NOVEL FORMS OF THROMBOMODULIN AND CD47 PROTEINS DISPLAYED ON SYNGENEIC ISLETS SYNERGISTICALLY MITIGATE IBMIR AND CONTRIBUTE TO THE ISLET ENGRAFTMENT

A Dissertation

Presented to

The Faculty of the Graduate School

at the

University of Missouri

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

in Immunology

By

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May 2023

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Dedication

To Mom and family, thank you for the enormous encouragement for my journey.

Acknowledgments

I would first like to thank my parents for always supporting me to do what makes me peaceful, encouraged, and happy and to follow my dreams. Without your love and support, I would not be the person or scientist I am today. I would also like to thank my mom, without your love and support, I would not be the person or scientist I am today. I would also like to thank my siblings for always listening to and supporting me. The biggest enormous thanks to my nephews or treasures, Berat and Eren, who always make me happy and peaceful.

A huge thanks to my friends are also necessary. I would not have made it without their uncountable support. Thank you for helping and accompanying me to relieve me even during stressful times and always being with me. A big thanks to Trang, Carlos, Nazende, Sumeyye, Esra, Orcun, Baris, Kelley, Hazel, Irina, Chris, Minhom, and Anne for always being cool with my ideas and obsessions and letting me be who I am.

I also owe a thank you to my fellow postdocs and graduate students, especially those at the University of Louisville and the University of Missouri. Thanks a lot for your friendships, teaching, support, and sharing. They were always there to discuss experiments and share protocols, experiences, and times whenever I needed them. Also, thanks for making my graduate journey more bearable.

I owe to thank my committee members and other faculties in MPT or UofL for their guidance and support of my Ph.D. career. I am lucky to have been in a scientific environment with supportive and thoughtful scientists for allowing me to be proud of my research and my abilities.

Last but not least, I would like to thank my mentor, Dr. Haval Shirwan, and my co-mentor, Dr. Esma S. Yolcu, for all your guidance and support during my time at the University of Louisville and the University of Missouri, Columbia. I am always glad to meet both of you, and you have taught me to “how to be a scientist and how to do science” and what those do research and my scientific career. Thanks for allowing me to work on extensive studies and collaborate with important scientists from top-notch institutes. Thank you for trusting in me and giving me this important chance to show my abilities and understanding of the bottom line of translational science. I have been a better scientist and person since our first interview.

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NOVEL FORMS OF THROMBOMODULIN AND CD47 PROTEINS DISPLAYED ON SYNGENEIC ISLETS SYNERGISTICALLY MITIGATE IBMIR AND CONTRIBUTE TO THE ISLET ENGRAFTMENT

Ali Turan

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ABSTRACT

The main aim of this dissertation was to display novel immunological ligands, Thrombomodulin (TM) and CD47, on the surface of islets to promote islet engraftment by controlling thrombotic/inflammatory reactions associated with an immediate blood-mediated inflammatory response (IBMIR). In chapter 3, syngeneic pancreatic islets are engineered with a novel form of TM (SA-TM) to maintain euglycemia by preventing the immediate loss of islet transplant grafts. In chapter 4, the pancreatic islet was engineered with SA-TM and CD47 combination to prevent IBMIR from contributing to islet engraftment in a model of syngeneic marginal mass islet transplantation.

Islet-blood interaction initiates IBMIR consisting of activation of the coagulation and complement systems and recruitment of myeloid cells, resulting in most initial graft loss. TM is an important regulator of coagulation and a cofactor of Activated Protein C (APC). TM and APC modulate the homeostasis of thrombosis, diminish the release of inflammatory mediators, and inhibit the immune cell infiltration into islet grafts. Moreover, CD47 binds to signal regulatory protein alpha (SIRPα),

delivering a “do not eat me signal” to suppress phagocytosis and inhibition of myeloid cell activation. Hence, we hypothesized that SA-TM and SA-CD47 combination on islets maintain islet engraftments in a syngeneic marginal mass model of intraportal transplantation by mitigating thrombotic/inflammatory reactions. 200 IEQ islet engineered with SA-TM and transplanted into syngeneic STZ-induced diabetic mice. Recipients with SA-TM-engineered islets demonstrated better outcomes compared to recipients whose-islets engineered with SA served as controls (83 vs 28%). There was a significant reduction in the graft infiltration immune cells and the gene expression of inflammatory genes, such as High mobility group box 1 (HMGB-1), Tissue Factor (TF), and Interleukin 6 (IL-6), associated with IBMIR.

To show the efficiency of combinational, we herein engineered 150 IEQ islets with SA-TM or SA-TM/SA-CD47. Importantly, SA-TM/SA-CD47-engineered islets showed improved engraftment, and long-term function in a syngeneic minimal mass model of intraportal islet transplantation compared to SA-TM engineered or unmodified islet served control (62.5 %, vs 50 % and 29% respectively). Enhanced survival revealed the decreased recruitment of myeloid cells and levels of numerous genes related to IBMIR and proinflammatory signals and hypoxia. Overall, SA-TM and SA-CD47 displayed on the islets serve as an effective platform to prevent IBMIR with important clinical implications for islet transplantation to treat type 1 diabetes and chronic pancreatitis.

Chapter 1

Introduction

Type 1 Diabetes

Type 1 diabetes (T1D) is an organ-specific and chronic autoimmune disease perpetuated by proinflammatory autoreactive T cells targeting insulin-producing pancreatic β cells via direct and indirect immune mechanisms [1]. According to the Centers for Disease Control and Prevention (CDC), more than half a million children have T1D worldwide, with incidence escalating yearly. In addition, the number of adult patients who have T1D is also rapidly escalating, making up forty percent of all new cases [1-3]. Altogether, the estimated medical costs of diabetes in the United States were about $327 billion in 2017 [3].

Technological developments in monitoring blood glucose levels and maintaining insulin release in circulation have recently contributed to the management of T1D. Still, the lack of preventive treatment protocols for T1D represents a critical unmet medical need for patients with T1D [3]. Exogenous insulin as a treatment for T1D is necessary to prevent fatal complications from hyperglycemia, but it cannot prevent recurrent hyperglycemic episodes and has serious long-term complications. Further, subcutaneous insulin delivery does not mimic the blood glucose level provided by insulin-producing pancreatic β-cells and other cells in the pancreas. Patient vigilance, the key to T1D management, may be hindered by complications such as hypoglycemic unawareness, in which patients are unable to perceive hypoglycemia symptoms [4]. For these reasons and more, it is critical

to develop better preventative treatments for T1D. The therapeutic ideal for T1D would maintain physiological glucose control without exogenous insulin administration [5].

Islet transplantation as a cure for *T1D* and major challenges

While the discovery of insulin over a century ago has proved to be a life-saving intervention for patients with T1D, there is no treatment to intercept the loss or dysfunction of insulin-producing pancreatic β cells. Hence, β cell replacement therapies are promising to cure diabetes [5]. An effective β-cell replacement therapy, pancreatic islet transplantation maintains glucose homeostasis and prevents hypoglycemic episodes without dependence on exogenous insulin administration [5, 6]. Additionally, islet transplantation decreases the level of hemoglobin A1C (HbA1c) and relieves microvascular complications. Thus, islet transplantation is considered one of the safest procedures with a high potential to treat T1D to maintain euglycemia. Recent progress in techniques regarding isolation, islet process, transplantation methods, and immunosuppressors has contributed to substantial achievements in treatment and safety outcomes. Thus, islet transplantation is considered one of the safest procedures with a high potential to treat T1D to maintain euglycemia. Furthermore, recent progress in techniques regarding isolation, islet process, transplantation methods, and immunosuppressors has contributed to substantial achievements in treatment and safety outcomes for patients [5-8].

Major barriers to successful allogeneic islet transplantation

While significant improvements have recently been made in protecting islet grafts from immune-mediated destruction both pre- and post-transplantation, several obstacles remain in maintaining adequate pancreatic β cell mass to retain glycemic control [5, 8]. For example, significant obstacles to successful pancreatic islet transplantation include i) instant blood-mediated inflammatory reactions (IBMIR), ii) donor-specific adaptive immunity, iii) shortage of cadaveric donor islets, and iv) adverse effects of immunosuppression to modulate the immune response to the grafts [5].

IBMIR, discussed in further detail in the next section, is responsible for significant islet damage following islet-blood interaction for autologous and allogeneic islet transplants. During islet isolation, processing, and transplantation, islets get stressed and produce inflammatory mediators, which can initiate the recruitment of innate immune cells upon infusion of islets through the portal vein [9, 10]. In addition, coagulation, proinflammatory cytokines, and hypoxia can also mediate IBMIR. As a result, IBMIR can cause a 50-70% loss of initial islet mass in syngeneic and allogeneic islet transplantation, which already requires multiple donors per transplant [9].

The innate immune response regulates the adaptive immune response to islet grafts. For successful islet transplantation, multiple donors are required, which lowers the success of HLA-matching. In this case, a broad immune HLA profile

increases the grafts' susceptibility to host immune recognition via both direct and indirect pathways [5, 8]. Donor islet resident antigen-presenting cells, such as macrophages and dendritic cells (DC), present antigens to host T cells, called direct antigen presentation. For indirect antigen-presenting, recipient antigen-presenting cells present donor antigens to host adaptive immune cells [5, 11, 12].

It has been shown that indirectly activated effector T cells infiltrate graft sites and prompt immune response to allogeneic islets. Once cytotoxic T cells are activated, they infiltrate the graft sites and destroy the transplanted islets. Moreover, host CD4 helper T cells play vital roles in islet damage by stimulating innate immune responses via inflammatory cytokines and inducing antibody production by B cells. Innate immune cells, such as macrophages, neutrophils, and NK cells, contribute to islet damage via recognition of non-host major histocompatibility complex (MHC) class I, alloantibody, and SIRP alpha polymorphism. Because the endurance of allogeneic islets is limited by the host's immune response to the allograft, immune suppressants are needed to prevent graft rejection [10, 13].

Administration of broad-spectrum immunosuppressive drugs to prevent islet graft rejection can have detrimental effects on both the health of the patient and the survival of the grafts. The use of immunosuppressive agents can induce lymphopenia, impair revascularization, and inhibit the proliferation of β cells. Furthermore, prolonged administration of these drugs leads to significant and diverse adverse effects and increases the risk of opportunistic infections and

cancer. Hence, transient single-agent immunotherapy is becoming more important for clinical trials to treat, delay, or prevent T1D [14, 15]. Recent interventions have mostly failed to maintain or enhance β-cell function in clinical trials due to poor targeting strategies, low drug potency, or inadequate treatment duration [5].

Instant blood-mediated inflammatory reactions (IBMIR)

When the donor islet interacts with the recipient's blood following intraportal infusion, IBMIR is instigated, leading to clot formation and inflammation [9, 16, 17]. The typical features of this thrombotic/inflammatory reaction are rapid activation of complement/coagulation cascades and the recruitment of myeloid cells expressing various inflammatory mediators that cause islet damage [16, 18].

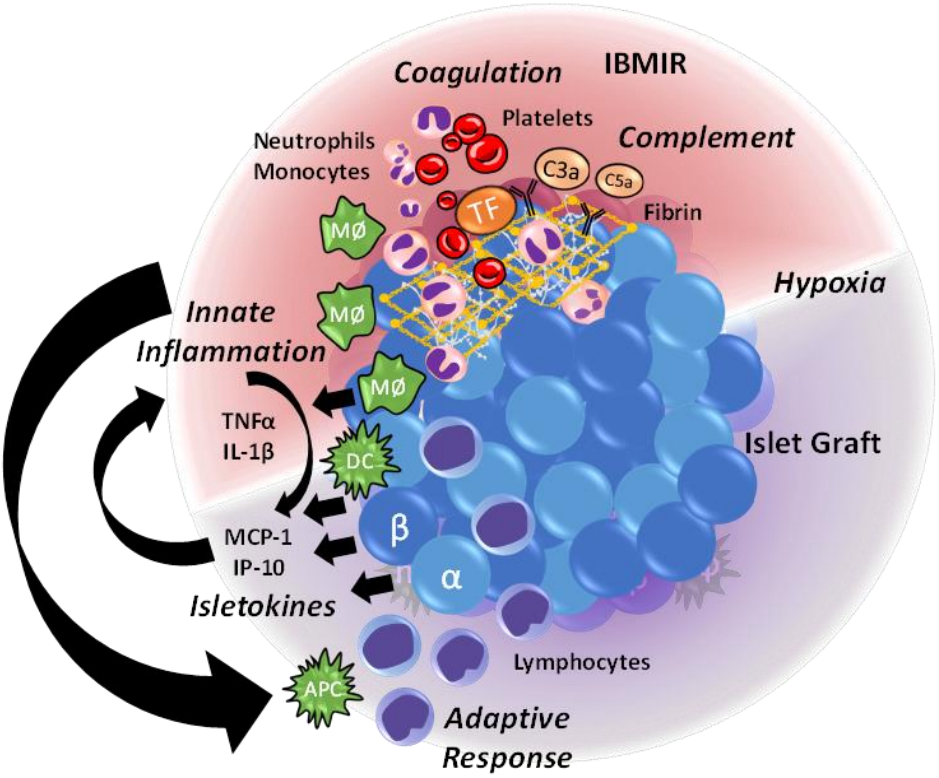


Figure 1: Several factors contribute to non-specific acute inflammation of the islets. IBMIR was initiated when damaged donor islet is exposed to recipient blood, leading to the initiation of coagulation and complement cascades, recruitment of platelets and leukocytes into islet grafts site which are highlighted in red. TF induces the infiltration of platelets and leukocytes, resulting in a fibrin clot causing coagulation. Then, innate immune cells, such as macrophages and neutrophil or DC activate adaptive immune cells, which are highlighted in blue. The figure was modified from [18].

Tissue factor

TF released by damaged or dead donor islets is one of the main triggers of IBMIR [17]. TF is a transmembrane protein and has both membrane-bound and soluble forms. The activation of TF sets off a cascade of reactions that converts prothrombin into thrombin, leading to a surge in thrombin production. Thrombin, in turn, promotes the development of fibrin clots and stimulates the activation of

platelets, facilitating the formation of a clot structure. [17, 19]. When islets get mechanically or chemically damaged during the isolation or transplantation process, they release after interacting with platelets, circulating TF can form a complex with Factor VIIa to initiate coagulation by activating the zymogen-to-enzyme transition. The complex enzyme comprising Factor IXa and Factor Xa initiates the production of thrombin, leading to the cleavage of fibrinogen and activation of Factor XIII to make fibrin clots. Transplanting a TF-expressing islet induces IBMIR via activation of coagulation and production of thrombin, which initiates inflammation. In addition to triggering blood coagulation, TF induces the expression of adhesion molecules to stimulate more leukocyte-endothelial cell adhesions, causing immune cell recruitment and inflammation [19-22]. Taken together, targeting TF may help to prevent IBMIR.

HMGB1

The release of HMGB1 from damaged or dying islet grafts can also trigger IBMIR. Under normal conditions, HMGB1 is a nuclear protein that interacts with nucleosomes to promote chromatin stability and regulate transcription. However, in the context of inflammation or injury, HMGB1 acts as a danger-associated molecular pattern (DAMP), which stimulates the recruitment of innate immune cells that play a crucial role in the early rejection of transplanted islets [5, 16, 22].

HMGB1 contributes to thrombosis by initiating platelet degranulation which results in the generation of thrombin, fibrin formation, and coagulation. In addition,

released HMGB1 can activate toll-like receptor 2 (TLR2), TLR4, and receptors for advanced glycation end products (RAGE) [23, 24]. Previously, it has been shown that HMGB1 engagement with TLR2 and TLR4 is affiliated with hypoxia-induced islet cell loss causing poor islet graft outcomes. Therefore, inhibition of HMGB1 release or neutralization of its function could mitigate inflammatory reactions and prevent the early loss of pancreatic islet graft [9, 23, 24].

Cytokines and chemokines

Besides TF and HMGB1, IBMIR is triggered by diverse proinflammatory cytokines and chemokines, such as IL-1 β, TNF-α, IFN-γ, IL-6, CXCL8, CXCL10, and CCL2 expressed at the graft site by the stressed islet, platelets, or graft infiltrates [9, 10, 16, 19, 22]. Inflammatory cytokines modulate the recruitment of immune cells and their inflammatory functions. Due to platelet aggregation hypoxia, both epithelial cells and islets produce IL-1β, IL-6, IL-8, and IFN-γ, playing important roles in the recruitment of macrophages and neutrophils and contributing to the inflammatory response to islet grafts [10, 25, 26].

Complement

IBMIR also initiates the activation of the complement cascade. Activation and accumulation of complement proteins such as C3a and C5a instigate recruitment and accumulation of leukocytes, production of inflammatory cytokines and reactive oxygen species (ROS), and overexpression of adhesion molecules on platelets and endothelial [27, 28]. In addition, C5a acts as an activator of coagulation and

TF-mediated inflammation, resulting in neutrophil activation. Studies showed that the immobilization of soluble complement receptors prevents the activation of the complement cascade [27, 29]. Therefore, effective control of IBMIR prevents immediate islet loss and positively impacts immunity for sustained graft survival [5, 10, 29, 30].

Mitigating IBMIR

IBMIR comprises coagulation, inflammatory responses, clot formation, and immune cell infiltration to the graft site causing islet loss and failure of islet engraftment [9, 16]. Several strategies for preventing and countering the effects of IBMIR are currently being pursued. For example, heparin is used in clinics to prevent coagulation. While islets were infused in media with 70 units of heparin per kg or systemic heparin infusion (3 U/Kg/hour) for 48 hours, heparin did not prevent myeloid cell infiltration and fibrin deposition. However, displayed heparin on the surface of islets (4 U heparin/ islet) via avidin-biotin interaction successfully attenuates IBMIR *in vivo* and *ex vivo* [31, 32]. In murine and porcine models, Cabric et al. found that the transplantation of marginal mass islets (300 islets/mouse) under the kidney capsule was successful, but the islet transplantation under the kidney capsule is not applicable in clinics. Hence, intraportal islet transplantation is the preferred method in clinical settings [32].

Besides heparin, other anti-coagulant strategies, such as using nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), have been performed to

weaken IBMIR [31, 33, 34]. Kuraya, D. et al. used dihydroxy-methylepoxyquinomicin (DHMEQ), an NF-κB inhibitor, to prevent early damage of islets post-transplantation and improve islet graft function in syngeneic marginal mass islet transplantation (175 islets/mouse) [33]. DHMEQ administration has been shown to reduce the release of HMGB1 and decrease inflammatory mediators, but it also has the potential to cause severe side effects, such as immunodeficiencies and an increased risk of malignancies, due to the inhibition of NF-κB. It is important to carefully evaluate the benefits and risks of any treatment before it is widely adopted [5, 35]. Moreover, anti-inflammatory molecules in combination with immunosuppressive regiments have been used in clinical islet transplantation. TNF-α inhibitors, such as etanercept, are utilized in a combination with immunosuppressive regimens in clinical islet transplantation and all patients have achieved euglycemia without exogenous insulin [36]. However, using immunosuppressants cause severe side effects including ulcers, malignancies, and serious infections [5]. The ultimate aim is to prevent coagulation, inhibit the release of inflammatory mediators and hinder graft infiltration cells in syngeneic islet transplantation, without excessive safety risk for the patient. Taken together, it is difficult to identify a specific approach or strategy to eliminate all inflammatory events in islet transplantation. To enhance the success of islet transplantation, a multi-faceted approach incorporating immunomodulatory strategies aimed at mitigating the underlying inflammatory processes involved in IBMIR is likely to be necessary [27, 37].

Immunomodulatory Proteins

Thrombomodulin

Thrombomodulin is a 557-amino acid transmembrane protein expressed on endothelial cells. It consists of 5 different domains: thrombomodulin domain 1 (TD1), a C peptide lectin-like domain; TMD2, an epidermal growth factor (EGF)-like domain; TMD3, a serine/threonine-rich domain; TMD4, a transmembrane domain, and TMD5, a cytosolic tail [38]. Thrombomodulin acts as an anticoagulant, cytoprotective, and anti-inflammatory protein, and modulates both innate and adaptive immune responses. Recombinant thrombomodulin has been used to treat disseminated intravascular coagulation (DIC) treatment in clinics in Japan since 2008 (Fig. 2) [39, 40].

