Toll-like receptor signaling in transplantation. *Curr Opin Organ Transplant.* 2008;13(4): 358-365.

252. Kolb M, Bonniaud P, Galt T, et al. Differences in the fibrogenic response after transfer of active transforming growth factor-beta1 gene to lungs of "fibrosis-prone" and "fibrosis-resistant" mouse strains. *Am J Respir Cell Mol Biol.* 2002;27(2): 141-150.

253. SoRelle JA, Itoh T, Peng H, et al. Withaferin A inhibits pro-inflammatory cytokine-induced damage to islets in culture and following transplantation. *Diabetologia.* 2013;56(4): 814-824.

254. Fort MM, Mozaffarian A, Stover AG, et al. A synthetic TLR4 antagonist has anti-inflammatory effects in two murine models of inflammatory bowel disease. *J Immunol.* 2005;174(10): 6416-6423.

255. Mullarkey M, Rose JR, Bristol J, et al. Inhibition of endotoxin response by e5564, a novel Toll-like receptor 4-directed endotoxin antagonist. *J Pharmacol Exp Ther.* 2003;304(3): 1093-1102.

256. Yamada M, Ichikawa T, Yamano T, et al. Optically active cyclohexene derivative as a new antisepsis agent: an efficient synthesis of ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242). *Chem Pharm Bull (Tokyo).* 2006;54(1): 58-62.

257. Ammazzalorso A, De Filippis B, Giampietro L, Amoroso R. N-acylsulfonamides: Synthetic routes and biological potential in medicinal chemistry. *Chem Biol Drug Des.* 2017.

258. Rautio J, Kumpulainen H, Heimbach T, et al. Prodrugs: design and clinical applications. *Nat Rev Drug Discov.* 2008;7(3): 255-270.

259. Kawamoto T, Ii M, Kitazaki T, Iizawa Y, Kimura H. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *Eur J Pharmacol.* 2008;584(1): 40-48.

260. Tsung A, Tohme S, Billiar TR. High-mobility group box-1 in sterile inflammation. *J Intern Med.* 2014;276(5): 425-443.

261. Ashley GW, Henise J, Reid R, Santi DV. Hydrogel drug delivery system with predictable and tunable drug release and degradation rates. *Proc Natl Acad Sci U S A.* 2013;110(6): 2318-2323.

262. Chang PV, Prescher JA, Sletten EM, et al. Copper-free click chemistry in living animals. *Proc Natl Acad Sci U S A.* 2010;107(5): 1821-1826.

263. Thirumurugan P, Matosiuk D, Jozwiak K. Click chemistry for drug development and diverse chemical-biology applications. *Chem Rev.* 2013;113(7): 4905-4979.

264. Huang S, Connolly PJ, Lin R, Emanuel S, Middleton SA. Synthesis and evaluation of N-acyl sulfonamides as potential prodrugs of cyclin-dependent kinase inhibitor JNJ-7706621. *Bioorg Med Chem Lett.* 2006;16(14): 3639-3641.

265. Meghani NM, Amin HH, Lee BJ. Mechanistic applications of click chemistry for pharmaceutical drug discovery and drug delivery. *Drug Discov Today.* 2017.

266. Trexler AJ, Taraska JW. Regulation of insulin exocytosis by calcium-dependent protein kinase C in beta cells. *Cell Calcium.* 2017;67: 1-10.

267. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. *Nature.* 2000;406(6797): 782-787.

268. Yang H, Hreggvidsdottir HS, Palmblad K, et al. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci U S A.* 2010;107(26): 11942-11947.

269. Reynolds JM, Martinez GJ, Chung Y, Dong C. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proc Natl Acad Sci U S A.* 2012;109(32): 13064-13069.

270. Merry HE, Phelan P, Hwang B, Mulligan MS. Validating the use of short interfering RNA as a novel technique for cell-specific target gene knockdown in lung ischemia–reperfusion injury. *The Journal of Thoracic and Cardiovascular Surgery.* 2016;151(2): 499-506.

271. Garate I, Garcia-Bueno B, Madrigal JL, et al. Toll-like 4 receptor inhibitor TAK-242 decreases neuroinflammation in rat brain frontal cortex after stress. *J Neuroinflammation.* 2014;11: 8.

272. Hua F, Tang H, Wang J, et al. TAK-242, an antagonist for Toll-like receptor 4, protects against acute cerebral ischemia/reperfusion injury in mice. *J Cereb Blood Flow Metab.* 2015;35(4): 536-542.

273. Piemonti L, Leone BE, Nano R, et al. Human pancreatic islets produce and secrete MCP-1/CCL2: relevance in human islet transplantation. *Diabetes.* 2002;51(1): 55-65.

274. Baker MS, Chen X, Rotramel AR, et al. Genetic deletion of chemokine receptor CXCR3 or antibody blockade of its ligand IP-10 modulates posttransplantation graft-site lymphocytic infiltrates and prolongs functional graft survival in pancreatic islet allograft recipients. *Surgery.* 2003;134(2): 126-133.

275. Jin SM, Oh SH, Oh BJ, et al. Benefits of PEGylation in the early post-transplant period of intraportal islet transplantation as assessed by magnetic resonance imaging of labeled islets. *Islets.* 2014;6(1): e27827.

276. Jung YS, Jeong JH, Yook S, et al. Surface modification of pancreatic islets using heparin-DOPA conjugate and anti-CD154 mAb for the prolonged survival of intrahepatic transplanted islets in a xenograft model. *Biomaterials.* 2012;33(1): 295-303.

277. Stabler CL, Sun XL, Cui W, Wilson JT, Haller CA, Chaikof EL. Surface re-engineering of pancreatic islets with recombinant azido-thrombomodulin. *Bioconjug Chem.* 2007;18(6): 1713-1715.

278. Hameed AM, Hawthorne WJ, Pleass HC. Advances in organ preservation for transplantation. *ANZ J Surg.* 2016.

279. Emamaullee JA, Shapiro AM. Factors influencing the loss of beta-cell mass in islet transplantation. *Cell Transplant.* 2007;16(1): 1-8.

280. Barbas AS, Knechtle SJ. Expanding the Donor Pool With Normothermic Ex Vivo Liver Perfusion: The Future Is Now. *Am J Transplant.* 2016;16(11): 3075-3076.

281. Hosgood SA, Saeb-Parsy K, Hamed MO, Nicholson ML. Successful Transplantation of Human Kidneys Deemed Untransplantable but Resuscitated by Ex Vivo Normothermic Machine Perfusion. *Am J Transplant.* 2016;16(11): 3282-3285.

282. Najarian JS, Sutherland DE, Matas AJ, Goetz FC. Human islet autotransplantation following pancreatectomy. *Transplant Proc.* 1979;11(1): 336-340.

283. Liapi E, Lee KH, Georgiades CC, Hong K, Geschwind JF. Drug-eluting particles for interventional pharmacology. *Tech Vasc Interv Radiol.* 2007;10(4): 261-269.

284. Wang P, Zhuo X, Chu W, Tang X. Exenatide-loaded microsphere/thermosensitive hydrogel long-acting delivery system with high drug bioactivity. *Int J Pharm.* 2017;528(1-2): 62-75.