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Development of Biomaterial Scaffolds to Modulate the Local Immune Microenvironment and Support Allogeneic Islet Transplantation

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Abstract

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Intrahepatic islet transplantation is a promising therapeutic option for the treatment of Type-1 diabetes that offers the ability to restore endogenous insulin production. Widespread use of islet transplantation is currently limited by poor survival of transplanted islets due to the harsh environment of the liver portal vein, prompting investigation into alternative transplantation sites. The Shea lab has been at the forefront of developing biomaterial scaffold platforms to support the engraftment of islets into intraperitoneal fat. However, the inefficacy of general immune suppression protocols required to prevent immune-mediated graft destruction remains an unsolved issue. This dissertation presents novel strategies seeking to use biomaterial-based immune intervention to extend allogeneic islet transplant survival. A layered poly(lactide-co-glycolide) (PLG) scaffold system was designed to incorporate the immunosuppressive cytokine TGF-β1. Localized delivery of TGF-β1 strongly reduced leukocyte infiltration into the scaffold environment seven days post-transplant, in addition to co-activation markers on antigen-presenting cells and inflammatory cytokine expression. TGF-β1 scaffolds showed biocompatibility with transplanted syngeneic islets and significantly extended survival of transplanted allogeneic islets, demonstrating the protective effects of limiting early leukocyte infiltration. The scaffold was then used to characterize the delivery of a novel immunomodulatory cytokine IL-33 to assess whether adipose tissue-specific anti-inflammatory immune cell lineages could be used to prevent graft rejection. IL-33 potently expanded local

CD4+ Foxp3+ regulatory T cells (Tregs) in a blank implantation model. IL-33 delivery also expanded Tregs expressing the IL-33 receptor ST2 while decreasing proliferation of graft-destructive CD8+ T cells in an allogeneic islet transplant model. We found IL-33 release was able to significantly extend allograft survival, demonstrating local allograft-protective effects. However, we also found that IL-33 delayed engraftment of transplanted islets in syngeneic and allogeneic models. IL-33 delivery induced a Type 2 cytokine response, specifically increasing expression of IL-4 and IL-5, which coincided with expansion of locally enriched eosinophils and Group 2 innate lymphoid cells (ILC2s). Finally, nondegradable poly(ethylene-glycol) (PEG) hydrogel designs were compared for their abilities to support islet engraftment and their interaction with the host immune system. Both nonporous macroencapsulation hydrogel and a microporous scaffold designs supported stable engraftment of islets that restored normoglycemia by three weeks post-transplantation. The microporous design restored normoglycemia in response to glucose challenge at the same rate as endogenous islets while the macroencapsulated hydrogel showed a delayed response. The microporous design provoked a foreign body response leading to a large population of neutrophils within the scaffold correlating to a period of hyperglycemia when transplanted with syngeneic islets. This dissertation demonstrates the promise of leveraging biomaterials to develop localized immunomodulatory strategies to control the local microenvironment and support survival of allogeneic islets.

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Chapter 1:

Introduction

1.1 Opening

Immune engineering is an exciting field in medicine that aims to use biological insight to develop precise technological solutions that can provide the necessary corrective signaling cues to overcome pathological deficiencies. A major field of interest for immune engineers is the selective induction of immune tolerance to foreign tissue, which represents a major barrier to the success of cell therapies like islet transplantation. The clinical future of islet transplantation as a therapeutic option for patients with Type-1 Diabetes Mellitus requires immune tolerance strategies that effectively protect transplanted cells long-term while preventing the off-target effects associated with general immune suppression [1-3]. Biomaterials are a crucial tool for successful tissue engineering and regenerative medicine approaches by promoting local environments conducive to cell survival and proliferation [4]. This dissertation seeks to take advantage of the natural interaction between transplanted biomaterial and the local immune microenvironment to develop novel immunomodulatory strategies [5, 6]. We describe advances made to a poly(lactide-co-glycolide) (PLG) scaffold platform aimed at modulating the local immune cell environment through factor release and test two mechanistically distinct factors that can improve allograft survival. We also test the viability of a different biomaterial poly(ethylene-glycol) (PEG) that may offer certain advantages to support islet transplantation in the future.