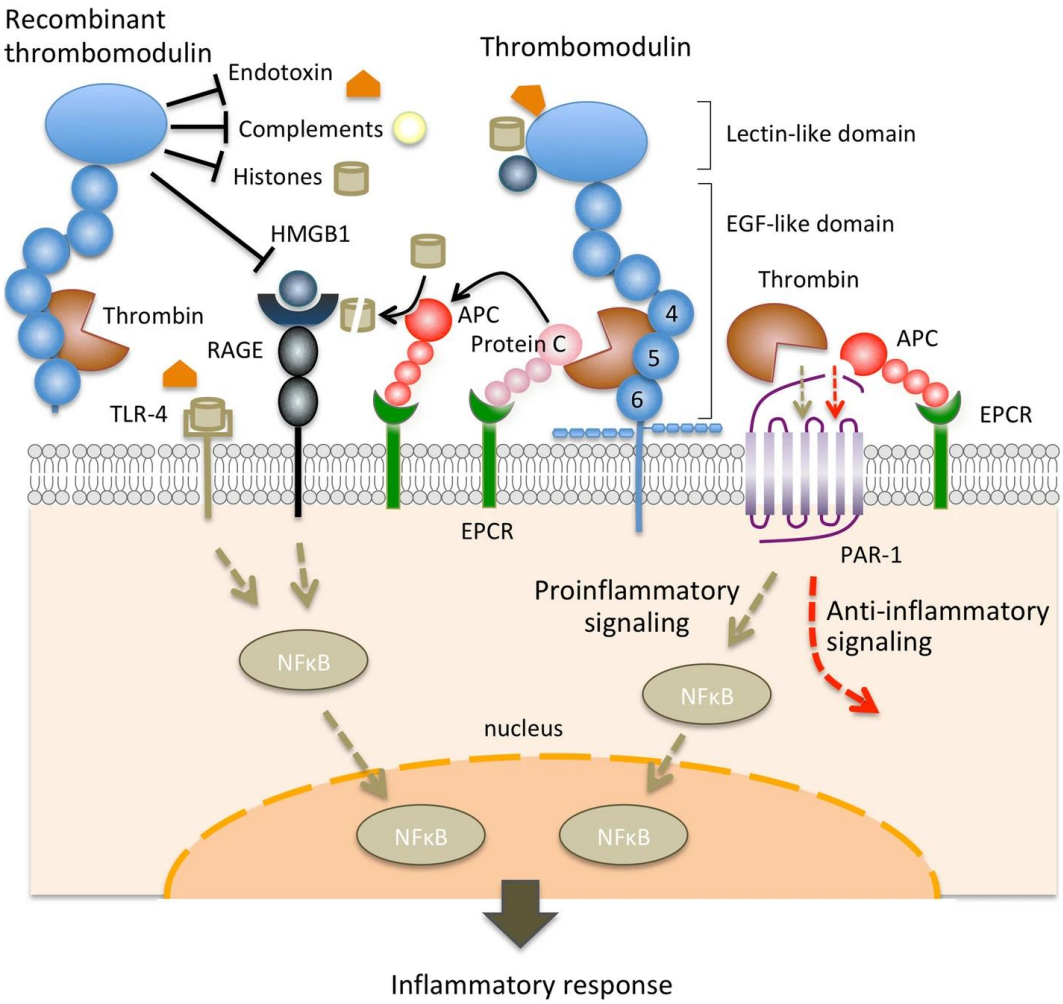


Figure 2: Thrombomodulin possesses anti-inflammatory properties through various mechanisms. Thrombomodulin binds thrombin and this complex stimulates protein C activation on endothelial cells through the endothelial protein C receptor (EPCR). Despite role of thrombin in promoting proinflammatory signaling by activating protease-activated receptor 1 (PAR1) (highlighted in brown dotted arrows), APC bound to EPCR cleaves PAR1 differently, triggering cell signaling that promotes anti-inflammatory effects (highlighted in a red dotted arrow). Additionally, the lectin-like domain of thrombomodulin binds to HMGB1, blocking its signals through RAGE. RAGE activation by HMGB1 initiates nuclear translocation of nuclear factor kappa B (NF-κB), which causes an inflammatory response. Similarly, the lectin-like domain of thrombomodulin interferes with the interaction between TLR-4 and its ligands, such as endotoxin and histones, to prevent proinflammatory reactions. The figure was modified from [39].

The lectin-like domain of TM and its immunomodulatory role

The lectin-like domain of TM (TMD1) neutralizes inflammatory mediators such as HMGB1 and prevents inflammatory signaling. Lipopolysaccharide (LPS) binds TLR4, activating an innate immune reaction via NF-κB signaling pathway to produce excessive proinflammatory responses. The lectin-like domain of TM hinders LPS interaction with TLR4 which prevents the activation of the inflammatory signaling pathways [39, 41]. Moreover, Kadono K. et al. showed recombinant TM alleviates inflammation in liver ischemia-reperfusion injury (IRI) by downregulating TLR4-dependent pathways. Kadono K. et al. also showed TM decreases both the expression and release of HMGB1 upon hepatocellular damage. Blocking of TLR4 by TM prevents Kupffer cells and macrophages inflammatory functions [42].

TMD1 also acts as an anti-inflammatory mediator by suppressing complement system activation. The complement system plays a critical role in innate immune response and inflammatory disorders. The complement system consists of multiple proteins such as C3a, C3b, C5a, and C5b which contribute to oxidative burst and induces both the release of granulocytic enzymes in neutrophils and the phagocytic activity of macrophages [39-42]. Van De Wouwer, M. et al showed a mutation in TMD1 results in the activation of complement and enhanced monocyte infiltration, which are highly associated with inflammatory arthritis [43]. TMD1 controls complement system activation by dampening complement anaphylatoxins C3a and C5a and interfering with neutrophil infiltration. This set of data highlights

how the lectin-like domain of TM modulates the innate immune response by suppressing DAMPs, preventing the recruitment of innate immune cells, and altering immune cell functions [39-43].

Epidermal growth factor (EGF)-like repeats of TM and their immunomodulatory role

TM contains six EGF-like repeats which are *associated* with anticoagulation and fibrinolysis. TM acts as a thrombin receptor which decreases the ability of thrombin to assist in the conversion of fibrinogen to fibrin. In addition, when TM forms a complex with the anion-binding exosite of thrombin, it initiates the conversion of Protein C to Activated Protein C (APC), resulting in the activation of thrombin activatable fibrinolytic inhibitor (TAFI) for anticoagulation and fibrinolysis [44]. The fifth region of EGF-like domain of TM called TM5 binds to G-protein coupled receptor 15 (GPR15), blocking DC activation and inhibiting IL-6 production for treating Graft-versus-host disease (GVHD) in allogeneic hematopoietic stem cell transplantation [45].

CD47 and its ligands

CD47 protein, also known as Integrin Associated Protein (IAP), miniature inverted-repeat transposable element 6 (MER6), or antigenic surface determinant protein (OA3), consists of an N-terminal extracellular IgV domain, five membrane-spanning domains, and a short cytoplasmic domain. CD47 is a 47 kDa heavily glycosylated protein with four isoforms due to its alternative splicing of the C-

domain [46, 47]. CD47 is expressed on the surface of various cells, including red blood cells and hematopoietic cells, but its expression differs by cell type. CD47 expression is high in human stem cells and several other cancer cells [48, 49]. CD47 plays important roles in phagocytosis, apoptosis, immune cell migration, and homeostasis. CD47, identified by the immune system as a marker for self, is expressed on healthy and cancer cells while downregulated on damaged or old cells, which makes those damaged cells more susceptible to macrophage-mediated phagocytosis. CD47 interacts with signal-regulatory protein alpha (SIRPα), SIRPγ, matrix protein thrombospondin-1 (TSP-1), and SH2-domain bearing protein. Moreover, CD47 inhibits TSP-1 signals coming from T cells regarding T cell activation [50, 51].

CD47 interacts with SIRPα, a transmembrane glycoprotein expressed mostly on myeloid cells such as macrophages, DCs, hematopoietic stem cells, and neurons. The CD47-SIRPα axis delivers “do not eat me’ signals while on healthy cells and prevents healthy cells or tumors from macrophage-mediated phagocytosis [50, 52]. SIRPα, known as CD172, is a member of the immunoglobulin-like protein family of cell surface glycoproteins. SIRPα is comprised of two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Phosphorylated ITIMs bind the Src homology 2 (SH2) domain-containing protein-tyrosine phosphatases, SHP-1, and SHP-2. Activated SHP-1/2 prevents the accumulation of myosin-IIA at the phagocytic synapse, blocking phagocytosis [50, 52, 53]. Additionally, CD47/SIRPα axis downregulates the function and adhesion of macrophages, neutrophils, and platelets [10, 53]. Upon interaction with CD47, SIRPα on T cells hampers DC

activation, decreasing proinflammatory mediator release by DCs [53, 54]. Previous studies showed that an inability of donor CD47 to interact with recipient SIRPα induces the rapid clearance of hematopoietic cells by macrophages in xenogeneic models [55, 56]. Dai H. et al demonstrated that donor SIRPα polymorphisms play a key role in the innate immune response to allogeneic bone marrow grafts [55]. Additionally, Wong AS. et al indicated that CD47/SIRPα interaction is refractory to the pathogenesis of T1D in Non-Obese Diabetic (NOD) mice [57]. Shrestha P. et al showed CD47 displayed on the surface of syngeneic islets significantly reduces infiltration of intrahepatic inflammatory cells into grafts and hampers the inflammatory molecules associated with IBMIR [10]. In sum, CD47/SIRPα negatively modulates both innate and adaptive immune systems. Therefore, CD47/SIRPα axis becomes a key regulator of donor cell fate.

Besides SIRPα, Fas (CD95) is another ligand for CD47. The IgV domain of CD47 interacts with Fas and promotes the downstream activation of Fas-mediated apoptosis via caspase-dependent pathways defined by rapid mitochondrial dysfunction. Manna PP. showed that CD47 augmentation on Jurkat cells or mouse T cells can induce Fas clustering to induce apoptosis via cytochrome c release [58].

CD47 was first discovered by its interaction integrins. For example, CD47/αvβ3 integrin negatively modulates nitric oxide (NO) synthase, and CD47 selectively modulates β_2 integrin adhesive function in neutrophils. Wang et al. demonstrated that the disassociation of CD47 from αVβ3 induces the production of

proinflammatory cytokines including IL-1, IL-6, and TNF, causing joint inflammation and adverse osteoarthritis [59]. Moreover, CD47 interacts with vascular endothelial growth factor receptor 2 (VEGFR-2) and CD36, resulting in the inhibition of angiogenesis [60]. In conclusion, CD47 negatively modulates immune reactions in different diseases.

Protex™ technology is a safe and practical platform to modulate immune response followed by islet transplantation

Despite breathtaking improvements in the immunosuppressant drugs and immunomodulatory biomaterials targeting innate and adaptive immune responses to grafts, the long-term survival and engraftment of islets remain unachievable due to IBMIR, adaptive immunity, and chronic rejection [5]. Multiple donors are needed for islet transplantation because of early islet loss. Almost half of the patients become insulin-dependent due to IBMIR-associated early loss of autologously transplanted islets [10, 15]. Another major problem for successful islet engraftment is systemic immunosuppressive agents. The continuous and systemic administration of immunosuppressants can cause insulin-producing pancreatic β-cell destruction [15]. Further, long-term administration of comprehensive immunosuppressive agents leads to adverse side effects, such as susceptibility to infection and cancer [5]. Thus, Dr. Esma S. Yolcu and Dr. Haval Shirwan pioneered a novel approach called ProtEx™, allowing the display of one or more immunomodulatory proteins on biological or nonbiological surfaces to ensure a safe, practical, and localized treatment [8].

The ProtEx™ platform comprises the generation of chimeric proteins consisting of an extracellular immunological ligand linked to a modified form of streptavidin. These chimeric proteins are displayed on the biotinylated biological or nonbiological surfaces, taking advantage of the strong interaction ($K_d = 10^{-15}$ M) between the biotin and streptavidin complex. In this platform, the process of engineering cells or tissues with immunomodulatory protein is performed in 2.5 hours [8, 10, 61, 62]. Moreover, the treatment can be designed to the personalized treatment. Thanks to the advantages of The ProtEx™ platform, different doses of single or combination of immunomodulatory protein can be used to treat or prevent different diseases [8, 10, 61, 62].

Chapter 2

Materials and Methods

Animals

C57BL/6 and MHC class I- and II-deficient NSG mice were purchased from Jackson Laboratory. Mice were bred and housed in the specific pathogen-free animal facility of the University of Missouri, Columbia, MO, according to the NIH guideline for the Care and Use of Laboratory Animals. All protocols were approved by the University of Missouri Animal Care and Use Committee.

SA-TM protein production, structural and functional characterization, and transient display on the surface of biotinylated cells

A synthetic gene including the extracellular domain of human TM N-terminus to core SA with a 6xHis tag was synthesized and subcloned into the pMT/BiP/V5-His vector for inducible expression in Drosophila S2 cells. Stably transfected S2 cells were induced with 1 mM $CuSO_4$, and SA-TM protein was isolated from the supernatant using a metal-ion charged Sepharose column and then characterized using sulfate–polyacrylamide gel electrophoresis (SAS-PAGE) and Western blots as published [63]. The function of hSA-TM is assessed by measuring the conversion of protein C into its active form (APC) using a spectrozyme PCa assay (Haematologic Technologies and Sekisui Diagnostics). Briefly, increasing doses of soluble hSA-TM were incubated with lightly heparinized mouse blood for 1 hr at 37 °C in the presence of $CaCl_2$ and thrombin cofactors, followed by the addition of

antithrombin III to quench the reaction. APC activity was measured using spectrophotometry at OD 405 to determine spectrozyme cleavage. For cell surface engineering, mouse splenocytes were modified with biotin (15 µM EZ-LinkTM Sulfo-NHS-LC biotin) followed by incubation with various doses of SA-TM protein or equimolar amounts of control streptavidin (SA) as published [64]. The engineered expression level of hSA-TM was assessed via flow cytometry using an antibody to human TM.

A designed gene containing extracellular domain of CD47 to core SA with a 6xHis tag was synthesized and cl subcloned into the pMT/BiP/V5-His vector for inducible expression in Drosophila S2 cells. Stably transfected S2 cells were induced with 1 mM $CuSO_4$, and SA-CD47 protein was isolated as SA-TM was isolated using a metal-ion charged Sepharose column and characterized using SDS-PAGE as published [63].

In vitro phagocytosis, NETosis, and *in vivo* cell clearance assays

The function of SA-CD47 was assessed using a phagocytosis assay. Briefly, rat splenocytes were generously donated by Dr. Daniel Davis. Xenogeneic rat splenocytes were engineered with SA or SA-TM or SA-CD47 or a combination SA-TM with SA-CD47 (referred as SA-TM/SA-CD47) and labeled with 2.5 µM Carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific). A mouse macrophage cell line (RAW 264.7) was stimulated with 1 µg/ml LPS in RPMI medium supplemented with 10% FBS and used for co-culture ($5x10^5$ cells/well)

with engineered rat splenocytes (2.5 x 10^6/well) for 18 hours. After incubation, cells were stained with antibodies to CD11b and F4/80 using flow cytometry. Phagocytosis of engineered splenocytes was assessed by gating on mouse $CD11b^+F4/80^+$ cells and assessing the percentages of $CD11b^+F4/80^+CFSE^+$ cells.

Neutrophil extracellular trap (NET) formation (also called NETosis) studies were performed on mouse neutrophils isolated from bone marrow cells using a density gradient centrifugation [65]. Neutrophils were activated by 200 nM phorbol myristate acetate (PMA) in complete media supplemented with varying concentrations of SA-TM and SA proteins for 3 hr. To assess myeloperoxidase (MPO) expression, an indicator of NET formation, cells neutrophils were stained with $CD11b^+Ly6G^+MPO^+$. Mean intensity fluorescence and percentages MPO^+ cell was calculated via flow cytometry with gating on $CD11b^+Ly6G^+$ cells.

For *in vivo* cell clearance studies, MHC class I- and II-deficient NSG bone marrow cells were engineered with SA-TM/SA-CD47, SA-CD47 SA-TM or SA as control, then labeled with 2.5 μM CFSE and 2.5 μM Cell Trace Violet (CTV, Thermo Fisher Scientific), respectively. Cells were admixed at a 1:1 ratio and 20x10^6 cells were injected i.v. into C57BL/6. Mice were euthanized 24 hours later to collect splenocytes, and proportions of CFSE or CTV-positive NSG cells were determined using flow cytometry.

Pancreatic islet isolation, engineering, and functional analysis

Islets were isolated using Liberase^{TM} TL enzyme (Roche), biotinylated (15 μM), and then engineered with SA-TM (3.2 μg/500 islets) or an equimolar amount of SA control protein as previously described [66]. To assess the impact of engineering on islet metabolic activity, 50 unmodified or engineered islets were incubated in a complete islet medium (RPMI 1640 supplemented with 10% FBS, 10000 Unit Penicillin/ Streptomycin and 2 mM L-Glutamine) supplemented with 10% resazurin (the oxidized form of Alamar blue) in 96-well plates at 37 °C 8 hrs. The amount of resorufin (reduced form of resazurin) was measured at 562 nm using Biotek Synergy multi-mode reader.

Glucose-stimulated insulin secretion (GSIS) was performed to assess the impact of engineering with SA or SA-TM or SA-TM/SA-CD47 on islet function as published [7, 66]. Fifty unmodified islets and engineered islets were transferred into a transwell plate (Millicell, Merck) and cultured in 1 ml of low glucose (3.5 mM) in Krebs ringer bicarbonate buffer for 1 hour to permit equilibration. Then islets were incubated in 1 ml of low glucose (3 mM) solution for 1 hour and then transferred into 1 ml of high glucose (16.5 mM) in Krebs ringer bicarbonate buffer for 1 hour for equilibration. The solution was collected at 0 and 1 hours to measure the amount of insulin released by islets using murine insulin ELISA kit (Mercodia). The stimulation index (SI) was calculated by dividing the mean insulin secreted by the islets in the high-glucose medium by the mean insulin secreted from the same islets in the low-glucose medium.

In vitro blood loop assay and qRT-PCR

An in vitro tube model blood loop assay was used as an in vitro model of IBMIR to assess the impact of SA-TM or SA-TM/SA-CD47 on modulating innate immune responses and islet viability [10]. Briefly, 100 engineered C57BL/6 islets were incubated in 500 µl of fresh syngeneic C57BL/6 blood at a rotator at 37 ^{0}C for 3 hrs. A portion of the islet-thrombus complex was then collected in 10% neutral buffered formalin, embedded in paraffin, and cut into 5 µm thickness followed by Hematoxylin & Eosin (H&E) staining. Tissues were graded for islet structure using previously published [10].

Another portion of the islet-thrombus complex was used for total RNA isolation using a Qiagen kit and quantitative RT-PCR for various inflammatory cytokines and chemokines using TaqMan assay (Applied Biosystem) and primers shown in Table 1. Total RNA was purified from the liver tissues using TRIzol reagents (Invitrogen Corporation) according to the manufacturer's instructions. cDNA was reverse transcribed from total RNA (2 µg) using SuperScript IV VILO cDNA Master Mix (Thermo Fisher Scientific). Quantitative RT-PCR was performed using TaqMan Gene Expression Assays for indicated genes.

Table 1: PCR TaqMan primer panel

Gene symbol	Assay ID
Myeloperoxidase (MPO)	Mm01298424_m1
HMGB1	Mm00849805_gH
F3 (TF)	Mm00438855_m1

IL-1b	Mm00434228_m1
IFN-γ	Mm01168134_m1
MCP-1	Mm00441242_m1
NF-KB (p65)	Mm00501346_m1
GAPDH	Mm99999915_21
IL-6	Mm00446190_m1
Arginase-1	Mm00475988_m1
iNOS	Mm00440502_m1
Csf2/ GM-CSF	Mm01290062_m1
Csf1/M-CSF	Mm00432686_m1
Cxcl12/ SDF1	Mm00445553_m1
Arnt/ HIF-1B	Mm00507836_m1

Islet transplantation

C57BL/6 mice were intravenously injected with 200 mg/kg of STZ (Sigma-Aldrich). Animals with non-fasting blood glucose readings of > 300 mg/dl using a portable glucose meter (AccuCheck, Roche) for two consecutive days were considered diabetic and were transplanted with 200 IEQ syngeneic unmodified, SA-TM, or SA-engineered islets intraporally into the ileocolic vein, a terminal branch of portal vein. For the combination study, 150 IEQ syngeneic unmodified, SA-TM-islets, or SA-TM/SA-CD47-islets were transplanted into STZ-induced diabetic recipients.

Graft recipients were monitored for blood glucose levels twice per week for 60 days post-transplantation and subjected to intraperitoneal glucose tolerance test (IPGTT) as published [64].

Flow cytometry to assess intrahepatic immune infiltrates

Graft recipients were euthanized at the indicated times or experimental endpoint, the liver was perfused with 5 ml Phosphate Buffered Saline (PBS) from the main-intraportal vein and harvested. The liver was placed in a 6-well-plate in 2 mL of HBSS on ice. The liver was then minced by turning a sterile knob of a syringe until it lost its integrity. Then, liver was transferred into a 50-mL Falcon tube, and incubated in 0.5 mg/ml collagenase IV on a shaker (250 rpm) at 37℃ for 40 min. Digested tissue was filtered through a 40 µm nylon mesh into a clean 50-ml Falcon tube and cells were collected by centrifugation (180 g for 7 minutes). Cells ($1x10^6$ cells/tube) were incubated with Fc Block (CD16/CD32) (BD bioscience) followed by staining with antibodies shown in Supplementary Table S2 and analyzed by flow cytometry gating on live cells (Cytek Aurora). Distribution of immune cells on the viSNE (t-distributed stochastic

neighbor embedding-based visualization) plot of viable $CD45^+$ cells from graft recipients at the indicated times was analyzed with FCS Express 7 software (De Novo Software). Cell type annotation on the viSNE plot was identified by differential expression of individual lineage markers and the different colors depicting the annotated cell type were displayed (concatenated n = 2 per group with equal down sampling to 30000 total cells).

Supplementary Table S2. Antibody list

ANTIBODY	CLONE	COMPANY	CATALOG
V500 Syrian Hamster anti-mouse CD3e	500A2	BD Horizon	560771
BUV496 Anti-mouse CD4	GK1.5	BDBiosciences	612952
BUV615 Anti-mouse CD5	53-7.5	BDBiosciences	751298
SparkViolet538 Anti-mouseCD8	QA17A07	Biolegend	155019
PerCP5.5 Anti-mouse CD11b (B1)	M1/70	BD Pharmigen	550993
AF647 Anti-mouse CD11c	N418	Biolegend	117312
BV570 Anti-mouse CD19	6D5	Biolegend	115535
BB700 Anti-mouse CD44	IM7	BD Biosciences	566506
APC Fire810 Anti-mouse CD45	30-F11	Biolegend	103174
APC Fire750 Anti-mouse CD80	16-10A1	Biolegend	104740
PerCP-eFluor 710-CD103 (Integrin alpha E)	2.00E+07	Fischer Scientific	46-1031-82
BV785 Anti-mouse CD206	C068C2	Biolegend	141729
AF700Anti-mouse NK1.1	PK136	Biolegend	108730
BV605 Anti-mouse Ly6C (M5)	HK1.4	Biolegend	128036
BUV395 Anti-mouse Ly6G	1A8	BDBiosciences	563978
Pacific Blue Anti-mouse F4/80	BM8	Biolegend	123124
Spark Blue 550 Anti-mouse I-A/I-E(MHC-II)	M5/114.15.2	Biolegend	107662

Statistics

All data analysis was performed using GraphPad Prism v.8 (GraphPad Inc) and reported as mean ± SEM. Student t-test was employed for comparisons between two groups while a Kruskal–Wallis one-way ANOVA, Dunn's test and Dunnett's multiple comparison test were used for multiple comparisons among more than 2 groups. The log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon statistics were employed for graft survival between groups. *P* values <0.05 were considered statistically significant.

Chapter 3

Engineering pancreatic islets with a novel form of thrombomodulin protein to overcome early graft loss triggered by the instant blood-mediated inflammatory reaction

Introduction

TM as a potential protein in islet transplantation

Intraportal transplantation of islets is an effective treatment for recurrent acute and chronic pancreatitis and T1D, but IBMIR is a major cause of islet damage following intraportal transplantation for both autologous and allogeneic islet transplants [9, 16, 67]. Islets express various DAMPs, cytokines, and chemokines due to enzymatic and mechanical stress and hypoxia [68, 69]. These proinflammatory factors initiate a vicious cascade of thrombotic and innate proinflammatory reactions responsible for 50-70% of initial islet mass loss following intraportal transplantation. This significant peri-transplant islet loss is a major impediment for islet engraftment that translates into poor euglycemia in an autologous setting and necessitates islet transplant from multiple donors in the allogeneic setting.

TF is an integral membrane protein expressed by islet graft and is a major trigger of IBMIR [5, 9, 15, 17, 68, 70]. TF activates the coagulation protease cascade, leading to platelet and complement activation, coagulation, and infiltration of leukocytes into the graft that inflicts damage by various means [71]. Particularly, TF-mediated thrombin production plays a prominent role in IBMIR by activating

platelets and initiating a coagulation cascade, both of which are inhibited by TM [72, 73]. TM is a cell membrane glycoprotein with potent anticoagulant, cytoprotective, and anti-inflammatory activities as well as immunomodulatory function for both innate and adaptive immune responses [74, 75]. TM exerts its anticoagulant activity by inhibiting thrombin and facilitating the generation of APC, which is critical for the inhibition of inflammation and thrombosis. TM binds to HMGB1 expressed by islets and facilitates its cleavage by thrombin as a mechanism to inhibit its proinflammatory signaling through innate immune receptors TLR4 and RAGE [42, 44, 45, 71]. TM also mitigates the proinflammatory function of TF and complement system activation [74, 76]. The anticoagulatory and innate immune-modulatory functions of TM led us to generate a novel construct chimeric with SA-TM for transient display on the surface of biotinylated islets as a practical scheme to mitigate IBMIR in a syngeneic mouse minimal mass intraportal islet transplantation model. Our data demonstrate that islets can be efficiently engineered with the SA-TM protein without a negative impact on their viability and function. SA-TM-engineered islets showed enhanced engraftment and sustained survival by inhibiting various thrombotic and inflammatory mediators of IBMIR.

Results

Protex technology allows the transient display of recombinant proteins on the surface of biological and nonbiological surfaces.

ProtEx™ technology allows us to create a new generation of recombinant proteins and display them on biological and non-biological surfaces [10, 62, 77]. We

developed a novel approach of using immunomodulators with a modified form of core streptavidin and displayed them on the biotinylated biological or non-biological surfaces. We designed a chimeric protein consisting of extracellular domain of human Thrombomodulin ™ with a modified form of core SA and present SA-TM on biotinylated islets to modulates immune response to syngeneic islet grafts. Drosophila copper sulfate–inducible pMT/BiP/V5-HisA expression vector was transfected into Drosophila S2 cells for protein expression (Fig. 3A). SA-TM structure was designed in Swiss model (Fig. 3B). SA-TM was purified metal affinity chromatography thanks to hexahistidine tag for protein purification as described in previous papers [8, 10, 62]. SA-TM protein is at around a ≈70-kDa protein in denaturing SDS-PAGE after samples were heated at 100°C (Fig. 3C).

TM binds to thrombin protein, resulting in a conversion of Protein C into APC. We tested whether SA-TM induces APC activity in vitro. Fresh blood in heparinized tube incubated with indicated doses of SA-TM and serum was isolated after incubation and APC activity was measured. The result reveals that SA-TM induces APC activity (Fig. 4A). Then, to show SA-TM can be displayed on the surface of biological surfaces, C57BL/6 splenocytes were isolated, biotinylated (15 µM) and engineered with indicated amount of SA-TM (Fig. 4B-C). To test the turnover kinetics of SA-TM protein on splenocytes, engineered syngeneic splenocytes (CD45.1) were engineered with 3.2 µg of SA-TM protein and injected (i.v.) into C57BL\6 mice (CD45.2). Data revealed that half-life of SA-TM on the splenocytes was at 6 hours (Fig. 4D).

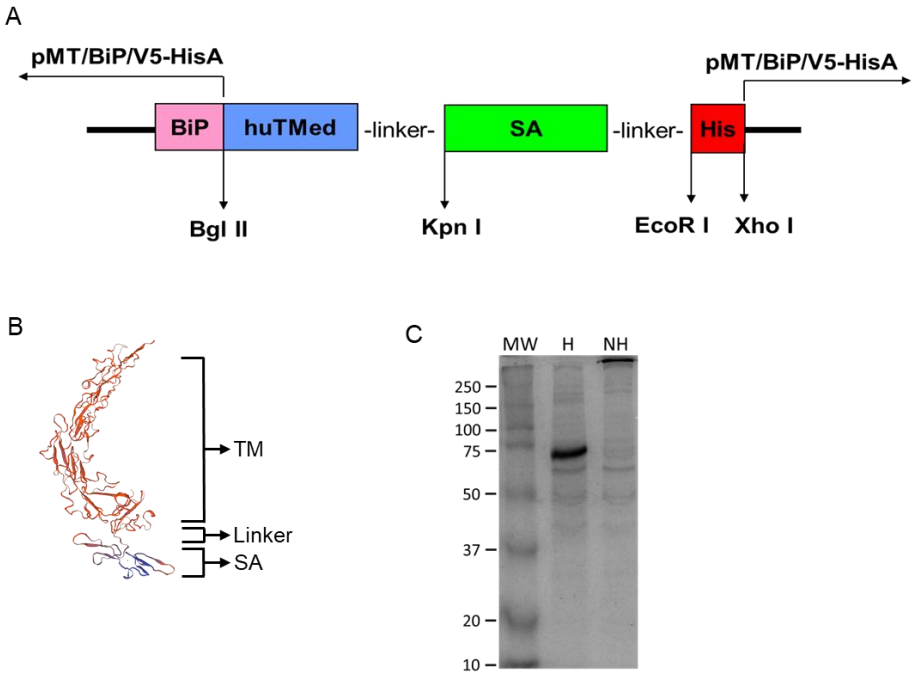


Figure 3:Generation of SA-TM protein and structural characterization. (A) Schematic representation of the SA-TM construct. A synthetic chimeric gene containing the coding sequences for the extracellular domain of human TM and core SA with flexible linkers and a 6xHis tag to facilitate protein purification was constructed and subcloned into the CuSO4-inducible pMT-Bip-V5-HisA S2 insect cell expression vector. (B) The anticipated three-dimensional structure of SA-TM protein using the SWISS-MODEL (https://swissmodel.expasy.org/interactive). (C) SDS-PAGE profile of SA-TM. The protein was produced in S2 cells, isolated from cell supernatant using metal affinity chromatography, left unheated (NH) or heated (H) at 100oC, and analyzed using denaturing SDS-PAGE for structure and purity.

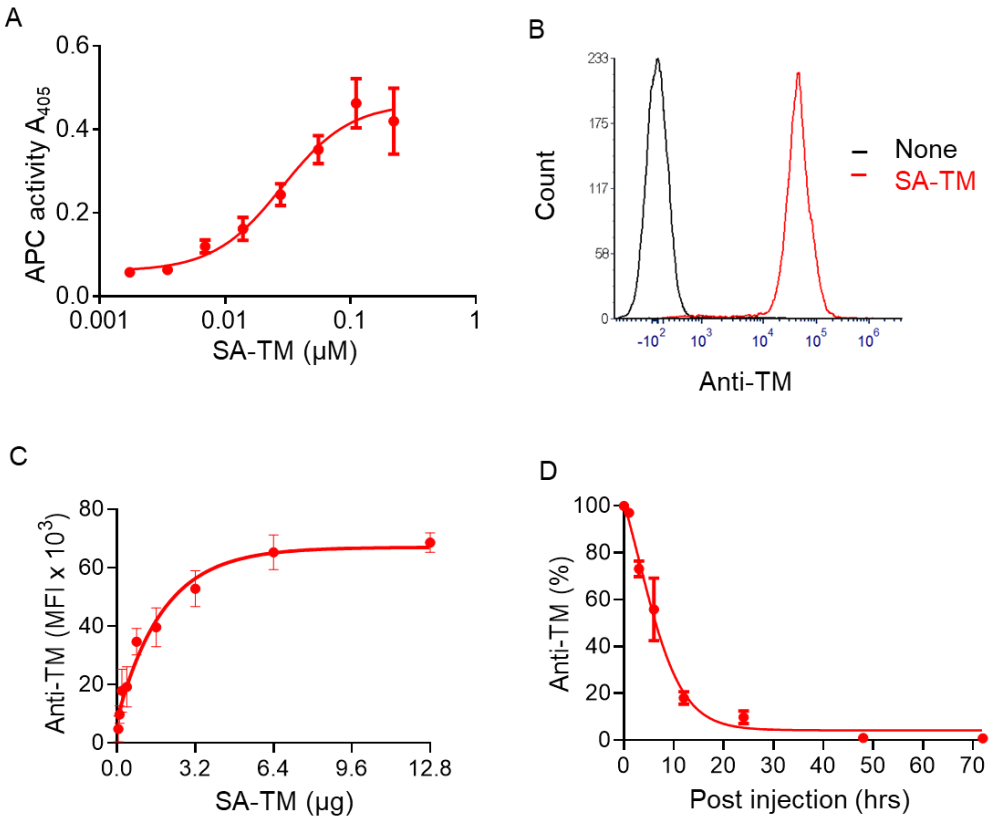


Figure 4: SA-TM protein functional characterization. (A) SA-TM converts protein C to APC. Lightly heparinized mouse blood was incubated with the indicated doses of soluble SA-TM for 1 hr and the activity of APC was measured at OD405. (B) Splenocytes engineered with SA-TM protein. Mouse splenocytes were biotinylated (15 μM), engineered with SA-TM protein (3.2 μg/106 cells), stained with an Ab to human TM (red line), and analyzed in flow cytometry with unmodified splenocytes (black line) serving as control. (C) Dose-dependent binding of SA-TM protein to biotinylated splenocytes. Data expressed as mean ± SD and representative of 3 independent experiments. (D) In vivo kinetics of SA-TM on the surface of splenocytes. C57BL/6.SJL (CD45.1) splenocytes were engineered with SA-TM protein as in (E) and injected i.v. into C57BL/6 (CD45.2) mice. Spleens were harvested at indicated time points and analyzed for the frequency of SA-TM positive cells in flow cytometry by gating on donor cells (CD45.1). Data from 3 separate experiments expressed as mean ± SD.

SA-TM blocks phagocytosis, NETosis, and clearance of allogeneic bone marrow cells *in vivo*

Macrophages and neutrophils play important roles as effectors of intrahepatic islet damage directly through the synthesis of nitric oxide or indirectly by secreting various proinflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ [78]. \We investigated whether SA-TM also impacts the phagocytic function of macrophages using an *in vitro* xenogeneic setting. CFSE-labeled rat splenocytes engineered with SA-TM or SA as control were cocultured with a mouse macrophage cell line which was activated with LPS and the percentage of macrophages positive for CFSE was assessed using flow cytometry. As shown in Fig. 5A, there was a significantly ($p = 0.0256$) higher percentage of macrophages engulfing SA-engineered rat splenocytes as compared to SA-TM-engineered cells. We next assessed whether SA-TM modulates neutrophil functions by assessing NETosis, formation of neutrophil extracellular traps (NETs), as a proinflammatory and defense mechanism against infections [79]. Treatment of PMA stimulated mouse bone marrow neutrophils with soluble SA-TM *ex vivo* resulted in a dose-dependent decrease in myeloperoxidase (MPO), a component of NETs, as compared with the SA proteins (Fig. 5B, C).

We next assessed the efficacy of SA-TM in modulating innate immune responses *in vivo.* To avoid allogeneic adaptive immune responses directed towards MHC molecules, we used bone marrow cells from NSG mice deficient for both MHC class I and class II for transplantation. CFSE-labelled, SA-TM-

engineered NSG bone marrow cells were comixed at 1:1 ratio with CTV-labelled-SA-engineered cells and injected i.v. into C57BL/6 allogeneic recipients. Flow cytometry analysis of NSG cells 24 hrs post infusion demonstrated significantly ($p < 0.0001$) reduced percentages of SA-engineered cells as compared with SA-TM-engineered cells (Fig. 5D). Taken together, these results demonstrate the efficacy of SA-TM in inhibiting the effector function of macrophages and neutrophils ex vivo and clearance of allogeneic cells by innate immunity *in vivo*.

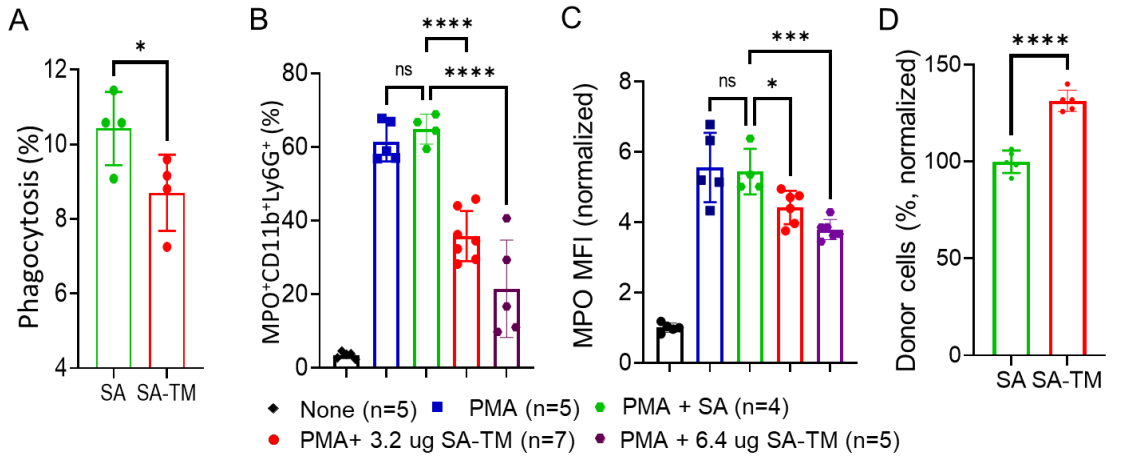
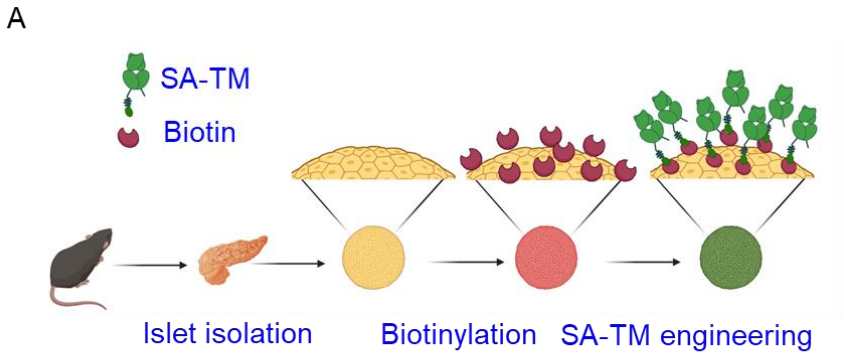


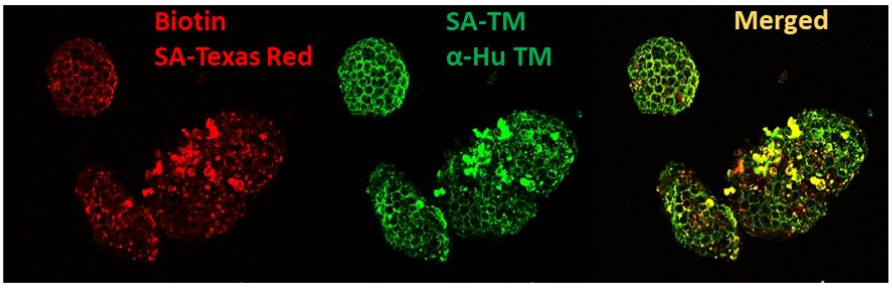
Figure 5: SA-TM mitigates effector function of macrophages and neutrophils and prevents clearance of allogeneic cells in vivo. (A) SA-TM on xenogeneic rat splenocytes inhibits phagocytosis by macrophages in vitro. CFSE labeled and SA-TM-engineered rat splenocytes (3.2 µg/10^6 cells) were cocultured with mouse RAW 264.7 macrophage cells at 1:5 ratio (splenocytes: macrophages) for 18 hrs. Phagocytosis of rat cells ($CD11b^+F4/80^+CFSE^+$) was assessed using flow cytometry. Data are shown as mean ± SD pooled from 3 separate experiments: Student t test. (B) SA-TM mitigates PMA-induced NETosis. C57BL/6 bone marrow neutrophils were stimulated with 200 nM PMA in the presence of the indicated doses of SA-TM and SA as control protein for 3 hrs. NETosis was assessed using an Ab to myeloperoxidase (MPO) in flow cytometry gating on $CD11b^+Gr\text{-}1^+MPO^+$ cells. (C) MFI values of MPO for panel (B). Data are shown as mean ± SD of 3 independent experiments; ANOVA with Bonferroni's post hoc test. (D) SA-TM inhibits in vivo clearance of allogeneic bone marrow cells. Bone marrow from NSG mice deficient for MHC class I and II were engineered with SA (0.8 µg/10^6 cells) or SA-TM (3.2 µg/10^6 cells) proteins followed by labeling with CTV and CFSE,

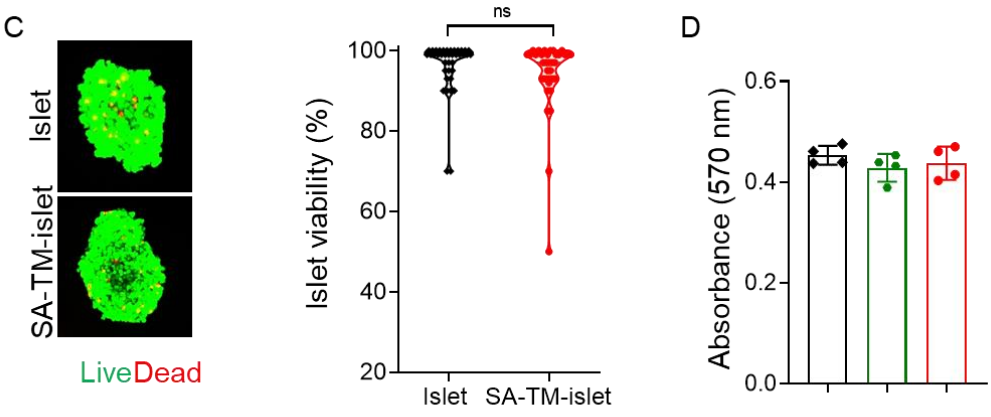
respectively. Cells were then mixed at 1:1 ratio and injected i.v. into allogeneic C57BL/6 recipients (in total $20x10^6$ cells/animal). Splenocytes were harvested 24 hrs post-transplantation and analyzed using flow cytometry by gating on $CFSE^+$ or CTV^+ donor cells. Data are shown as mean ± SD of 4 independent experiments: Student's t test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Engineering with SA-TM protein does not impact islet viability or function

We next assessed whether pancreatic islets can be successfully engineered with SA-TM protein without a negative impact on viability and function. Mouse islets were surface modified with biotin followed by engineering with SA-TM protein (Fig. 6A). Confocal microscopy analysis of the islets showed efficient engineering with SA-TM protein (Fig 6B). Engineered islets showed comparable viability to unmodified islets as assessed by a fluorescein diacetate (FDA)/ propidium iodide (PI) viability assay (Fig. 6C). Metabolic activity of the SA-TM-engineered islets was assessed using the Alamar blue assay and remained unaltered as compared with the unmodified islets (Fig. 6D). Engineering with SA-TM protein also did not impact the ability of islets to secrete insulin in response to glucose stimulation *in vitro* (Fig. 6E, F). Collectively, these results show that islets can be efficiently engineered with SA-TM protein without significantly altering their viability, metabolic activity, and insulin secretion function.