1.2 Contents of this dissertation

Chapter 2 establishes the current state of clinical islet transplantation and the need to find alternative extrahepatic islet transplantation sites. We then discuss tissue-specific immune environments and the mechanisms through which the immune system can cause damage to the islet graft. We review the immunosuppressive regimens in addition to novel cell based therapies

that have been used to preserve islet function. Finally, we review how biomaterials have been applied to enhance cell engraftment, provide an immune-isolating barrier, or deliver immunomodulation to enhance islet transplantation.

Chapters 3 and 4 describe how the existing PLG scaffold platform for islet transplantation can be designed to release soluble immunosuppressive factors specifically for the enhancement of allogeneic islet survival. We use a complete MHC-mismatch allogeneic model, where Balb/C donor islets are transplanted on scaffolds into the epididymal fat pad of streptozotocin-induced diabetic C57/Bl6 recipients. We detail the characterization of two factors that have distinct effects on the local immune microenvironment. **Chapter 3** tests the capability of scaffold-released TGF-β1 to protect transplanted islets. Th7is work was inspired by a previous project using scaffold co-transplantation of *in vitro* TGF-β1 induced Tregs to extend graft survival. Scaffold-incorporated TGF-β1 severely decreased infiltration of leukocytes in the post-transplant environment. Allogeneic islets seeded on TGF-β1 scaffolds showed an extended survival, demonstrating that limiting local leukocyte interactions with transplanted cells delayed graft rejection. These studies validate our strategy of using the biomaterial scaffold environment to deliver highly localized immune intervention of extend islet allograft survival.

Chapter 4 uses the scaffold to characterize a novel immunomodulatory factor IL-33, which was chosen due to the enrichment of a number of putative regulatory immune cell lineages that express the IL-33 receptor ST2, including regulatory T cells. We attempt to bridge insight between IL-33's role suppressing inflammation in adipose tissue and its usage as a tolerogenic factor for allogeneic graft survival by deploying it locally within adipose tissue and assessing its ability to protect allogeneic cells. We identify a robust expansion of Tregs in response to local IL-33 release, particularly with the addition of alloantigen, leading to extended allograft survival.

We also characterize polarization of a local Th2 environment leading to expansion of innate cell populations that may affect the graft independently of the alloimmune response. We demonstrate that the anti-inflammatory pathways induced by IL-33 in the adipose tissue do not neatly fit into the graft rejection/acceptance paradigm.

Chapter 5 details work developing and optimizing a PEG hydrogel platform for islet transplantation. Two nondegradable hydrogel architectures, an encapsulating nonporous structure designed to limit leukocyte infiltration and a porous scaffold promoting tissue infiltration based off of the PLG scaffolds, are tested for their support of islet engraftment and interaction with the local immune response. Both architectures successfully engraft syngeneic islets, though the encapsulating hydrogel delays glucose-stimulated insulin secretion and the microporous gel provokes a large neutrophil response seven days after transplant. This project lays the groundwork for future optimization and further modification to support islet transplantation in more complex transplantation models.

1.3 Conclusions and Future Directions

In the final chapter, findings of the dissertation are summarized and the progress developing localized biomaterial-based immunomodulatory strategies is discussed. Future directions are also proposed to continue the work detailed in this dissertation in addition to a general discussion of the future of clinical islet transplantation.

Chapter 2:

Background

2.1 State of Clinical Islet Transplantation

Type 1 Diabetes Mellitus (T1DM) afflicts roughly 1.25 million Americans and is characterized by the loss of endogenous insulin production due to the autoimmune-mediated destruction of the pancreatic β-cells and the inability of the patient to control blood glucose levels. The current clinical standard of care to treat T1DM is known as intensive insulin therapy, where strict blood glucose monitoring and frequent injections of insulin are used to maintain near-normoglycemia. Despite the introduction of automated insulin pumps and glucose sensors that can provide continuous blood glucose control, constant and invasive self-monitoring of the disease is still required to prevent severe hyper- and hypo-glycemic episodes. Poor management of T1DM can also cause a number of secondary complications including vascular damage to the eyes, nervous system, feet, kidneys and an increased risk of suffering a stroke or heart attack. The health care costs associated with insulin therapy are also extremely high due to the emergence of the disease during adolescence and the lack of a permanent cure.