A
SA-TM
Biotin
Islet isolation
Biotinylation
SA-TM engineering


B
Biotin
SA-Texas Red
SA-TM
α-Hu TM
Merged


C
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SA-TM-islet
LiveDead
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Islet viability (%)
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80
60
40
20
Islet
SA-TM-islet
D
Absorbance (570 nm)
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0.4
0.2
0.0

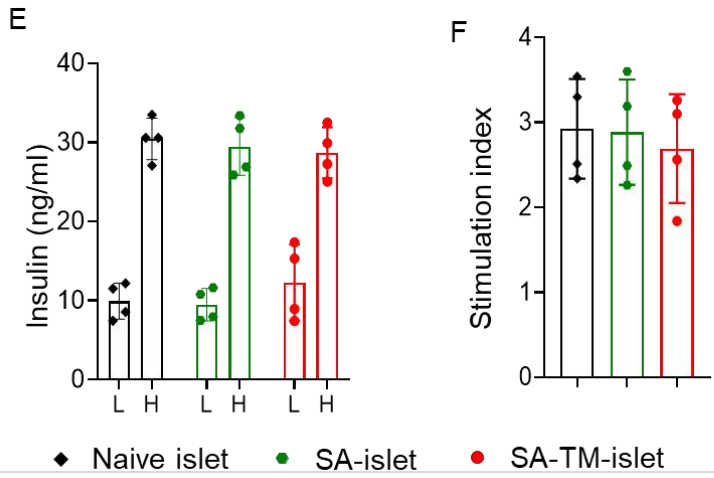

E
Insulin (ng/ml)
40
30
20
10
0
L
H
L
H
L
H
F
Stimulation index
4
3
2
1
0
Naive islet
SA-islet
SA-TM-islet

Figure 6: Engineering islets with the SA-TM protein does not impact islet viability and function. (A) Schematic drawing of islet engineering with the SA-TM protein. (B) A representative image of SA-TM-engineered islets (15 µM biotin and 3.2 µg/500 islets). Biotinylation and engineering were assessed using fluorescence labeled streptavidin (red) and an antibody to human TM (green) in confocal microscopy. (C) FDA/PI islet viability test showing no negative effect of engineering. Data are shown as mean ± SD pooled from 2 separate experiments: Student's t test. (D) Islet metabolic activity. The islets were left unmodified or engineered with SA-TM or an equimolar of SA proteins and metabolic function was assessed using Alamar Blue cell viability assay. (E) Insulin stimulated glucose secretion assay performed using unmodified and SA-TM or SA-engineered islets. Islets were cultured in low (L, 3.5 mM) and high (H, 16.5 mM) doses of glucose and secreted insulin levels in the supernatant were measured using ELISA. (F) Stimulation index of data shown in (E). For panel C, data are shown as mean ± SD pooled from a minimum of 2 separate experiments: using Student's 2-tailed unpaired t test with; ns, not significant. For panel D and E, data are shown as mean ± SD pooled from a minimum of 4 separate experiments: ANOVA with Bonferroni's post hoc test.

SA-TM on islets inhibits various innate immune inflammatory responses in an ex vivo model of IBMIR

TM is a crucial regulator of coagulation and inflammation that are major culprits of IBMIR responsible for massive graft loss immediate post islet transplantation into the liver [80, 81]. To investigate the efficacy of SA-TM in mitigating IBMIR, we used an *in vitro* blood loop assay as a practical approach to simulate innate immune responses occurring following intraportal islet transplantation [66, 82]. Mouse islets were engineered with SA-TM protein and incubated in autologous fresh blood in heparin-coated tubes at 37^{o}C for 3 hrs. Histological analysis of islet-thrombus revealed over 50% of SA-TM-engineered islets with intact structure, while the majority of SA-engineered islets (>70%) showing severe damage (Fig. 7A, B). HMGB1 as a danger associated molecular pattern playing a significant role

in early graft loss [83] was also expressed at reduced levels in the SA-TM-engineered islet group both at the transcript and protein levels as compared to the SA-engineered islet group (Fig. 7C, E). There were also higher levels of activated protein C in the serum of the SA-TM-engineered group as compared with the SA-engineered control islet group (Fig. 7D). Quantitative RT-PCR analysis also showed significantly ($p < 0.05$) lower levels of transcripts for proinflammatory cytokines IFN-γ, IL-1β, and IL-6 in the SA-TM-engineered group as compared to those in the SA-engineered islet group (Fig. 7E). Taken together, SA-TM on the surface of islets significantly reduces the levels of proinflammatory cytokines and damage-associated molecular patterns that translate into protection in an ex vivo model of IBMIR.

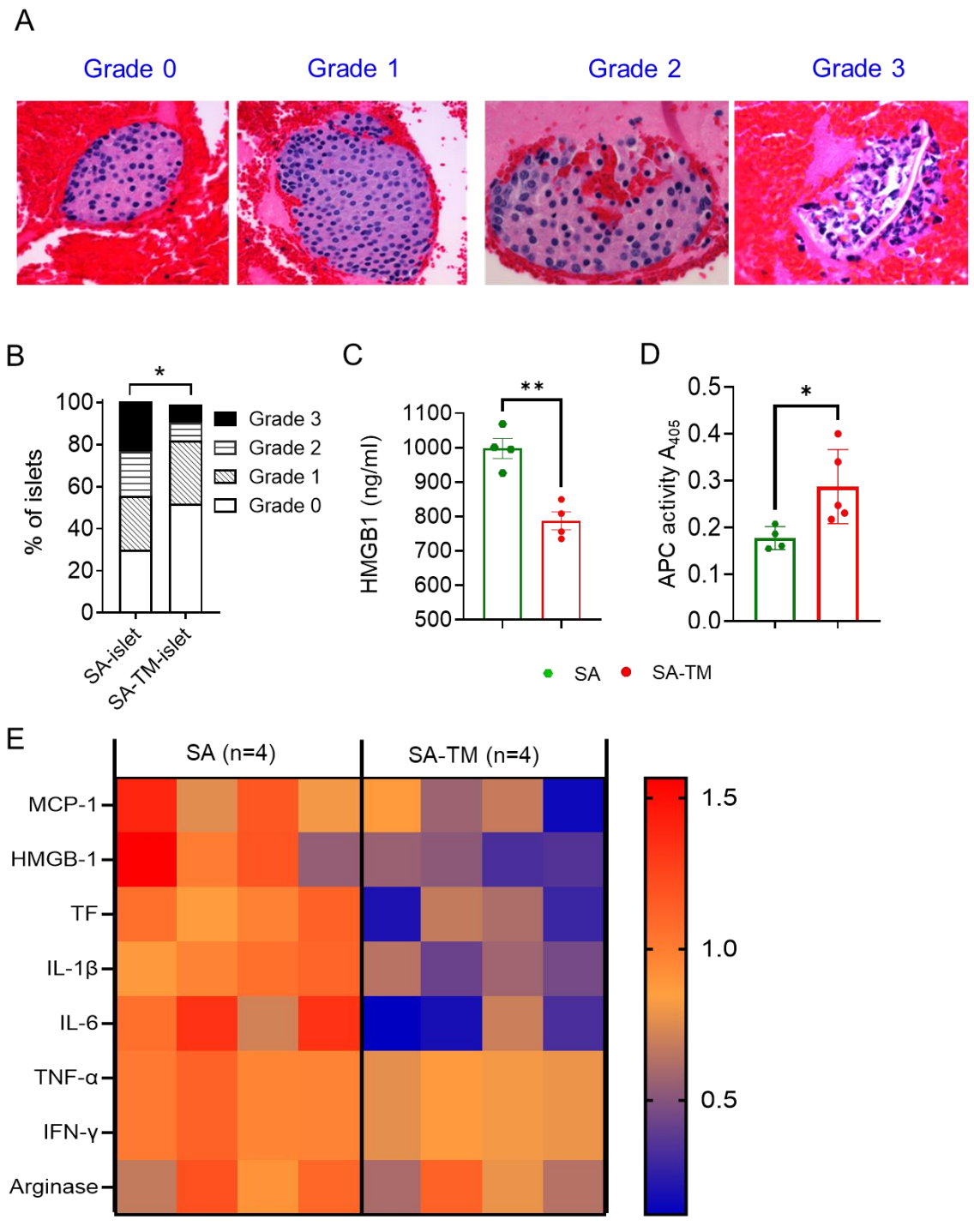


Figure 7: SA-TM on islets inhibits various innate immune inflammatory responses. An in vitro blood loop assay mimicking IBMIR was performed by culturing 100 SA-TM- or SA-engineered islets in fresh autologous blood at 37 ^{0}C for 3 hrs. Islet thrombus was used for H&E staining and qPCR, while serum was

analyzed using ELISA. (A, B) Histological assessment of islet thrombus. SA-TM maintains islet structure and protects from IBMIR as compared to SA. Data from at least 3 independent experiments. Statistical differences were assessed using Student's 2-tailed unpaired t test. (C) HMGB1 levels in the serum of islet thrombus using ELISA. (D) APC activity in the serum of islet thrombus using. SA-TM activates APC activity and (D) degrades the release of HMGB1. (E) Heatmap of proinflammatory factors. TaqMan qPCR were performed on total RNA extracted from islet thrombus. Data from 4 independent experiments. E Data shown as mean ± SEM of at least 3 independent experiments. Statistical differences were assessed using Student's 2-tailed unpaired t test with *P < 0.05, **P < 0.01.

SA-TM-engineered islets achieve improved engraftment and sustained function in a minimal mass intraportal transplantation setting

Inasmuch as TM is an effective regulator of coagulation and inflammation [84], we assessed the efficacy of SA-TM protein transiently displayed on the surface of islets in preventing peri-transplant graft loss in a syngeneic minimal mass intraportal transplant model. C57BL/6 islets were engineered with SA-TM and transplanted (200 IEQ/recipient) intraporally into diabetic syngeneic recipients. Over 80% of recipients of SA-TM-engineered islets established euglycemia for a 60-day observation period as compared to less than 30% of recipients of unmodified or SA-engineered control islets (Fig. 8A, B). Importantly, intraperitoneal glucose tolerance test (IPGTT) at the experimental endpoint showed glucose response in SA-TM group at similar levels to naïve mice without transplantation (Fig. 8C, D). In marked contrast, recipients of unmodified or SA-engineered control islets showed statistically significant compromised response as compared to the naïve animals (Fig. 8D).

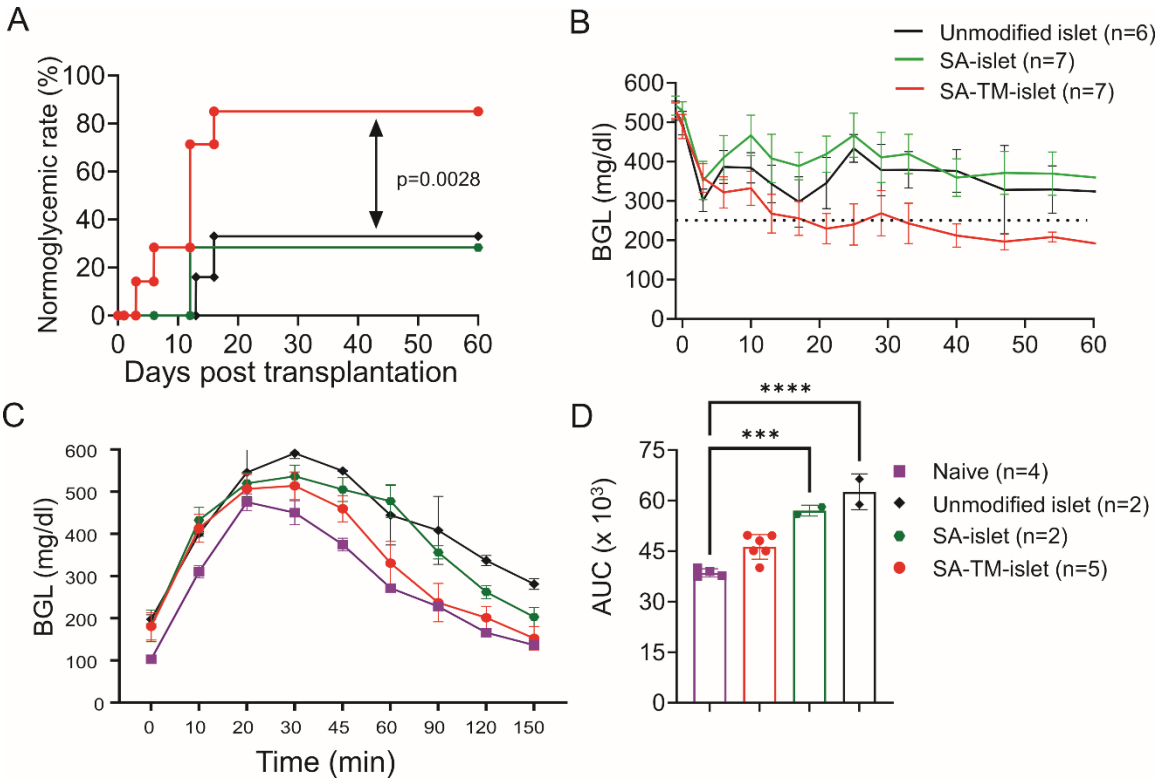
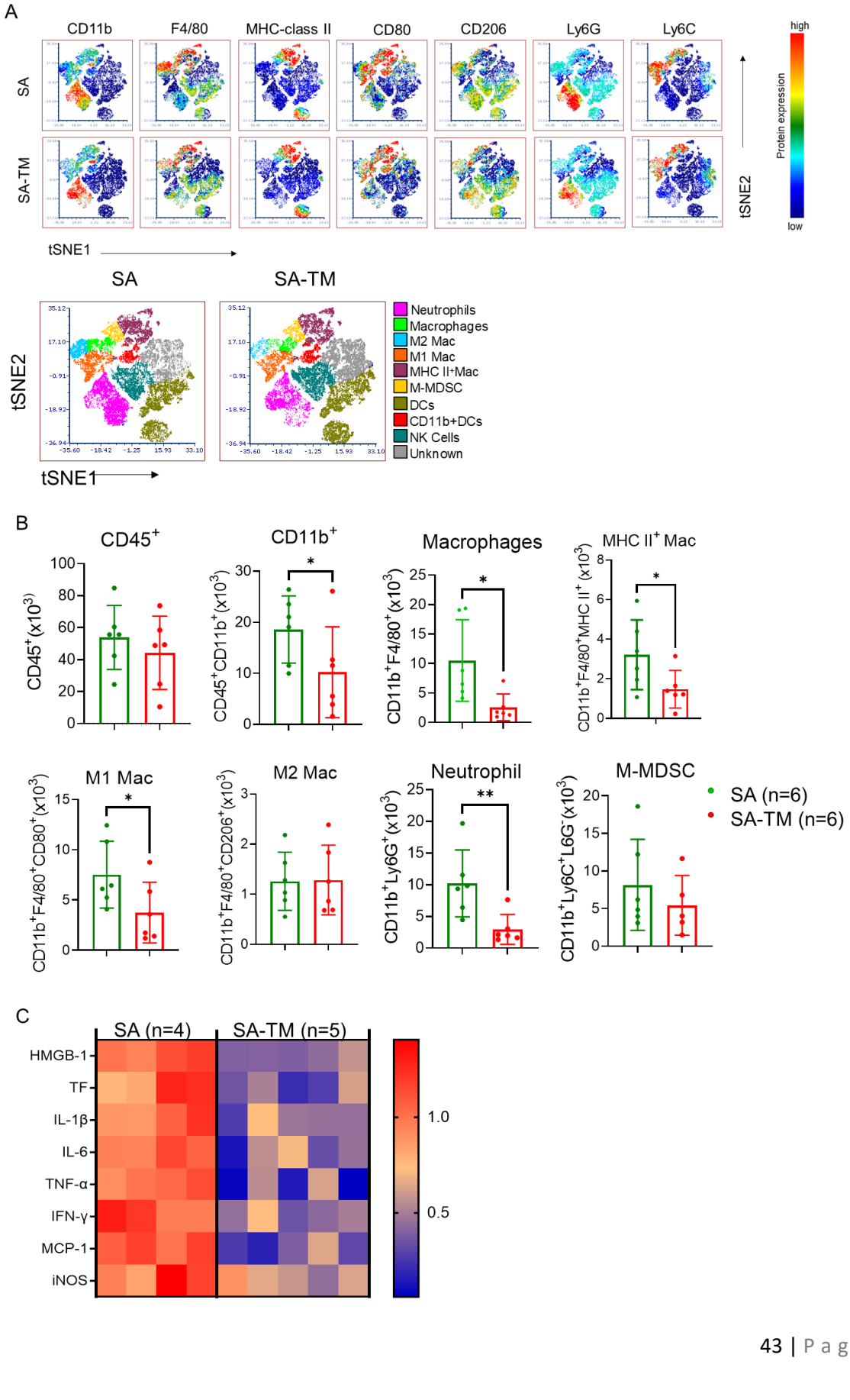


Figure 8: SA-TM improves islet engraftment and function in a syngeneic marginal mass intraportal transplantation model. Islets from C57BL/6 mice were left unmodified or engineered with SA-TM or SA as control protein and 200 IEQ were transplanted intraportally into streptozotocin diabetic syngeneic recipients. (A) Rate of euglycemia over a 60-day observation period. Log-rank (Mantel-Cox), P < 0.0028. (B) Non-fasting blood glucose levels of transplant recipients in (A). (C) Intraperitoneal glucose tolerance test (IPGTT) on long-term (> 60 days) euglycemic mice in the indicated groups. Naïve C57BL/6 mice were used as controls. (D) Area under cure (AUC) analysis for (C). Data expressed as mean ± SD. Statistical differences were assessed using a one-way ANOVA, Dunn`s test with *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

SA-TM-engineered islets inhibits various innate immune inflammatory responses at the graft site *in vivo*

The release of inflammatory molecules, such as HMGB1, and recruitment of macrophages and neutrophils into the graft site are the distinctive features of IBMIR-associated islet loss [9, 85]. To elucidate the mechanistic basis of SA-TM-mediated prevention of islet graft loss, intrahepatic immune cells were analyzed by

flow cytometry 3 hrs post-transplantation. There was significant ($p < 0.05$) reduction in the absolute numbers of graft infiltrating myeloid cells, especially M1 macrophages ($CD11b^{+}F4/80^{+}CD80^{+}$) and neutrophils ($CD11b^{+}Ly6G^{hi}$), in the SA-TM-engineered islet graft recipients as compared with those transplanted with SA-engineered control islets (Fig. 9A, B, 10A, B). Moreover, quantitative RT-PCR analysis demonstrated significantly ($p < 0.05$) decreased levels of transcripts for pro-inflammatory TF, HMGB1, MCP-1, IL-1β, IL-6, TNF-α, and IFN-γ at the site of SA-TM-engineered islet graft as compared to the SA-engineered islets (Fig 9C). Although we observed a similar trend for intragraft infiltrates and transcripts at 24 hrs post-transplantation, the differences were not significant, implying that the innate immune responses occur immediately post-transplantation (Fig. 10C). These results demonstrate that localized presentation of SA-TM by islet graft is effective in mitigating IBMIR by inhibiting the mediators of coagulation and innate inflammation.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CD11b
F4/80
MHC-class II
CD80
CD206
Ly6G
Ly6C
SA
SA-TM
high
Protein expression
low
tSNE2
tSNE1
SA
SA-TM
tSNE2
tSNE1
Neutrophils
Macrophages
M2 Mac
M1 Mac
MHC II+Mac
M-MDSC
DCs
CD11b+DCs
NK Cells
Unknown
B
CD45+
CD11b+
Macrophages
MHC II+ Mac
M1 Mac
M2 Mac
Neutrophil
M-MDSC
SA (n=6)
SA-TM (n=6)
C
SA (n=4)
SA-TM (n=5)
HMGB-1
TF
IL-1β
IL-6
TNF-α
IFN-γ
MCP-1
iNOS
1.0
0.5

Figure 9: Engraftment of SA-TM-engineered islets are associated with decreased intragraft proinflammatory innate immune mediators of IBMIR. Chemically diabetic C57BL/6 mice were transplanted with 200 IEQ engineered with control SA and SA-TM proteins. Intrahepatic islet infiltrates were harvested 3 hrs post-transplantation and analyzed in flow cytometry using antibodies to various cell surface markers demarking the indicated innate immune cells. (A) tSNE plot of immune infiltrates. (B) Absolute number of the indicated immune cell type plotted per gram of liver per animal. Data shown as mean ± SEM of 2 independent experiments. (C) Heatmap of proinflammatory intrahepatic transcripts. Total RNA was harvested from the indicated transplant groups 3 hrs post-transplantation and analyzed for quantitative assessment of the transcript levels for the indicated proinflammatory mediators using the TaqMan Gene Expression Assay. Data shown as mean ± SEM of 2 independent experiments. Statistical differences for B and C were assessed using student t-test (unpaired, one tailed) with *P < 0.05, **P < 0.01.