A subset of T1DM patients are unable to maintain optimal blood glucose levels and are at high risk of severe hypoglycemic events (SHE), which are defined as episodes where the patient requires the intervention of an outside person to actively administer carbohydrates, glucagon or take corrective action. SHEs are a leading cause of hospitalization and morbidity due to T1DM [7]. Moreover, up to 1/3 of T1DM patients suffer from impaired hypoglycemic awareness (IAH) caused by sustained hypoglycemia, increasing the likelihood of SHEs sixfold [8]. For these patients, islet transplantation has now become a common experimental procedure aimed at preventing SHEs and restoring endogenous insulin production and normoglycemia [2, 9, 10].

Over 90% of human islet transplantations have been performed into the liver sinusoids [11]. Upon receiving the purified islet product, the transplant team can directly infuse the islets into

the hepatic portal vein, wherein the islets will proceed to lodge and remain in the hepatic vasculature. There are a number of characteristics of the site that are beneficial for islet performance. The liver portal vein is the physiological site of insulin injection for the pancreas and thus transplanted islets are able to mimic a natural response to changes in blood glucose. Additionally, infusion of transplanted cells into the hepatic portal vein is minimally invasive and can be considered the safest cell transplantation technique. Despite the almost exclusive use of the liver vasculature as the site of islet transplantation, there a number of obvious drawbacks that represent hurdles to the widespread use of islet transplantation to treat T1DM. First, the introduction of cells into the bloodstream invokes a potent response known as the instant blood mediated inflammatory reaction (IBMIR) [12, 13]. Immediately upon entry into the blood stream, cells become covered by platelets, tagged by the complement system, and are surrounded by monocytes and other components of the immune system. Trapping the islets within the liver vasculature and obstructing blood flow can also cause liver ischemia or thrombosis [14, 15]. Loss of the pancreatic vasculature during the cell isolation process also leads to a persistent hypoxia experienced by the transplanted islets [16]. The liver also accumulates drugs and toxins, leading high local concentrations of immunosuppressants that themselves are toxic to islets and limiting the suppressive dosing that can be delivered to the patient [17]. These factors combine to result in massive islet death during the early days of islet engraftment. While post-portal infusion monitoring of the grant is extremely difficult, it is estimated that anywhere from 60-80% of the graft is lost within days of transplantation [18, 19]. This engraftment inefficiency likely represents the greatest hurdle to widespread use of islet transplantation. In regards to long-term survival, the liver vasculature does not recapitulate the native pancreatic ECM environment, which can lead to islet death through anoikis [20]. The hepatic portal vasculature also does not

provide a sufficiently oxygenated environment to meet the high metabolic demands of islets. Lacking the natural perfusion of arterial blood, intraportally transplanted islets are thus reliant on passive diffusion of oxygen and undergo a state of hypoxia during the early stages of transplant [16]. Endogenous islets have a pO_2 of 40 mmHg and have been measured to be no more than 1 cell diameter away from capillaries in the pancreas [21]. While technical difficulties have inhibited accurate measurements of pO_2 in transplanted islets, studies have estimated that islets lodged in the hepatic vasculature have pO_2 values ranging from 5-25 mmHg. Using an oxygen-sensitive dye, one study showed that as many as 60% of transplanted intraportal islets remain at pO_2 values under 10 mmHg one month after transplant [22]. Finally, grafts that escape initial graft destruction most clear two additional barriers to tolerance: the allogeneic immune response invoked due to the use of cadaveric islets in addition to the autoimmune response against β-cells that led to the initial disorder and will remain active in the patient. Once islets are lodged into the hepatic vasculature, it is impossible to retrieve the graft or perform biopsies to check on graft viability or detect early signs of failure, though imaging technologies have been developed to try and provide noninvasive monitoring [19].

Currently, islet transplantation cannot be performed at a 1:1 donor to recipient ratio. Given the supply of islets is limited to deceased donors, islet transplantation as a clinical option is currently limited to patients whose glycemic episodes cannot be controlled by intensive insulin therapy. In a 2014 position statement, the American Diabetes Association still regarded islet transplantation as an experimental therapy inferior to the clinical efficacy shown by whole-pancreas transplantation, despite the decreased risk of surgical complications [23]. In the ensuing years, the CITR published phase III clinical trial results on multi-center standardization for islet transplantation, concluding that 71% of patients successfully met the study endpoints of avoiding

SHEs and maintaining normal HbA1c levels below 7% (HbA1c is an alternate measure of blood glucose levels that tracks the natural binding of glucose to hemoglobin to form glycated hemoglobin, which allows for longer term assessment of blood glucose control given the 8-12 week circulation of erythrocytes) [10].