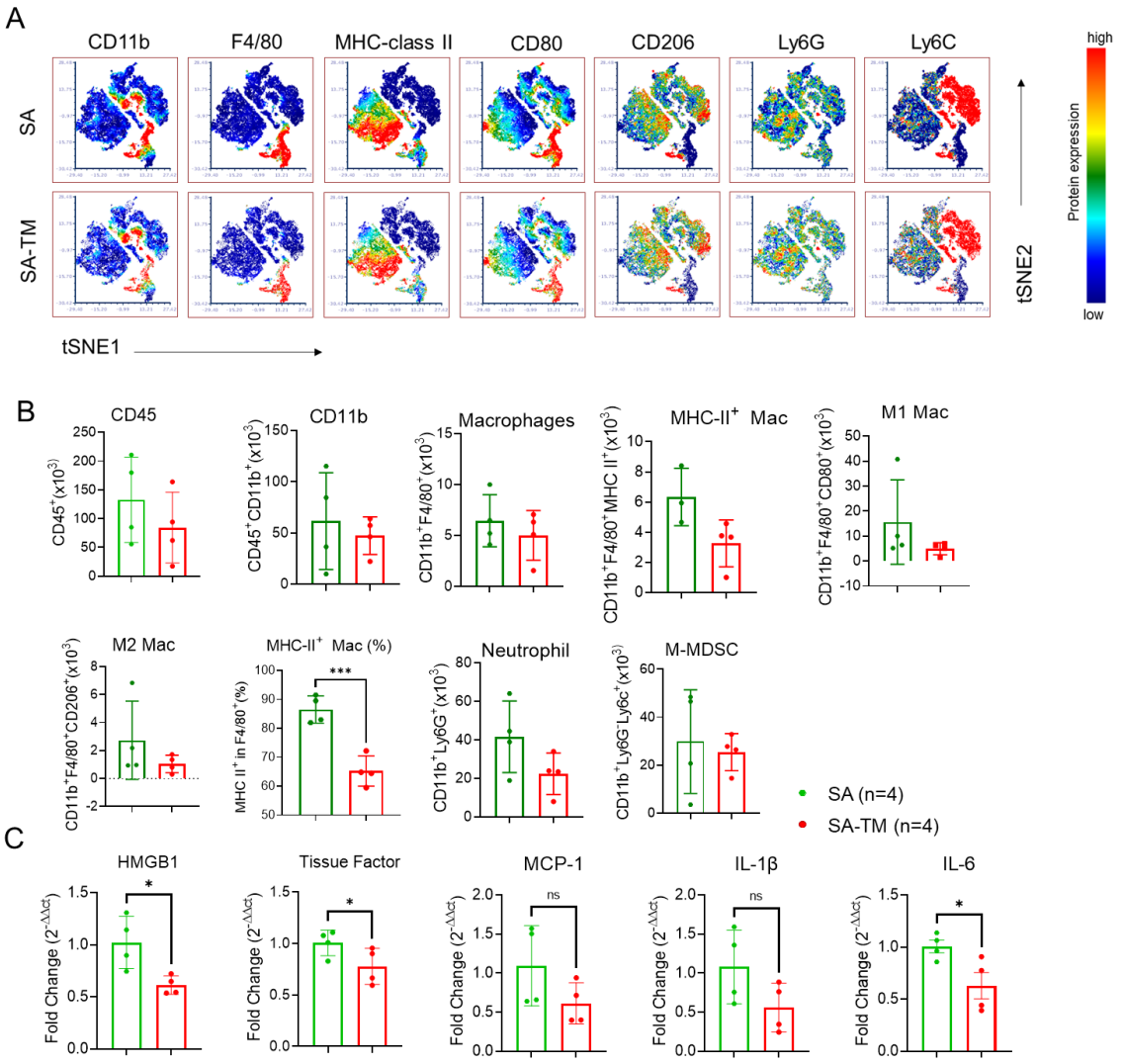


Figure 10: SA-TM on islets reduces intragraft proinflammatory mediators of IBMIR. Chemically diabetic C57BL/6 mice were transplanted intraportally with SA-TM or SA-engineered 200 IEQ. Intrahepatic immune cells were harvested 24 hours post-transplantation and analyzed using flow cytometry. (A) viSNE display of flow cytometry analysis by individual lineage identification markers of immune infiltrates of intragraft immune cells. (B) Absolute number of the indicated intragraft immune cell types. Data shown as mean ± SD of 2 independent experiments. (C). Intrahepatic transcripts for proinflammatory mediators 24 hours post-transplantation. Total RNA was isolated from the perfused liver and analyzed for quantitative assessment of the transcript levels for the indicated proinflammatory mediators using the TaqMan Gene Expression Assay. Data shown as mean ± SD of 2 independent experiments. Statistical differences for B and C were assessed using student t-test (unpaired, one tailed) with *P < 0.05, **P < 0.01 and ***P < 0.001.

Discussion

IBMIR initiated and perpetuated by innate immune responses is a major impediment for the broad use of pancreatic islet transplantation as a beta cell replacement therapy for refractory chronic pancreatitis and autoimmune T1D [9, 80, 85, 86]. Pancreatic islets express various proinflammatory mediators because of hypoxia and stressed associated with the isolation process [17, 86-90]. Following intraportal transplantation, these proinflammatory mediators initiate a cascade of nonspecific-thrombotic/inflammatory reactions that results in significant islet mass loss immediately post-transplantation, leading to lack of engraftment and inability to establish euglycemia [9, 16, 86-88]. Thrombomodulin (TM) is a cell membrane protein expressed by multiple cells and a multi-faceted molecule that regulates procoagulant and proinflammatory responses initiating and perpetuating IBMIR [80, 91, 92]. In the present study, we generated a novel form of TM, SA-TM, and transiently displayed on the surface of islets without a major impact on islet viability and function. SA-TM-engineered islets showed enhanced engraftment in a minimal mass syngeneic intraportal transplantation model that was associated with the downregulation of procoagulant and inflammatory pathways.

Inasmuch as islet graft contributes to its demise by initiating various procoagulants and inflammatory mediators that trigger and perpetuate IBMIR [9, 16, 17, 86-90, 93], pre-transplant *ex vivo* manipulation of the graft presents an attractive, safe, and potentially efficacious alternative to the treatment of graft recipient for

mitigating peri-transplant islet loss. Towards this objective, we generated a novel recombinant form of TM chimeric with SA-TM with the anticipated physical and functional characteristics. SA-TM was effectively displayed on the surface of biotinylated cells in a dose-dependent manner by taking the advantage of high affinity interaction between biotin and streptavidin [94, 95]. SA-TM protein showed a half-life of ~6 hrs on the surface of splenocytes *in vivo* as compared with significantly longer *in vivo* kinetics for various other immunomodulatory molecules we previously reported, including SA-CD47 ($t_{1/2}$ ~ 48 hrs), SA-FasL ($t_{1/2}$ > 3.5 days), and SA-CD80 ($t_{1/2}$ > 10 days) [66, 95, 96]. The mechanistic basis of the rapid turnover kinetics of SA-TM from the cell surface *in vivo* remains to be investigated and may be impacted by the structural characteristics and size of the protein, sensitivity to proteases, and potential interaction with other endogenous membranous proteins.

Islets were effectively engineered to display SA-TM on their surface without a negative impact on their viability and function consistent with the previous studies using various other immunomodulatory proteins for engineering [64, 66, 97, 98]. Islets engineered with SA-TM showed enhanced engraftment and sustained survival in a minimal mass intraportal transplantation model. SA-TM on islets is expected to enhance islet engraftment by two distinct mechanisms; i) inhibiting TF initiated coagulation by binding thrombin and blocking fibrin generation, platelet activation, and clot formation, and ii) thrombomodulin/thrombin complex converting protein C into the activated form that in turn further inhibits coagulation by

inactivation of clotting factors Va and VIIIa and also blocks expression of proinflammatory cytokines by interfering with NF-κB signaling [84, 99-103]. There was significant reduction in transcript levels for TF, IL-1β, IL-6, IFN-γ, TNF-α, MCP-1, and HMGB1, implicated in the initiation and perpetuation of IBMIR [80, 86, 87, 92, 104], in SA-TM-engineered islet graft. SA-TM-prevented damage to islets in an *ex vivo* blood loop assay that was associated with decreased levels of HMGB1 protein and increased activity of APC, consistent with studies reporting TM on the surface of islets effectively converts protein C into its active form [105, 106] . Pancreatic islets were shown to express over 50 coagulators and proinflammatory factors in response to isolation induced stress and hypoxia and actively contribute to IBMIR [16, 17, 89, 90, 93]. In particular, TF expressed by islets is a critical trigger of IBMIR by initiating extrinsic and intrinsic coagulation pathways [17] and release of various proinflammatory chemokines and cytokines that results in activation of neutrophils and macrophages and infiltration into the islet graft to inflict damage [9, 93]. The decreased level of TF in SA-TM-engineered islets *in vitro* and *in vivo* implicates this molecule in the observed islet engraftment efficacy, consistent with a study demonstrating prolonged intrahepatic islet allograft survival in a non-human primates (NHP) model treated with an Ab to TF [87]. Blocking TF with an Ab was shown to prevent the clotting reaction triggered by human islets *ex vivo* [86]. Furthermore, α-1 antitrypsin, a serine protease inhibitor, was shown to enhance islet engraftment by inhibiting intragraft levels of TF and recruitment of neutrophils and macrophages [107].

Immunophenotyping studies demonstrated that SA-TM mitigates the recruitment of myeloid cells into islet graft and their expression of inflammatory mediators. SA-TM-engineered islet grafts had significantly reduced number of $CD11b^{+}$ myeloid cells, MHC class II and CD80 expressing macrophages ($CD11b^{+}$, $F4/80^{+}$), and neutrophils ($CD11b^{+}$, $Ly6G^{hi}$) as compared to the control group. Although there was no reduction in the number of $CD206^{+}$ macrophages between the SA-TM group and control, increased number of M1 macrophages in the control group resulted in a higher ratio of M2 to M1 macrophages. These finding are consistent with the reported role of TM in blocking procoagulant and inflammatory reactions by converting protein C into APC, which in turn blocks activation and extravasation of leukocytes at sites of tissue injury and inhibits innate immune responses [100-103]. SA-TM displayed on the surface of rat splenocytes blocked their phagocytosis by mouse macrophages and inhibited activation of neutrophils in a dose dependent manner under *in vitro* settings. SA-TM was also effective in blocking the clearance of MHC deficient bone marrow cells in immunocompetent allogeneic recipients. These findings are consistent with the reported function of recombinant soluble TM to inhibit neutrophil function by preventing the formation of neutrophil extracellular trap in settings of intravascular injury and intestinal ischemia-reperfusion [108, 109]. TM polarizes macrophages to M2 with anti-inflammatory phenotype and convert protein C into APC that limits procoagulant and proinflammatory responses, such as inhibition of mononuclear phagocyte activation and production of inflammatory cytokines, as major culprit of IBMIR and peri-transplant islet graft damage [92, 110, 111]. Our findings that SA-TM

enhances islet engraftment are consistent with various reports targeting TM for the inhibition of IBMIR. The delivery of TM using a liposome formulation was effective in inhibiting IBMIR in an *ex vivo* model of human islets and enhancing engraftment of survival of islets in a mouse model of intraportal islet transplantation by reducing fibrin deposition, neutrophil infiltration, and expression of TNF-α and IL-1β [92]. Similarly, inhibition of thrombin was shown to inhibit HMGB1-induced IFN-γ by neutrophils, leading to enhanced engraftment and survival in a syngeneic intraportal transplantation model [83]. TM also has cytoprotective effect as treatment of mice with recombinant human thrombomodulin resulted in the prevention of beta cell apoptosis and increased frequency of regulatory T and tolerogenic DCs in an allogeneic setting [112].

In conclusion, our results demonstrate that SA-TM on the surface of islet graft effectively mitigates various procoagulants and proinflammatory mediators of IBMIR (Fig. 11), resulting in enhanced engraftment and sustained survival in a minimal mass intraportal transplantation model. The facile and efficient aspects of engineering islets to transiently display on their surface exogenous immune ligands for localized immunomodulation offer significant translational potential for syngeneic as well as allogeneic islet transplantation.

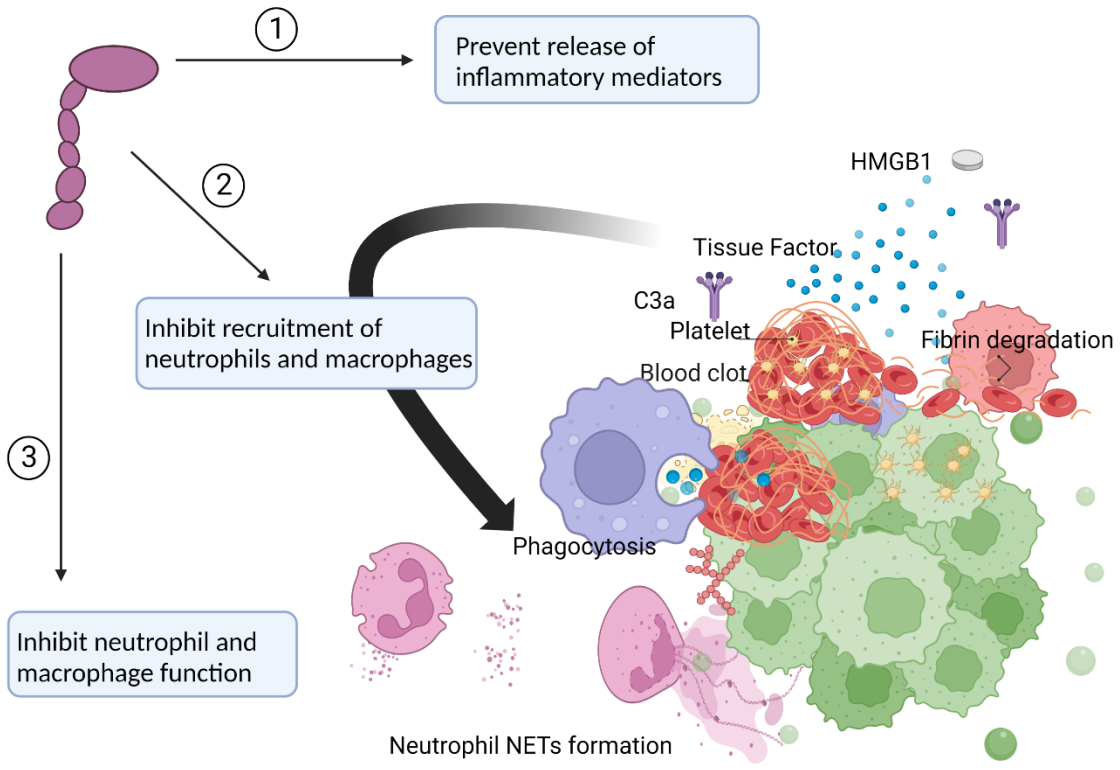


Figure 11: SA-TM modulates IBMIR. SA-TM basically shows its efficiency i) preventing the release of inflammatory mediators, ii) inhibiting the recruitment of neutrophils and macrophages and iii) inhibiting neutrophil and macrophage functions.

Chapter 4

SA-TM combination with SA-CD47 diminish IBMIR

CD47 as a potential protein in islet transplantation

A major challenge for successful islet transplantation is IBMIR due to various immunological and coagulation pathways causing inflammation and resulting in islet loss [9, 15]. IBMIR is associated with various immune responses to grafts including activation of the complement system, activation of the coagulation cascade, and infiltration of innate cells into graft sides. Upon islet-blood interaction followed by intrahepatic islet infusion, numerous inflammatory molecules such as HMGB1, TF, IL-1β, and IL-6, playing a significant role in immune response are released by islets and recipient cells to trigger IBMIR [5, 9, 10, 15, 16, 23].

The lack of CD47 on pancreatic islets makes islets more susceptible to being phagocyted by macrophages. Zhang J. showed that STZ administration declines CD47 expression on islets, promoting macrophage infiltration and phagocytosis of pancreatic islets in T1D [113]. Following syngeneic hepatocyte transplantation, CD47 KO cells significantly induce mac1 and CD16/32 protein-express on myeloid cells. Mac1 is a marker for myeloid cell activation. That demonstrates that CD47 can modulate innate immune cell activation and immune response to donor cells [114]. Moreover, Pradeep S. demonstrated that CD47 displayed on the surface of the islet diminishes IBMIR in syngeneic islet transplantation [10]. CD47 inhibits the expression level of IBMIR-associated genes, including HMGB1, TF, and IL-1β, and prevents graft infiltration of immune cells in the liver 3 hours post-islet transplantation [10].

SA-TM and SA-CD47 maintain enhanced islet engraftment by decreasing IBMIR and numerous proinflammatory signaling.

IBMIR consists of numerous reactions including the release of inflammatory mediators, coagulation, complement system activation, myeloid cell infiltration, and platelet activation which are major culprits of early islet loss following by intraportal islet transplantation [5, 9]. Recently, strong immunosuppressants combined with different anti-inflammatory drugs were used to overcome IBMIR reactions, however using immunosuppressant therapies has serious side effects [5]. Hence, the main goals are to mitigate non-specific inflammatory reactions and establish localized anti-inflammatory microenvironments for islet grafts without using immunosuppressants. Thanks to the multifaceted anti-inflammatory roles of TM and CD47 in modulating both innate and adaptive immune responses, we propose that both TM and CD47 displayed on the surface of islets synergistically inhibit IBMIR and provide a localized anti-inflammatory microenvironment for islet graft.

Islet-recipient blood interaction induces coagulation, complement system activation, platelet activation, and the release of various pro-inflammatory mediators causing a strong innate immune response to islet grafts [9, 10, 27]. TM is known as an anti-coagulant inhibiting the release of TF and platelet activation [16, 39, 42, 72]. Moreover, both TM and CD47 have been shown to inhibit the release of inflammatory mediators such as HMGB-1, TF, IL-1β, and IFN-γ [10,

115]. TM combined with CD47 modulates macrophages. CD47-SIRPα signaling pathway mitigates phagocytosis while TM induces M2 macrophage polarization [10, 56, 110, 116]. Both proteins also play important roles in macrophage migration and cytokine production [112, 117]. The release of dangerous signals and activation of coagulation and complement systems lead to the infiltration of neutrophils. Shrestha demonstrated that CD47 inhibits neutrophil infiltration in syngeneic islet transplantation, and other studies have shown TM inhibits NETosis [10, 112, 116, 118]. Moreover, CD47 inhibits NK cell-mediated cell cytotoxicity via SIRPα signaling pathway [56]. To sum up, CD47 and TM synergistically regulate various inflammatory reactions associated with IBMIR.

SA-TM and SA-CD47 transiently display on the surface of biological and nonbiological surfaces.

ProtEx™ technology enables the display of different immunomodulatory proteins on biological and non-biological surfaces. Our lab previously showed that a novel form of extracellular CD47 with a modified form of core SA displayed on a biotinylated islet shows enhanced engraftment in syngeneic islet [10]. SA-TM or SA-CD47 was designed in the copper sulfate–inducible pMT/BiP/V5-HisA expression vector and transfected into Drosophila S2 cells for protein expression (Fig. 12A). SA-TM and SA-CD47 were around ≈70-kDa and ≈37-kDa, respectively, and showed their size in denaturing SDS-PAGE after samples were heated at 100°C (Fig. 12B). Previously we showed 400 ng of SA-CD47 were efficiently displayed on the surfaces of biotinylated islets and splenocytes. In the combination of SA-TM with

SA-CD47, 3.2 µg SA-TM and 400 ng of SA-CD47 proteins can be transiently on the surface of biotinylated splenocyte (15 µM) (Fig. 12C).