2.2 Alternative sites for transplantation

In order to advance the efficacy of islet transplantation, it is important to identify a transplant site that minimizes the number of islets necessary to fully restore insulin independence long-term. There are a number of characteristics desirable for the selection of an optimal transplant site that will enable the most efficient engraftment of islets possible [24, 25]. First, the transplant site should reduce direct contact between blood and islets to avoid induction of the aforementioned IBMIR. The site needs to have sufficient vascularization and oxygen tension to support the high metabolic and nutritional demands of an islet that are normally met by the native pancreatic environment. The site should be minimally invasive and accessible for transplantation and subsequent follow-up inspections in case of post-surgery complications. Preferably, the site will also be located at a portal draining site that mimics the physiological release of insulin. Finally, the site chosen would be relatively tolerant to foreign antigen and be relatively immunoprotective to minimize immune-related graft destruction.

The most prevalent site for islet transplantation used in experimental rodent models is the kidney capsule [26, 27]. The renal subcapsular space was shown to require as little as 25% of the islet mass required for the intraportal site, demonstrating the clear survival advantage of avoiding provocation of the IBMIR [28]. These minimal mass advantages seen in rodent models were not successfully translated to humans. Sub-optimal local revascularization conditions appear to be a

more significant issue with the engraftment and survival of human islets, which are less tolerant of the isolation process and are morphologically distinct from rodent islets [29]. Additionally while the kidney capsule in rodents is easily surgically accessible, the equivalent human site is much more difficult to access.

The omentum and gastric submucosa are two transplant sites that offer a physiologically relevant site for insulin release while avoiding direct transplantation into the vasculature. The omentum is the large double layer membrane covering a layer of adipose tissue that lies over the abdominal cavity and the intestines. Islets transplanted into omentum show superior revascularization and local hypoxia rapidly decreases 7 days after transplant compared to islets infused into intraportal site [30]. The omentum provides an extremely large transplant area. Some studies have also suggested the omentum has locally tolerogenic properties [31]. A Phase I/II clinical trial at University of Miami is currently being run to assess the clinical viability of the omentum (NCT02213003). In May 2017, results from a single patient were released, showing that through 12 months, omental islet transplantation was able to eliminate the need for exogenous insulin and prevent severe hypoglycemic episodes, though metabolic tests of islet function showed deteriorating performance [32]. While the murine omentum is too small and poorly vascularized to support islet transplantation, the epididymal or peri-gonadal fat pad shares a number of properties with the human omentum such as large transplant area and high content of adipose tissue that make it a useful model system for translational research [33]. The gastric submucosa is accessible via endoscopy and is viewed as an accessible surgical site [34, 35]. The gastric submucosa provides a much larger transplant space to infuse islets compared to the kidney capsule. There has been less preclinical work comparing islet engraftment between the gastric submucosal wall and the intraportal site. Nonetheless, there is currently a phase 1 clinical

trial being run at UCSF testing the safety of islet transplantation into the gastric submucosal wall (NCT02402439).

Immunoprivileged sites have been studied to avoid allo- and auto-recognition of transplanted tissue [36]. The anterior eye chamber, brain, and testis have shown in rodent models to tolerate the transplantation of islets without immune suppression due to endogenous tolerogenic mechanisms that exist locally to inhibit the development of an autoimmune response against local antigens [37-39]. The anterior eye chamber and testis are not clinically translatable due to the large number of islets required to restore normoglycemia while the brain represents a surgically inaccessible site. The thymus is the site of central tolerance and T cell deletion. Exposure of islet antigens to the developing thymocytes could promote acceptance of the graft. The bone marrow has been used successfully in human autologous islet transplantation and is home to a number of stem cell and precursor populations that promote local tissue repair and engraftment while suppressing T cell activation [40, 41].

Intramuscular and subcutaneous provides ample area for minimally invasive transplantation and are extremely accessible for post-surgical follow-up [42-44]. Many macroencapsulation devices use the subcutaneous space as the site of administration due to the ease of surgical accessibility, with the devices typically being sutured into the skin [45]. Both spaces tend to require a large numbers of islets to restore insulin control, due to the systemic site of insulin secretion.