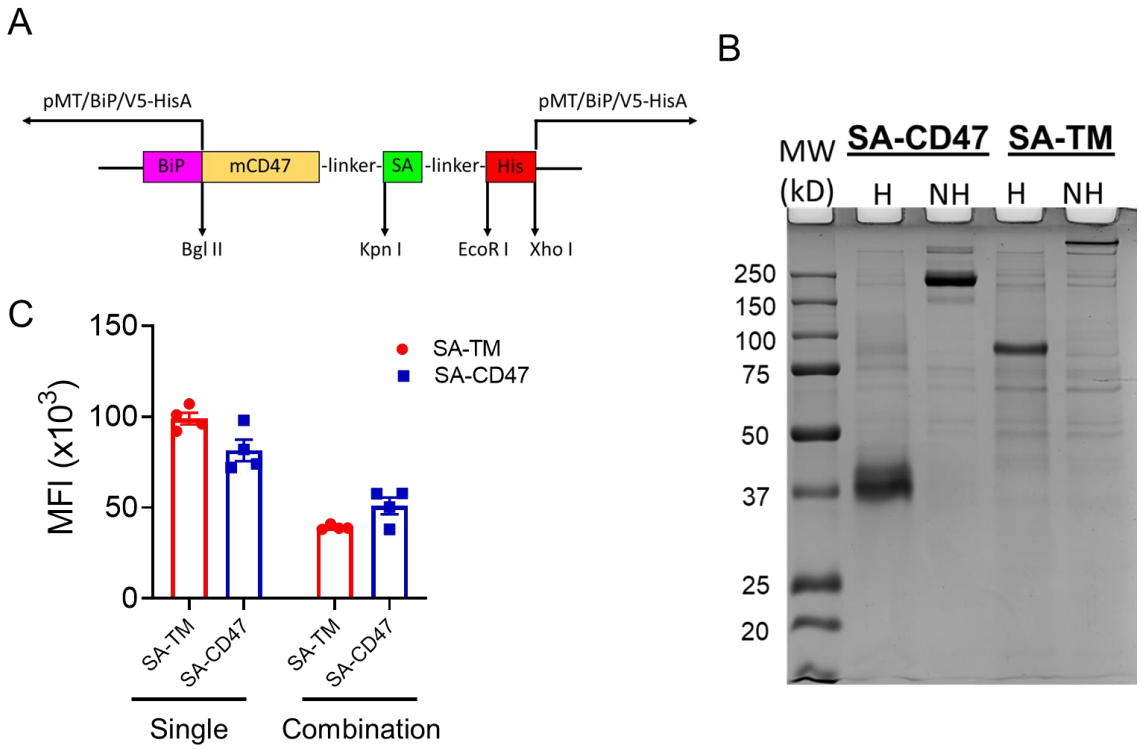


Figure 12:Generation of SA-CD47 chimeric protein, structural and establishing co-engineering condition of SA-TM and SA-CD47. (A) Schematic representation of the SA-TM construct. A synthetic chimeric gene containing the coding sequences for the extracellular domain of mouse CD47 and core streptavidin (SA) with flexible linkers and a 6xHis tag to facilitate protein purification was constructed and subcloned into the CuSO4-inducible pMT-Bip-V5-HisA S2 insect cell expression vector. (B) SDS-PAGE profile of SA-TM and SA-CD47. The protein was produced in S2 cells, isolated from cell supernatant using metal affinity chromatography, left unheated (NH) or heated at 100^{o}C, and analyzed using denaturing SDS-PAGE for structure and purity. (C) Splenocytes engineered with SA-TM or SA-CD47 protein. Mouse splenocytes were biotinylated (15 µM), engineered with SA-TM protein (3.2 µg/10^6 cells), or SA-CD (400 ng/10^6 cells), or SA-TM/SA-CD47 and stained with an Ab to human TM, or CD47 and analyzed MFI values. Data expressed as mean ± SD and representative of 3 independent experiments.

SA-TM and SA-CD47 inhibit phagocytosis by macrophages.

Myeloid cells are mainly responsible for islet damage in intrahepatic islet transplantation. Shrestha P. showed a novel form of CD47 inhibits graft infiltrating myeloid cells and their pro-inflammatory functions in syngeneic islet transplantation while Okano Y. demonstrated recombinant Thrombomodulin maintains pancreatic islet by decreasing the macrophage infiltration in diabetes mellitus [10, 112]. We evaluated whether the combination of SA-TM and SA-CD47 inhibits phagocytosis using an *in vitro* xenogeneic setting. CFSE-labeled rat splenocytes engineered with SA, SA-TM, SA-CD47, or SA-TM/SA-CD47 were cocultured with mouse macrophage RAW264.7 cell line activated with LPS (Fig. 13). The proportion of CFSE-positive macrophages was assessed using flow cytometry. As shown in Figure 13, SA-TM or SA-CD47 or SA-TM/SA-CD47 reduced the macrophages engulfing, compared to SA, which served as a control. However, we did not observe a more enhanced inhibition when both molecules are displayed on the cells as compared to single agents, which may be due to the limitation of the *in vitro* system utilized.

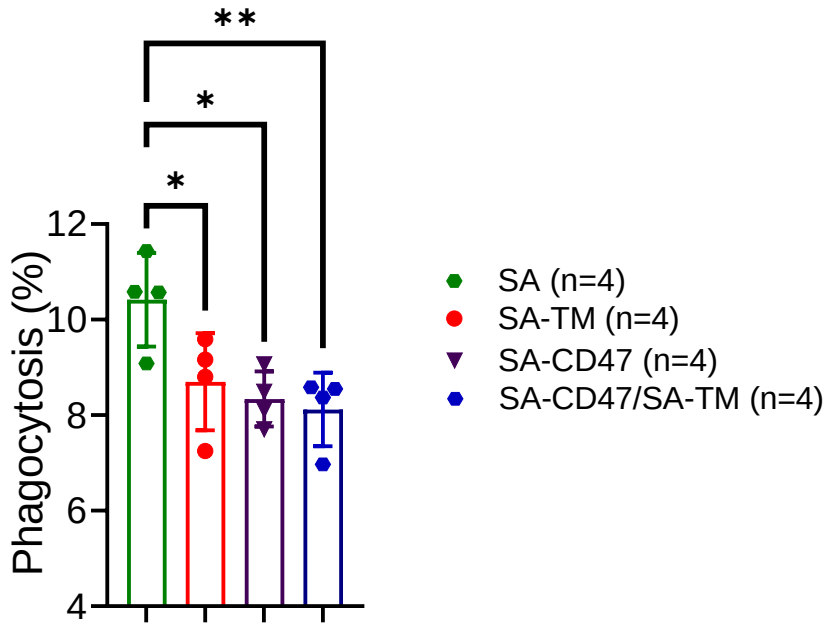


Figure 13: SA-TM and SA-CD47 prevent phagocytosis in vitro. SA-TM, SA-CD47 or SA-TM/SA-CD47 on xenogeneic rat splenocytes inhibits phagocytosis by macrophages in vitro. CFSE labeled SA-TM or SA-CD47 or SA-TM/SA-CD47 engineered rat splenocytes were cocultured with mouse RAW 264.7 macrophage cells at a 1:5 ratio for 18 hrs. Phagocytosis of rat cells ($CD11b^{+}F4/80^{+}CFSE^{+}$) was assessed using flow cytometry. Data are shown as mean ± SD pooled from 3 separate experiments: Statistical differences were assessed using ANOVA followed by Dunnett's multiple comparison test with *P < 0.05, **P < 0.01.

Next, we assessed the efficiency of SA-TM and SA-CD47 effects on allogeneic innate immune response *in vivo*. To avoid allogeneic adaptive immune responses directed towards MHC molecules, we used bone marrow cells from NSG mice deficient for both MHC class I and class II for transplantation. CFSE-labeled SA-TM, SA-CD47, or SA-TM/SA-CD47 labeled bone marrow cells were mixed at a 1:1 ratio with CTV-labelled-SA-engineered cells and injected i.v. into C57BL/6 allogeneic recipients. Flow cytometry analysis of NSG cells 24 hrs post-infusion demonstrated significantly ($p < 0.005$) reduced percentages of SA-engineered cells

as compared with SA-TM, SA-CD47, or SA-TM/SA-CD47-engineered cells (Fig. 14 A-C). Taken together, these results demonstrate the efficacy of SA-TM and CD47 in inhibiting the effector function of macrophages and neutrophils ex vivo and clearance of allogeneic cells by innate immunity *in vivo*.

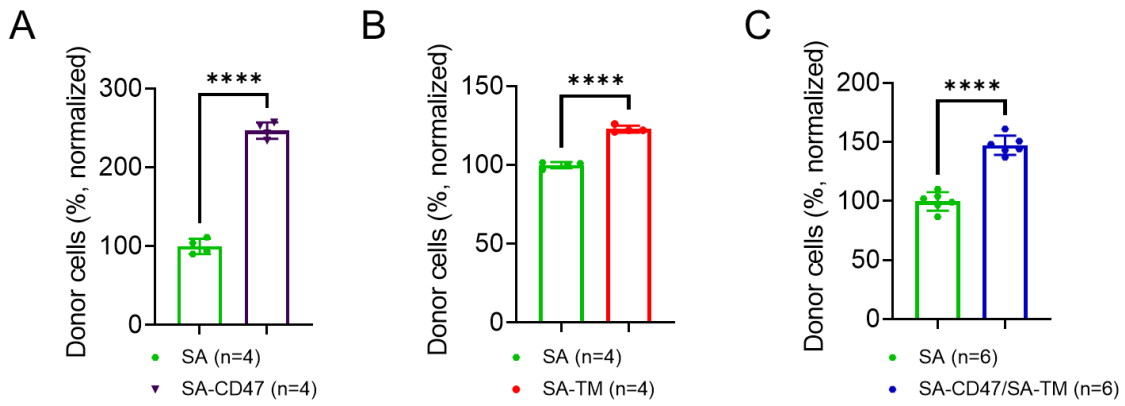


Figure 14: SA-TM and SA-CD47 prevent the clearance of bone marrow against allogeneic innate immunity. SA-TM inhibits in vivo clearance of allogeneic bone marrow cells. Bone marrow from NSG mice deficient for MHC class I and II was engineered with SA (0.8 µg/10^6 cells) or SA-TM (3.2 µg/10^6 cells) proteins followed by labeling with CTV and CFSE, respectively. Cells were then mixed at a 1:1 ratio and injected i.v. into allogeneic C57BL/6 recipients (in total 20x10^6 cells/animal). Splenocytes were harvested 24 hrs post-transplantation and analyzed using flow cytometry by gating on $CFSE^+$ or CTV^+ donor cells. Data are shown as mean ± SD of 4 independent experiments: Statistical differences were assessed using Student's 2-tailed unpaired t-test with *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

3.3 Co-engineering with SA-TM and SA-CD47 proteins does not adversely impact islet viability or function

We next evaluated that co-engineering islets with SA-TM and SA-CD47 do not adversely impact islet viability and functionality. Mouse islets were biotinylated (15 µM) and co-engineered with 3200 ng of SA-TM and 400 ng of SA-CD47 (Fig. 15A,

B). Confocal microscopy results showed islets were co-engineered with SA-TM and SA-CD47 proteins. Alamar blue assay was used for the metabolic activity of co-engineered islets (Fig. 16A). The metabolic activity of co-engineering of islets was unaltered, compared to unmodified islets. GSIS assay results demonstrated that engineering islets with SA-TM and SA-CD47 do not have any adverse impact on insulin secretion in response to glucose stimulation *in vitro* (Fig. 16B, C). Altogether, both SA-TM and SA-CD47 were efficiently displayed on the surface of islets and the engineering process remained unaltered in islet viability, metabolism, and functionality.

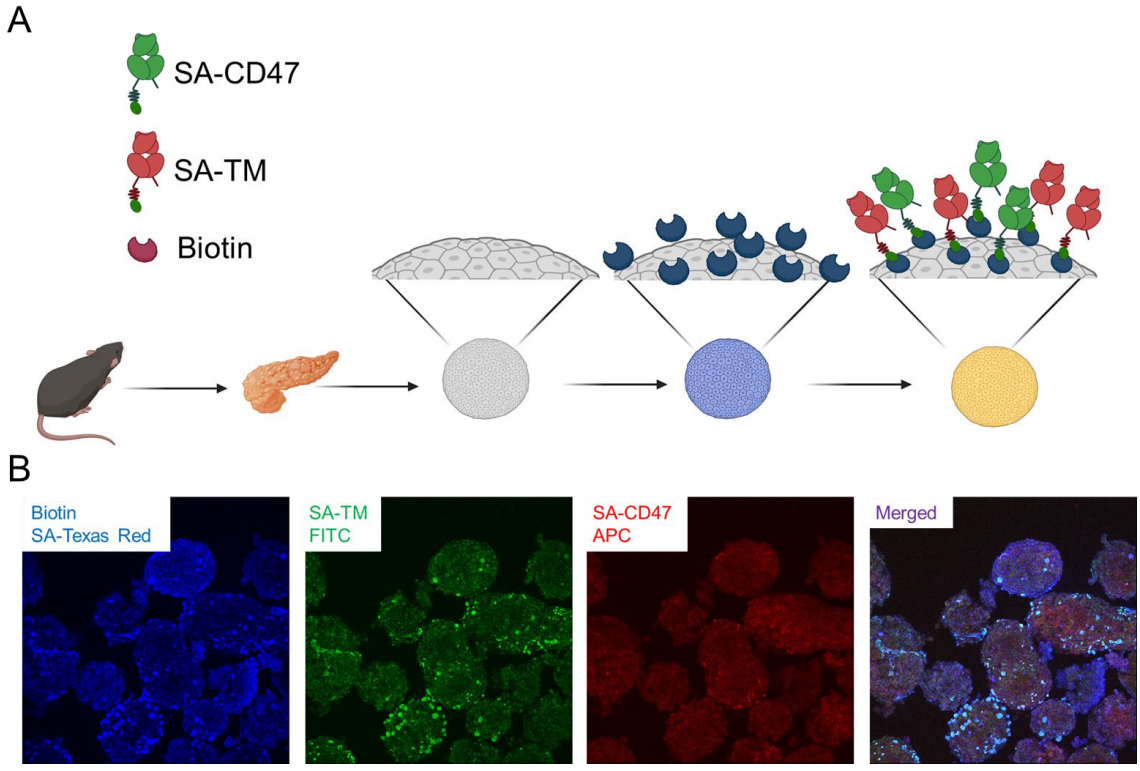


Figure 15:Co-engineering islets with the SA-TM and SA-CD47 proteins does not impact islet viability and function. (A) Schematic drawing of islet engineering with the SA-TM and SA-CD47 proteins. (B) A representative image of SA-TM/SA-CD47-engineered islets (15 µM biotin and 3.2 µg SA-TM and 400 ng SA-CD47/500 islets). Biotinylation and engineering were assessed using

fluorescence-labeled streptavidin (blue) and an antibody to human TM (green) and an antibody to mouse CD47 (red) in confocal microscopy.

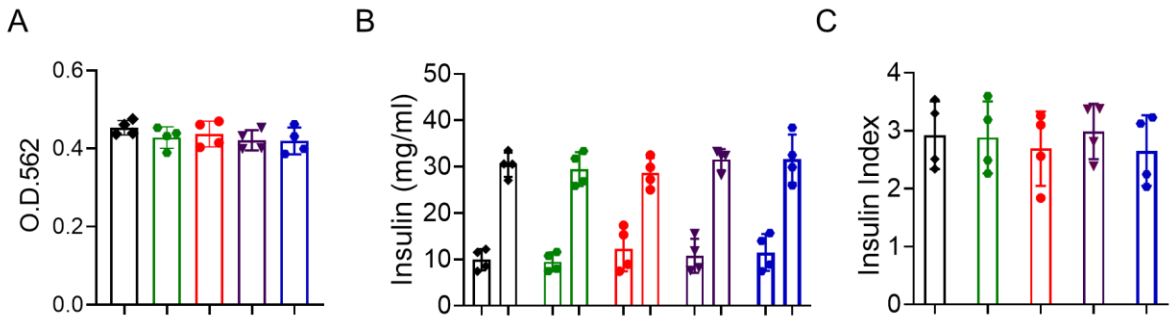


Figure 16: Co-engineering islets with the SA-TM and SA-CD47 proteins does not impact islet viability and function. A) Alamar blue assay performed for metabolic activity of co-engineered islets. Islets were unmodified or engineered with single or co-engineered SA-TM and SA-CD47, or an equimolar of SA proteins. Alamar blue reagent was added into each well. 8 hours later, optical density (OD562) value was measured by a microplate reader with a 570nm filter. B) Insulin stimulated glucose secretion assay performed for the functionality of unmodified or engineered islets with SA-TM and SA-CD47. 50 unmodified or SA- or SA-TM or SA-CD47 or SA-TM/SA-CD47-engineered islets were cultured in cultured in Kreb`s buffer containing low (L,3.5 mM) and high (H, 16.5 mM) doses. Secreted insulin levels in the supernatants were measured using ELISA. C) Stimulation index of insulin secretion in high/low doses of groups as shown in (B). For panel A, data are shown as mean ± SD pooled from a minimum of 2 separate experiments: Statistical differences were assessed using ANOVA followed by Dunnett's multiple comparison test.

SA-TM and SA-CD47 displayed on the surface of islets maintain islet integrity and diminish various innate immune responses

SA-TM and SA-CD47 are immunoregulatory proteins that regulate coagulation and induce anti-inflammatory signals resulting in mitigating IBMIR [10, 44, 70, 110, 112, 114, 115]. To test the combination of SA-TM and SA-CD47 efficiently inhibits IBMIR, we used *in vitro* blood loop assay to mimic innate immune responses

occurring following intrahepatic islet infusion [66, 82]. 100 IEQ C57BL/6 islets engineered with SA or 3.2μg of SA-TM and 400 ng of SA-CD47 and cocultured in autologous fresh blood in heparin-coated tubes at 37^{o}C for 3 hrs. Histological analysis of islet-thrombus demonstrated that SA-TM and SA-CD47 combination maintains islet intact structure (>70%) while the majority of islets were damaged in SA-engineered islets (Fig. 17A, B). There was also a significantly lower level of HMGB-1 in the serum of the SA-TM/SA-CD47-engineered group as compared with the SA-engineered control islet group (Fig. 17C). Gene expression results revealed that SA-TM and SA-CD47 synergistically reduced pro-inflammatory mediators, cytokines, and chemokines associated with IBMIR and myeloid cell functions and migration. HMGB-1, TF, IL-1β, INF-γ, and TNF-α playing important roles in IBMIR were significantly reduced in the SA-TM and SA-CD47 group, compared to SA. Moreover, SA-TM and SA-CD47 decreased the inflammatory cytokines and chemokines associated with macrophage and neutrophil functionality and infiltration, such as arginase, inducible nitric oxide synthase (iNOS), CXCL-12, and MCP-1 (Fig. 17D). Overall, co-engineering islets with SA-TM and SA-CD47 significantly reduces the levels of proinflammatory cytokines, chemokines, and DAMPs that translate into protection in an ex vivo model of IBMIR.

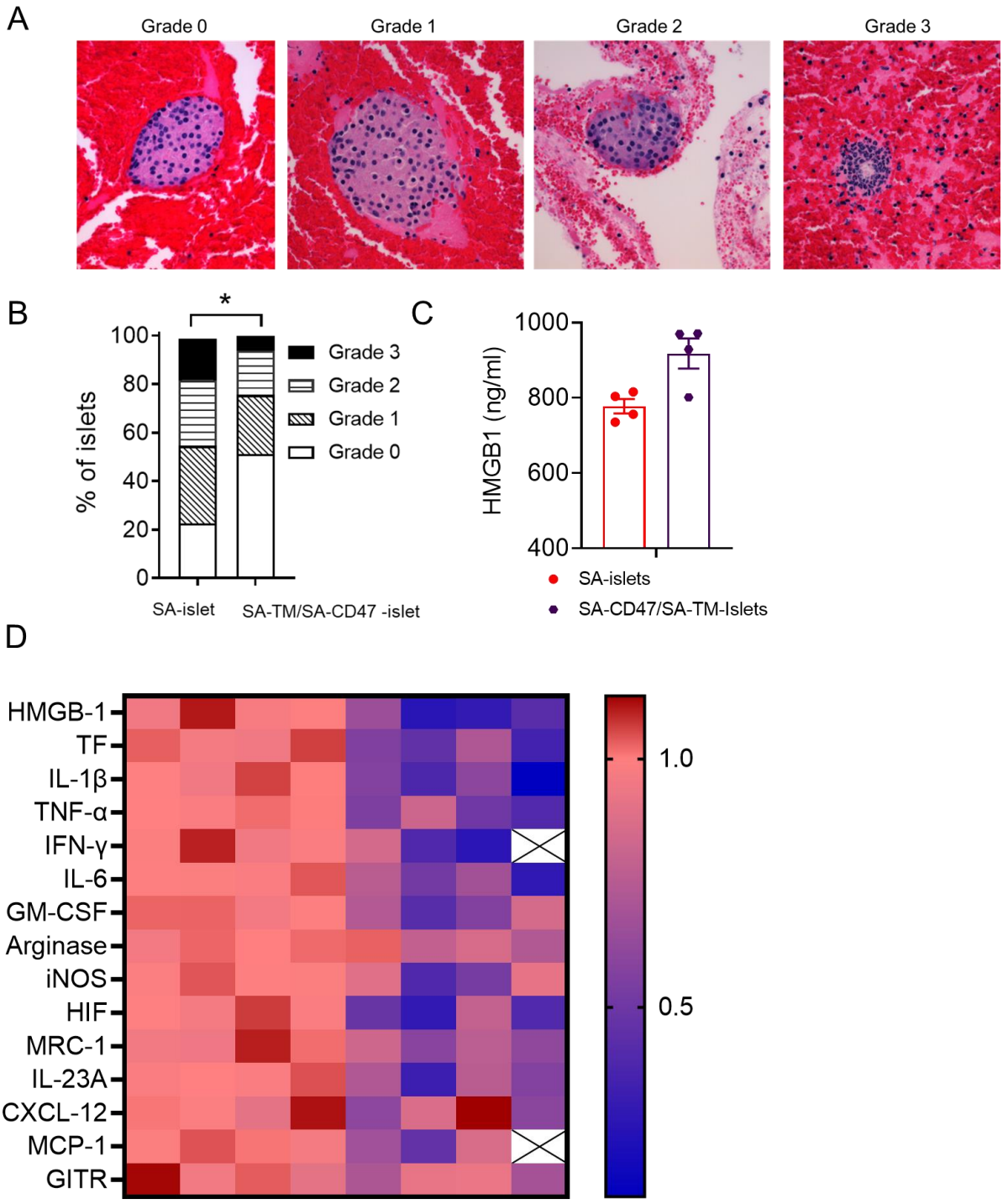


Figure 17: SA-TM and SA-CD47 maintain islets integrity by inhibiting numerous proinflammatory signaling pathways. An in vitro blood loop assay was performed to mimic IBMIR. 100 islets engineered with SA or SA-TM/SA-CD47 were cultured in fresh autologous blood at 37^0C for 3 hrs. Islet-thrombus was

utilized for histological assessment and gene expression assays while serum was used for the level of released HMGB1. (A) Islet thrombus was used for H&E staining. (B) Based on islet structure and quality, islet thrombus was assessed. Data are shown as mean ± SEM of at least 3 independent experiments. (C) ELISA was performed to evaluate the levels of released HMGB1. (D) Heatmap of proinflammatory factors related IBMIR. TaqMan qPCR was performed on the total RNA extracted from the islet-thrombus. Data from 4 independent experiments. (C) HMGB1 levels in the serum of islet thrombus using ELISA. Data are shown as mean ± SEM of at least 3 independent experiments. Statistical differences were assessed using Student's 2-tailed unpaired t-test with *P < 0.05, **P < 0.01.

SA-TM/SA-CD47-engineered islets contribute to islet engraftment and maintain euglycemia in a minimal mass intraportal transplantation setting

TM is an effective and physiological modulator of inflammation and CD47 prevents phagocytosis by macrophages and neutrophil migration. Previously, Shrestha showed 125 (≈175-200 IEQ) islets engineered with 400 ng of SA-CD47 maintain enhanced islet engraftment [10]. We next tested the synergistic efficiency of SA-TM and SA-CD47 proteins transiently displayed on the surface of islets in early islet graft loss in the syngeneic minimal mass intraportal transplant model. C57BL/6 islets were biotinylated (15 µM) followed by engineered with 3.2 µg of SA-TM and 400 ng of SA-CD47 and then transplanted into (150 IEQ/recipient) intraportal into diabetic syngeneic recipients. Over 60% of recipients of SA-TM/SA-CD47-engineered islets reached and maintained euglycemia for a 60-day observation period as compared to 50% of recipients of SA-TM -engineered islets and less than 20% of recipients of unmodified islets, served as a control (Fig. 18A, B). Moreover, Recipients who received SA-TM/SA-CD47-engineered islets displayed better outcomes in IPGTT compared to those who received unmodified

islets or SA-TM-islets. The combination treatment demonstrates levels that are comparable to those observed in non-transplanted naïve mice. On the other hand, there IPGTT levels of SA-TM-engineered recipients are significantly higher than naïve mice without transplantation ($p<0.05$). Moreover, IPGTT outcome recipients with naïve islets showed a significantly worse outcome ($p<0.01$) (Fig. 18C, D).

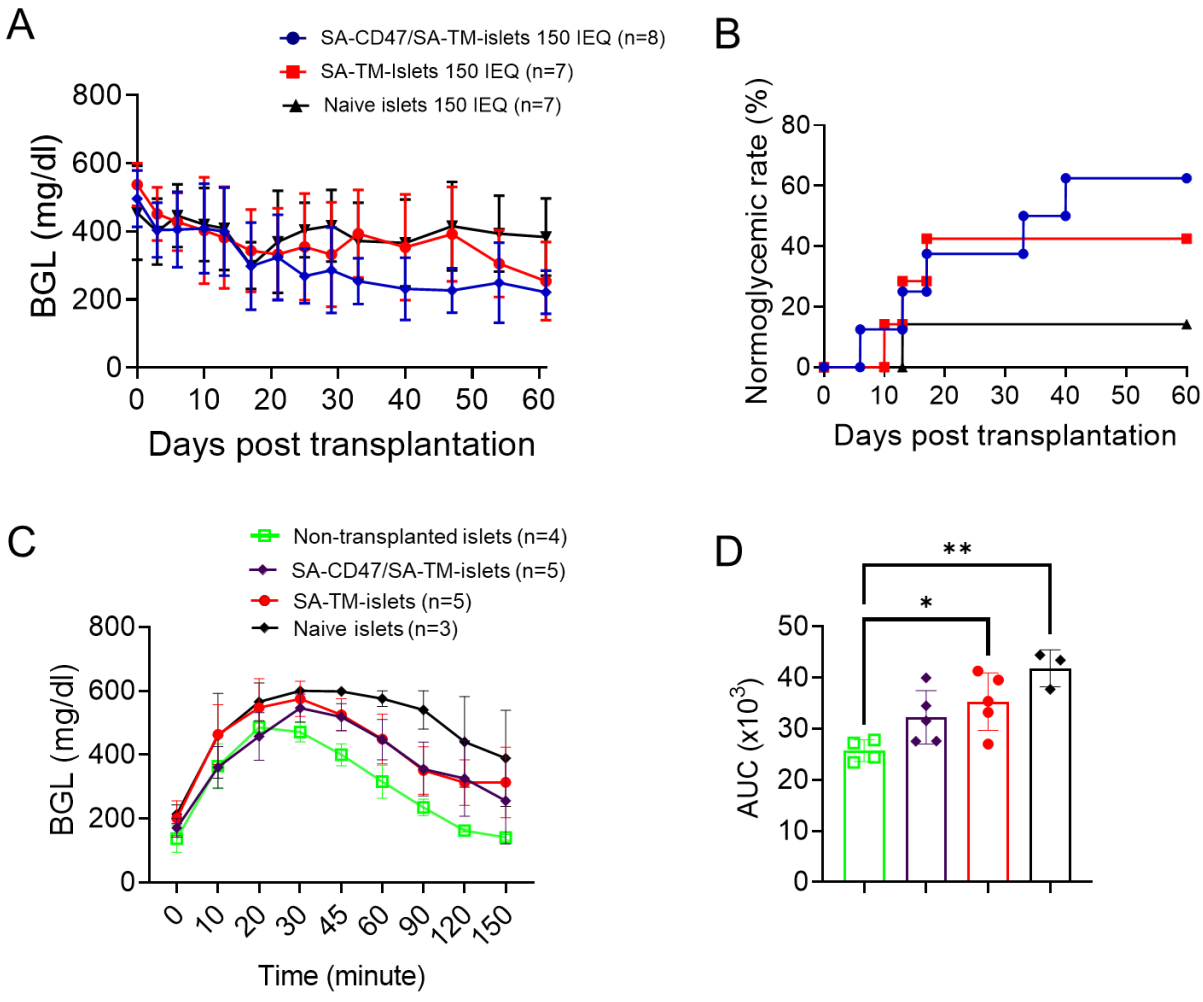
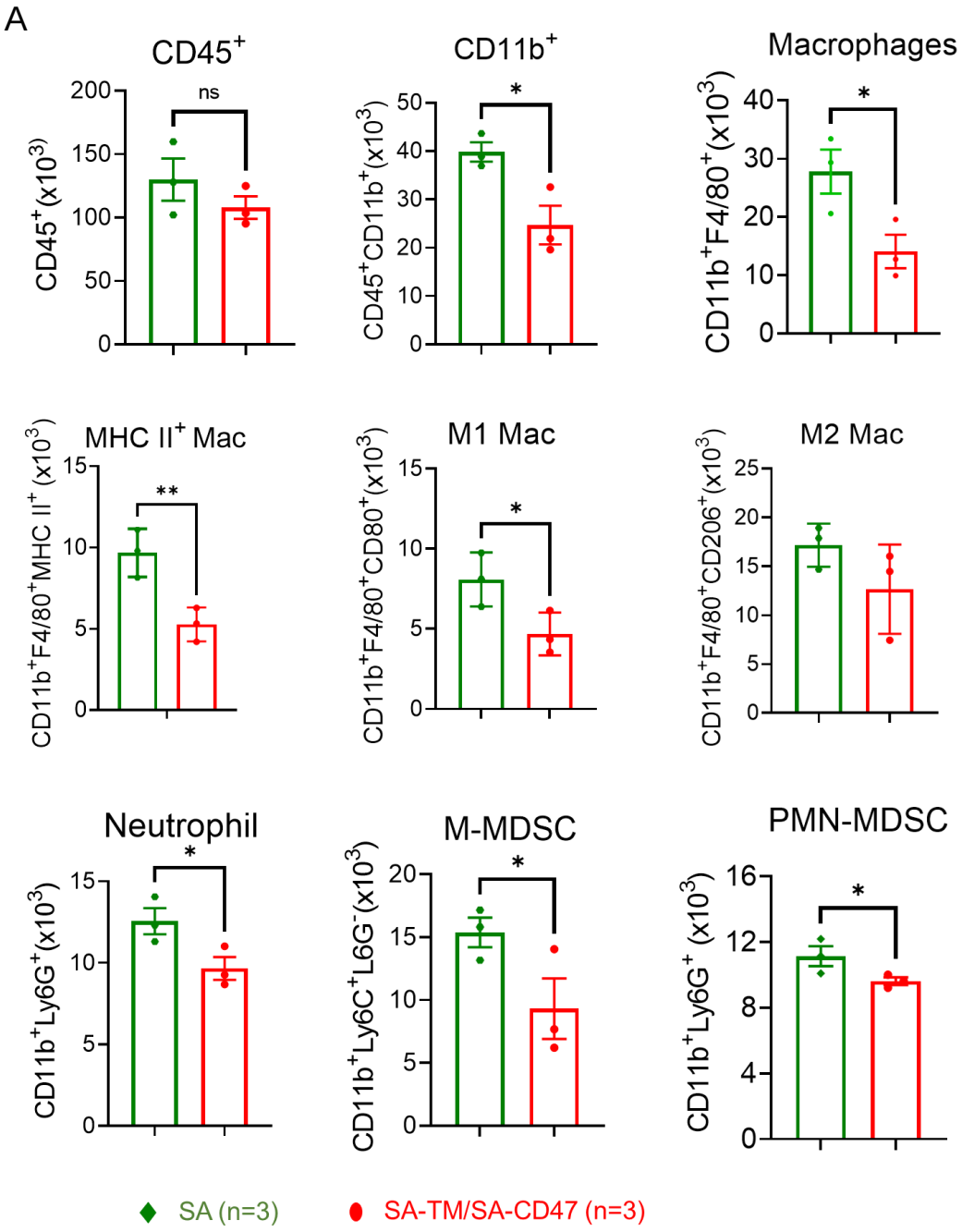


Figure 18: SA-TM/SA-CD47 improves islet engraftment and function in a syngeneic marginal mass intraportal transplantation model. Islets from C57BL/6 mice were left unmodified or engineered with SA-TM or SA-TM/SA-CD47 as the control protein. Streptozotocin diabetic syngeneic recipients were intraportally transplanted with 150 IEQ islets. (A) Rate of euglycemia over a 60-day observation period. Log-rank (Mantel-Cox), $P < 0.0028$. (B) Non-fasting blood glucose levels of transplant recipients in (A). (C) Intraperitoneal glucose tolerance

test (IPGTT) on long-term (>60 days) euglycemic mice in the indicated groups. Naïve C57BL/6 mice were used as controls. (D) Area under curve (AUC) analysis for (C). Data expressed as mean ± SD. Statistical differences were assessed using a one-way ANOVA with *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

SA-TM and SA-CD47 diminish innate immune response and levels of numerous innate immune inflammatory gene expressions in vivo

The release of DAMPS and inflammatory mediators, such as HMGB1 or TF, and immune cell infiltration into the graft site cause the majority of islet loss [5, 9, 15, 16]. To understand the mechanism of the SA-TM/SA-CD47 combination preventing IBMIR and maintaining grafts, intrahepatic immune cells were harvested 3 hours post-transplantation. There are no significant differences in $CD45^+$ immune cells while $CD11b^+$ myeloid cells reduced significantly in SA-TM/SA-CD47 recipients, compared to SA. SA-TM combined with SA-CD47 inhibits both macrophages ($CD11b^+F4/80^+$), especially M1 inflammatory macrophages ($CD11b^+F4/80^+Mhc\text{-}II^+CD80^+$) ($p<0.01$), while there are not significantly differences in M2 macrophages, as compared to SA. There was significantly ($p <0.05$) reduction in the absolute numbers of graft infiltrating Neutrophil, PMN-MDSC and M-MDSC in recipient with islets engineered with SA-TM and SA-CD47, as compared with those transplanted with SA-engineered control islets (Fig. 19A). Moreover, quantitative RT-PCR analysis demonstrated significantly ($p <0.05$) decreased levels of transcripts for DAMPS, such as TF, HMGB1, pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IFN-γ, GM-CSF, hypoxia (HIF) at the site of SA-TM/SA-CD47-engineered islet graft as compared to the SA-engineered islets (Fig. 19B).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CD11b+F4/80+CD206+(x10^3)
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M-MDSC
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CD11b+Ly6C+L6G-(x10^3)
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SA (n=3)
SA-TM/SA-CD47 (n=3)

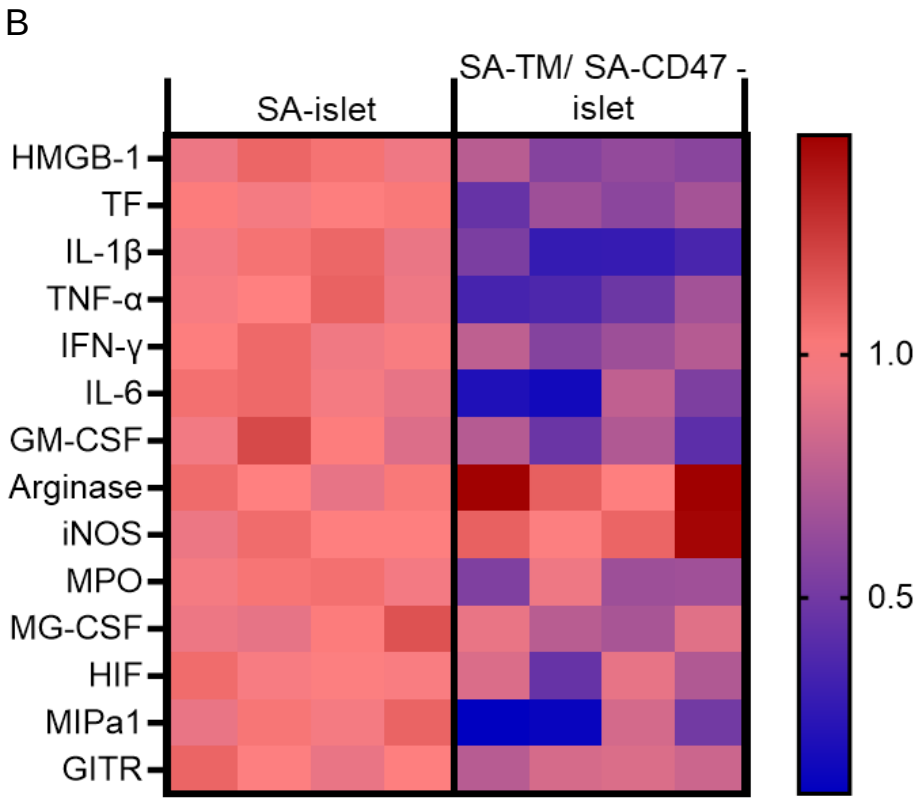

Figure 19: SA-TM/SA-CD47-engineered islets modulate intrahepatic immune cells profile and intragraft proinflammatory innate immune mediators of IBMIR. STZ-administered diabetic mice were transplanted with 150 IEQ engineered with SA-TM/SA-CD47 proteins or SA served as a control. 3 hours post-transplantation, the liver was harvested and used for flow cytometry and qPCR. Intrahepatic islet infiltrates were labeled with antibodies to various cell surface markers and analyzed in flow cytometry. (A) The absolute number of the indicated immune cell type plotted per liver cells per animal. Data are shown as mean ± SEM of 1 independent experiment. Statistical differences were assessed using a student t-test (unpaired, one-tailed) with *P < 0.05, **P < 0.01. (C) Heatmap of proinflammatory intrahepatic transcripts. Total RNA was harvested from the indicated transplant groups 3 hrs post-transplantation and analyzed for quantitative assessment of the transcript levels for the indicated proinflammatory mediators using the TaqMan Gene Expression Assay

Discussion

TM is a transmembrane glycoprotein expressed on endothelial cells and many cells that initiates anti-inflammatory and procoagulant signals [22, 44, 45]. CD47 sends “do not eat me” signals and regulates anti-inflammatory signals by interacting with different molecules, such as SIRPα, on myeloid cells [10, 46, 49, 56].

Due to the canonical features of Thrombomodulin and CD47, both proteins can perpetuate IBMIR. To overcome IBMIR, we generated novel forms of Thrombomodulin (SA-TM) and CD47 (SA-CD47), and both chimeric proteins were successfully transiently displayed on the cells and islets. Islet engineering with SA-TM and SA-CD47 proteins has no adverse impacts on islet viability and functionality. Both immunomodulatory proteins, SA-TM and SA-CD47, demonstrated their anti-inflammatory features in phagocytosis in vivo and inhibiting clearance of allogeneic bone marrow cells in vivo. SA-TM (3.2 µg/ 500 islets) and SA-CD47 (400 ng/ 500 islets) were successfully displayed on the surface of the islets. Previously, Shrestha P et al. showed 125 islets (175-200 IEQ islets) SA-CD47 maintains enhanced engraftment and decreases graft infiltrating cells and the expression levels of inflammatory genes [10]. We also showed that 200 IEQ SA-TM contributed to islet engraftment and supported euglycemia by modulating myeloid cells and decreasing inflammatory signals.

In this project, SA-TM and SA-CD47 synergistically perpetuated the engraftment and maintained euglycemia in a minimal mass syngeneic intraportal

transplantation model related to a reduction in graft infiltration myeloid cell numbers and the downregulation of inflammatory pathways. The combination of SA-TM and SA-CD47 on islets is expected to improve islet engraftment by four distinct mechanisms 1) inhibiting coagulation and thrombosis by TM-binding Thrombin, activating APC, and binding thrombin and congesting fibrin generation, 2) TM and APC, a product of TM-Thrombin complex, blocking coagulation, 3) preventing phagocytosis by both proteins, 4) blocking expression of proinflammatory cytokines and graft-infiltrating cells. There was a significant reduction in transcripts for MCP1, HMGB1, TF, IL-1β, IL-6 (all playing critical roles for early islet graft mass loss), GM-CSF, ins, MPO, MIP1α, GITR (playing essential roles in the proinflammatory functions of macrophages and neutrophils), and HIF (a hypoxia marker) in recipients with SA-TM/SA-CD47 engineered-islets, compared to recipients whose those islet engineered with SA. SA-TM/SA-SA-CD47- intercepted islet loss in an *ex vivo* blood loop assay was related to reduced levels of HMGB1 protein in the serum, consistent with studies reporting TM function [42, 116].

Pancreatic islets produce various coagulators and proinflammatory mediators in response to isolation-induced stress, transplantation process, coagulation, and hypoxia: all contribute to IBMIR. Both proteins, particularly TM, inhibit TF and HMGB1, which induce various proinflammatory chemokines and cytokines that initiate activation and infiltration of neutrophils, and macrophages resulting in adverse islet damage. Reduction expression levels of TF and HMGB1 in SA-

TM/SA-CD47-engineered islets in *ex vivo* and *in vivo* reveals the efficiency of combinational treatment in islet engraftment, in harmony with prolonged intrahepatic islet allograft survival in an NHP model treated with anti-TF antibody in or sepsis treatment with HMGB1 antagonists.

Furthermore, Blocking HMGB1-mediated RAGE or TLR4 signaling pathways diminished the release of inflammatory cytokines, particularly TNF-α and IL-1β, attenuating peri-transplant islet graft damage [16, 23, 24, 119]. Additionally, hypoxia-induced NF-*κ*B signaling pathways induce overexpression of iNOS and MCP-1 on islets and result in the recruitment of macrophages and the destruction of islets [35]. *In vitro* and *in vivo* gene expression data revealed that SA-TM and SA-CD47 on the surface of the islet significantly decreased the expression of IBMIR-associated genes and protected islet damage from early graft loss.

Immunophenotyping studies showed that SA-TM and SA-CD47 prevent the recruitment of myeloid cells into the graft. There is a significantly reduced in the number of myeloid cells (CD11+), MHC class II and CD80 expressing M1 macrophages (CD11b+, F4/80+), and neutrophils (CD11b+, Ly6Ghi), PMN-MDSC (CD11b+, Ly6Ghi, Ly6Clow) and M-MDSC (CD11b+, Ly6G-, Ly6Chigh) in SA-TM/SA-CD47 engineered group as compared to the control group. There was no significant reduction in anti-inflammatory M2 macrophages (CD11b+, F4/80+, CD206+). This immunophenotyping study is consistent with both SA-CD47 on the islet reduces graft infiltrating myeloid cells, and TM inhibits the activation and infiltration of neutrophils and macrophages into inflammation sites via blocking

coagulation and inflammatory reactions by TM or APC-mediated signals [10, 110, 112, 116]. The importance of using a combination of SA-TM with SA-CD47 on macrophages is that CD47 prevents phagocytosis of islets by macrophages while TM polarizes macrophages to M2 macrophages. Moreover, TM and CD47 prevent neutrophil infiltration into the graft site [120].

Our findings revealed that combining SA-TM and SA-CD47 contributes to enhanced islet engraftment by decreasing immune cell infiltration and mitigating various procoagulatory and proinflammatory mediators associated with IBMIR. Our data are consistent with different reports showing using TM and CD47 for mitigating IBMIR [10, 116]. Transiently displaying immune ligands on the surface of islets for localized immunomodulation provides the enormous translational potential for syngeneic, allogeneic, and xenogeneic islet transplantation.

Chapter 5

Discussion

Type 1 diabetes (T1D) is a chronic autoimmune disease in which the immune system attacks and destroys the insulin-producing cells in the pancreas, leading to an inability to produce insulin [12, 15]. This results in elevated blood sugar levels and requires individuals with T1D to regularly monitor their blood sugar and receive insulin through injections or an insulin pump [4, 5]. T1D typically develops in childhood or adolescence, and management of the disease requires a daily balancing act of monitoring blood sugar levels, eating a healthy diet, and receiving insulin to maintain proper blood sugar control [3, 15, 52]. T1D can lead to a range of long-term complications if not effectively managed. For a century since insulin's discovery, T1D still presents a major challenge in patients' lives, sometimes even threatening their survival [5]. Insulin, though crucial, fails to prevent the progression of microvascular complications in many cases and inconsistent delivery leads to low blood sugar and unstable blood sugar levels. While advancements in insulin delivery systems like insulin pumps, continuous glucose monitoring and glucose-responsive systems exist, they still fail to prevent hypoglycemia, do not always allow patients to maintain optimal blood sugar levels, are uncomfortable to use for extended periods, and can sometimes break down [5, 15, 37]. Despite careful insulin administration, long-term complications remain common. Islet immunotherapy is a promising approach for the treatment of T1D that aims to modify the body's immune response to prevent destruction of insulin-producing cells (islets) [5, 8, 95, 98]. The idea behind islet immunotherapy is to either

suppress the immune system or modify it so that it stops attacking the islets, allowing them to function normally within the body. There are several forms of islet immunotherapy, including the use of immunosuppressive drugs, anti-inflammatory agents, and cellular therapies such as regulatory T cell therapy [37, 97]. The goal of these therapies is to prevent or reverse islet destruction, preserve insulin production, and improve long-term glycemic control for individuals with T1D [37, 97]. While early results have been encouraging, further research is needed to determine the safety and efficacy of these approaches. Intraportal islet transplantation is a procedure used in the treatment of T1D in which insulin-producing cells (islets) are transplanted into the portal vein, which supplies blood to the liver. The goal of this procedure is to restore insulin production and improve glycemic control in individuals with T1D. During the procedure, the islets are isolated from a donor pancreas and then infused into the portal vein [7]. The islets then settle into the liver where they can produce insulin in response to changes in blood glucose levels. Intraportal islet transplantation has been shown to be effective in restoring insulin production and improving glycemic control, but it is still considered experimental and is typically reserved for individuals with T1D who have not responded well to other treatments. The procedure is usually performed in combination with immunosuppressive therapy to prevent the body from rejecting the transplanted islets. Intraportal islet transplantation is the clinically only treatment for type 1 diabetes and chronic pancreatitis [5, 9, 11, 15, 16]. During islet isolation, modification, and transplantation, islets release numerous inflammatory signaling. Upon Islet infusion into the portal vein, islet grafts undergo rapid

destruction connected as a part of non-specific thrombotic/inflammatory reactions called IBMIR [5, 9, 16, 109, 114]. The basic features of IBMIR are expeditious activation of coagulation, complement system, hypoxia, and infiltration of innate immune cells; all contribute to excessive islet destruction, inability to successful engraftment, and euglycemia [5, 8-10, 16, 36, 72, 109, 114]. Mitigating IBMIR enables 1) using fewer donors for each transplant, 2) decreasing innate immunity e during the initial stages of transplantation, 3) regulating adaptive immunity during the late stages of transplantation, and 4) no need to use immunosuppressants [8].

IBMIR is a phenomenon that occurs during islet transplantation and can negatively impact the success of the procedure. IBMIR is a rapid immune response that occurs within minutes of transplanting insulin-producing cells (islets) into the recipient's bloodstream. This response causes an influx of immune cells and release of cytokines and other inflammatory factors, which can lead to the destruction of the transplanted islets and poor engraftment. IBMIR is a significant challenge in islet transplantation, and researchers are developing strategies to prevent or reduce this reaction's occurrence to improve the procedure's success rate. These strategies include modifying the islets to make them less susceptible to IBMIR, using immunosuppressive drugs to prevent the immune response, and improving the methods used to isolate and transplant the islets. As an alternative to gene therapy, encapsulation provides practical immunomodulatory approaches that enable donor islets to regulate metabolic fuel homeostasis while modulating inflammatory immune cells and inducing localized anti-inflammatory microenvironments for islet grafts [8]. Additionally, encapsulation contributes to

long-term islet survival without long-term-using immunosuppressants [5]. To achieve this goal, our lab pioneered a novel platform called ProtExTM that allows displaying extracellular domains of immunomodulatory proteins on surfaces of cells and tissues on macromolecules. The advantages of the Protex^{TM} platform are to 1) localize immunomodulation, 2) display the different amounts of or combination of immunomodulatory proteins 3) make the engineering process done in two hours. Inasmuch as the ProtexTM platform is an attractive, safe, and potentially more effective approach to treat graft recipients for preventing graft loss in early islet transplantation.

Chronic Pancreatitis (CP) is a disease characterized by progressive inflammation of the pancreas that leads to irreversible damage of the pancreatic parenchyma. In the initial stages of the disease, the pancreatic exocrine function is impaired, which can subsequently lead to impaired endocrine function and the onset of diabetes mellitus[16, 121]. To treat CP, the first successful autologous islet transplantation was performed at the University of Minnesota in 1977 [122]. Basically, the islet transplantation procedure involved removing the pancreas from a patient with severe chronic pancreatitis, isolating the islet cells from the pancreas, and then transplanting them back into the liver of patient [5, 9, 15, 16].

A three-year post-transplant outcomes have shown that, one third of patients were insulin-independent while one third had partial islet function. Patients who received autologous islets demonstrated higher survival rates compared to those who received similar doses of allogeneic islets [122]. In term of autologous islet transplantation, autologous islets do not trigger an autoimmune response or

alloimmune rejection, and recipients who received autologous islets do not need to take immunosuppressive drugs that harm beta cells [16, 122]. However, the presence of hypoxia, coagulation, complement system, and innate inflammatory damage is strongly linked to islet graft failure caused by IBMIR, which can pose significant obstacles to the success of autologous islet transplantation [9, 16, 17, 22, 32, 70, 72, 119]. The ProtexTM platform has the potential to address major issues related to IBMIR, as it can enhance islet engraftment by modulating non-specific reactions in various ways [37].

In this study, the combination of SA-TM and SA-CD47 was found to improve islet engraftment in a syngeneic transplantation model. This was achieved through several mechanisms including inhibition of coagulation and thrombosis, prevention of phagocytosis, and suppression of proinflammatory cytokine expression. Results showed a significant reduction in the transcripts of several proinflammatory genes and a decrease in HMGB1 protein levels in recipients with SA-TM/SA-CD47 engineered islets compared to those with only SA-engineered islets. The results suggest that the combination of SA-TM and SA-CD47 can reduce early islet loss in transplantation.

The protein CD47, which is expressed on the surface of various cells, acts as an inhibitor of phagocytosis by immune cells through its "don't eat me" signal. In islet transplantation, research has shown that CD47 can minimize immune reactions peri-transplant [10, 49, 113]. IBMIR is a rapid immune response that can occur within minutes of transplanting insulin-producing islets into the recipient's

bloodstream, resulting in the failure of the transplantation and hindrance of engraftment [5, 15, 16, 27]. By expressing CD47 on the surface of the islets, the likelihood of phagocytosis by immune cells is reduced, leading to a reduced occurrence of IBMIR and improved engraftment of the transplanted islets [108]. Shrestha et al. showed displaying CD47 on islets can significantly improve islet engraftment and survival in animal models of islet transplantation. Shrestha et al. also demonstrated that SA-CD47 reduce phagocytosis in a xenogeneic in vitro experimental setting [108]. Our study demonstrated that the displayed of SA-CD47 on xenogeneic rat splenocytes, either alone or in combination with SA-TM, significantly reduces phagocytosis by LPS-stimulated mouse macrophages. Moreover, we showed that the presence of SA-CD47 on allogeneic bone marrow cells, either alone or in combination with SA-TM, effectively inhibits clearance of the transplanted islets through modulation of the recipient's immune responses. In conclusion, these findings have paved the way for clinical trials to assess the safety and effectiveness of using CD47 and TM as surface markers on islets for patients with T1D.

TM expressed on endothelial cells plays a role in the regulation of blood coagulation and inflammation [39, 71, 74, 92, 110]. TM plays as an anticoagulant by converting thrombin into an enzyme that stimulates activated protein C production, which helps prevent coagulation [44, 103]. Also, TM regulates immune responses by reducing the activation and migration of neutrophils, limiting their potential to cause inflammation and tissue damage [109]. In the context of islet

transplantation, TM has been shown to reduce the occurrence of IBMIR by inhibiting inflammatory molecules, such as IL-1β, TNF-α and iNOS perpetuating the inflammatory responses and leading to the destruction of transplanted islets [92]. Our study also demonstrated TM displayed on the islet surface, either alone or in combination with SA-CD47 can reduce IBMIR not only inhibiting the release of inflammatory mediators, but also modulating functions of neutrophils and macrophages.

The advantages of combining SA-CD47 and SA-TM treatment includes adaptive effects of both proteins. The combination treatment includes improved inhibition of phagocytosis by macrophages, prevention of NETosis and enhanced reduction IBMIR in islet transplantation and increased preservation of transplanted islets leading to improved islet engraftment and function [10, 55, 56, 74, 92, 115]. Moreover, combining SA-CD47 and SA-TM treatment decreased the release of inflammatory cytokines and chemokines and DAMPs associated with IBMIR. The combination treatment showed enhanced islet viability decreasing expression level of hypoxia-associated gene HIF-1α. In conclusion, our treatment demonstrated the efficiency of combination treatment of CD47 and TM which perpetuate syngeneic islets and improved islet viability during transplantation reducing the occurrence of IBMIR. Our aim of syngeneic islet transplantation is to minimize the loss of initial islet mass peri-transplant and eliminate the need for exogenous insulin. However, for clinical autologous islet transplantation, IBMIR can lead to a high rate of insulin dependence within five years. The use of a combination treatment that targets

IBMIR may improve the engraftment and function of transplanted autologous islets in clinics, leading to a higher rate of successful outcomes and reduced dependence on exogenous insulin.

In the manner of allogeneic islet transplantation, danger associated molecules released by damaged islets and other cells induce the recruitment of innate immune cells [27, 28, 70, 104]. Both macrophages and neutrophil lead to the release of cytokines and chemokines which are subjected to prolonged or excessive inflammation. Innate immune cells uptake the islet antigens followed by return to the lymphoid tissue for antigen presentation and initiate T cell activation [106]. Activated T cell migrates to target allogeneic islets. Modulating innate immune response can also shape adaptive immunes [5, 27, 42, 56]. All evidence highlights the significance of the combination of CD47 and TM treatment in reducing IBMIR in allogenic islet transplantation (Fig. 20).

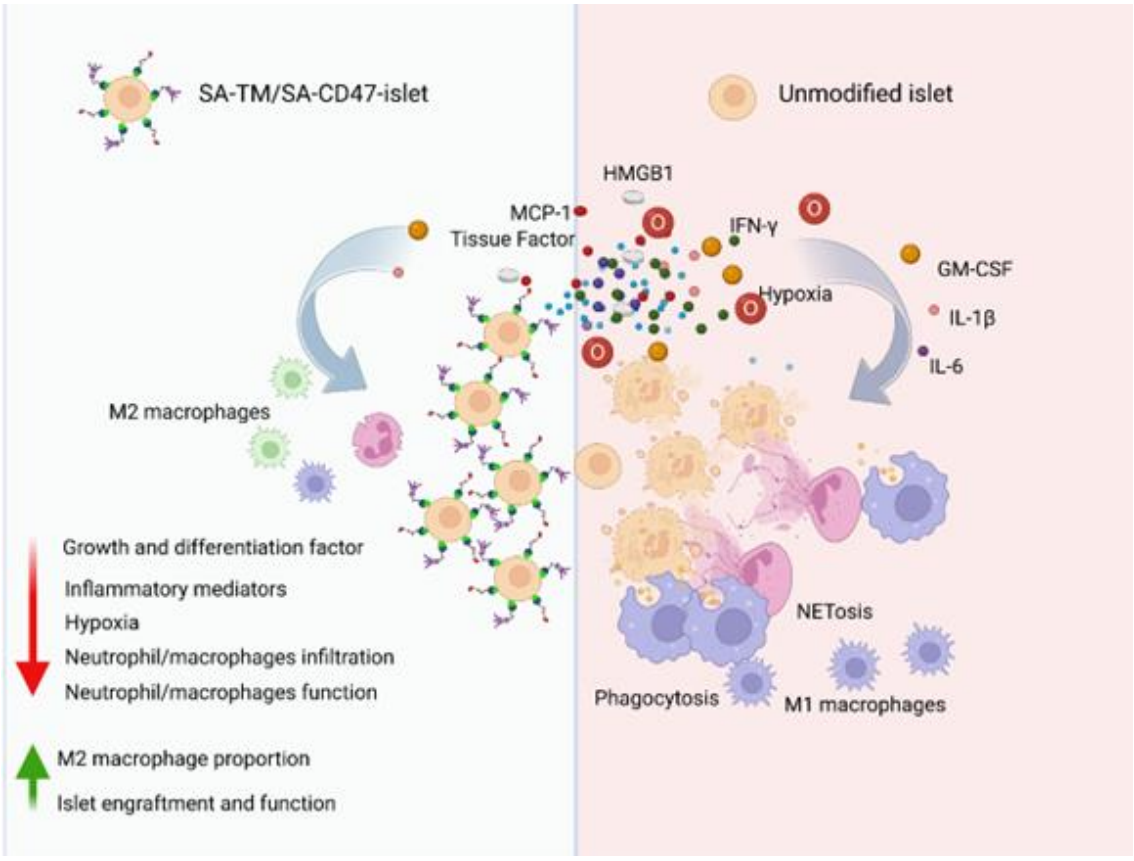

Figure 20: Summary of efficiency of SA-TM/SA-TM combinational treatment. Engineering islets with SA-CD47 and SA-TM improve their efficiency by suppressing the release of inflammatory cytokines, chemokines, and DAMPs. Additionally, these engineered islets mitigate hypoxia, a major cause of islet mass loss during transplantation. The presence of SA-TM/SA-CD47 also decreases the infiltration of myeloid cells and prevents the inflammatory properties of macrophages and neutrophils by decreasing NETosis and phagocytosis. As a result, the combination of SA-TM and SA-CD47 maintains islet mass and protects the islet engraftment.

Both TM and CD47 proteins have been shown to regulate both the innate and adaptive immune responses, which can further enhance the improvement of allogeneic islet engraftment outcomes [10, 13, 45, 84, 123]. Pan et al. demonstrated that TM bins G-protein coupled receptor 15 (GPR15) which in turn regulates the migration of FOXP3-expressing regulatory T cells (Treg) resulting in weaking inflammation [45]. Additionally, TM promotes the polarization of anti-

inflammatory macrophages even though in the presence of inflammatory IFN-γ and LPS [110]. Adamczyk et al. showed that the expression of GPR15, a receptor for TM, leads to the migration of Tregs into colonic tumor tissue and identifies a subset of Tregs that may have a tumor-promoting role similar [124]. As Tregs and M2 macrophages play a crucial role in promoting tolerance and modulating T cell-mediated adaptive immune responses for a successful outcome in transplantation.

Besides TM, CD47 plays a key role in the modulation of adaptive immune responses. Zhang et al. showed that CD47 on donor cells regulates T cell responses allogeneic hepatocyte post-transplantation [113, 123]. CD47 expressing on bone marrow cells regulates the innate immune response of NK cells to bone marrow in syngeneic, allogeneic, and xenogeneic contexts [55, 56, 123]. Also, CD47-SIRPα axis block phagocytosis. CD47-SIRPα interaction can also prevent endocytosis by DCs what present allogeneic antigens to T cells for adaptive immune responses [54, 59, 113, 123]. Zhang et al. also showed CD47 KO hepatocyte transplantation induces allogeneic T cells [123]. Additionally, CD47 binds the Fas receptor, leading to apoptosis in various cells [58]. In the context of allogeneic islet transplantation, CD47 binding to the Fas receptor can result in apoptosis of T cells and neutrophils. In conclusion, the combination treatment of CD47 and TM displayed on surface of islets effectively regulates both innate and adaptive immune responses, resulting in a heightened state of tolerance and

improved success of islet engraftment without the use of immunosuppressant drugs.

Future studies

In this study, we showed SA-TM alone or in combination with SA-CD47 when transiently displayed on islet surface showed enhanced engraftment and the combination showed better efficacy in a minimal mass syngeneic intraportal islet transplantation model. This concept has clinical potential in autologous clinical islet transplantation as only 30% recipients maintain their islet grafts over 5 years [122]. In the context of allogeneic islet transplantation, the efficacy of single chimeric proteins, SA-CD47 and SA-TM, as well as their combination treatment will be established based on allogeneic immune response to allografts. In the future, researchers may also need to optimize the combination treatment to achieve better results and to determine the optimal dosing and engineering conditions. Due to multifaced features of TM and CD47, future studies can be conducted to determine the immunomodulation mechanisms by which CD47 and TM modulate the immune response, which could lead to the development of new therapeutic strategies for islet transplantation and other medical conditions.

The Protex^{TM} platform provides a flexible and versatile solution for islet transplantation and for localized immunomodulation. We have shown the efficacy of this technology to co-display two immune modulators that mitigate early innate immune responses. Molecules that target adaptoive immunity for regulation, such as SA-FasL that showed efficacy in a nonhuman primate setting as a singel agent,

can be combined with those targeting innate immunity for a much efficacious outcome [61]. This approach may enhance the tolerance and contribute to engraftment of islets, reducing the risk of rejection and improving the long-term outcomes of transplantation in allogeneic transplant settings. Protex^{TM} platform provides a promising approach for the successful islet transplantation and the development of new therapies for the treatment of diabetes and other medical conditions.

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Vita

Ali Turan earned his undergraduate degree in Molecular Biology and Genetics from Istanbul University in 2016, where he gained a strong foundation in biological research. He went on to pursue his master's degree in Medical Biology at Erciyes University in 2016, further deepening his knowledge and understanding of molecular mechanisms and biological processes. In 2018, Ali was accepted into the Microbiology and Immunology program at the University of Louisville, where he began to specialize in immunology and transplantation. After completing two year of study, he transferred to the Molecular Pathogenesis and Therapeutics graduate program at the University of Missouri in 2020, seeking further opportunities to expand his expertise in the field. Under the guidance of Dr. Haval Shirwan, a renowned expert in the field of immunology, Ali`s work focused on exploring novel therapeutic strategies to prevent transplant rejection and improve outcomes for transplant recipients. Ali has published several research papers in reputable scientific journals and has presented his work at numerous conferences and scientific meetings. Ali Turan is a highly accomplished scientist with extensive experience in the field of immunology and transplantation.