2.3 Tissue resident immunity

Each tissue constitutes a unique immune microenvironment that is suited to function. While the immune system is generally responsible for responding to infectious agents, it also is

responsible for maintaining local homeostasis with parenchymal cells. Certain physiological environments are suited towards controlling local immune responses and thus may offer lessons for engineering a tolerogenic environment.

The traditional designation of immune privilege is applied to organs that are separated by an impenetrable blood-brain barrier, thus normally not allowing leukocyte migration and surveillance. The traditional organs associated with immune privilege are the testis, eyes, and CNS [46, 47]. These immune privileged sites often contain sensitive cell types that may be damaged in an inflammatory immune response. Common features of these sites include high constitutive expression of the apoptosis-inducing ligands FasL and PD-L1 and immunosuppressive cytokines such as TGF-β1 and IDO capable of suppressing Th1 responses. The testis environment contains a large population of locally immunosuppressive Sertoli cells, which can protect islets in co-transplantation studies [48, 49]. The anterior eye chamber can also impose systemic tolerance on escaping antigen through a process called anterior chamber associated immune deviation (ACAID), which can be linked to migration of tolerogenic local antigen presenting cells (APCs) carrying antigen from the transplant site to the spleen [50].

There are a number of anatomic sites that are tolerogenic to prevent naturally high exposure to foreign antigen from causing aberrant immune response. Mucosal and oral tolerance must be maintained in the gut and pulmonary immune systems, which frequently come into contact with foreign antigen through consumption of food and inhalation of particles in the air [51, 52]. Allergies to food and inhaled substances are often due to a break in tolerance. To prevent an infectious response, these sites are naturally high in immunosuppressive cytokines and factors like retinoic acid and TGF-β1 that prevent immune activation, APCs that lack costimulatory molecules, and large induced CD4+ regulatory T cell (Treg) populations that can control T cell

responses [53]. Ironically, the liver as a whole organ is considered a highly tolerogenic environment, making it one of the easiest organs to induce tolerance after transplantation [54, 55]. Like the gastrointestinal tract, the liver experiences high amounts of foreign and neo-antigen through the digestion of food. The liver utilizes similar tolerogenic mechanisms, including low costimulatory marker expression on APCs, high expression of anti-inflammatory cytokines by resident immune, endothelial, and parenchymal cells, and large resident populations of Tregs.

While adipose tissue is primarily composed of its namesake adipocytes, it is also home to a diverse network of cells collectively known as the stromal vascular fraction (SVF). In addition to endothelial and stromal cell types, the SVF has a significant population of immune cells, ranging from 25 to 45% of the total cells [56]. In healthy adipose tissue, the immune microenvironment is maintained in an anti-inflammatory state ideal for normal adipocyte metabolic processes [57]. Typically the largest immune cell population in adipose tissue is macrophages, which adopt a CD11b+ F4/80+ phenotype. In lean tissue, the majority of macrophages adopt a non-active M2 phenotype that has been identified by the upregulation of CD301, CD206, and arginase 1 [58, 59]. Lean adipose tissue is also high in eosinophils and Group 2 innate lymphoid cells (ILC2s), which secrete Th2 polarizing cytokines [60, 61]. Foxp3+ resident Tregs are the dominant T cell lineage present in lean adipose tissue, particularly in older individuals [62]. Transcriptional profiling has identified VAT Tregs as a unique subset with a characteristically high expression of IL-10 and TGF-β1, implying a heavily immunosuppressive phenotype. Obesity and metabolic disorders can trigger chronic inflammation of adipose tissue, leading to local macrophages adopting M1 CD11c+ phenotype with upregulation of costimulatory molecules [58]. Inflammation also leads to increased secretion of pro-inflammatory cytokines and chemokines, an infiltration of neutrophils, and hypoxic local conditions, all of which may contribute to

exacerbation of metabolic pathologies such as insulin resistance [63]. The local inflammatory environment also causes a dramatic polarization of the local T cell compartment to Th1, Th17, and CD8 effector cells lineages [64, 65].

2.4 Immune recognition of transplanted islets

With the exception of immune privileged sites, any alternative islet transplantation sites will still require an immunosuppressive or tolerogenic regimen to protect transplanted allogeneic tissue. Islet transplantation tolerance is also particularly tricky to attain because both the allogeneic immune response against MHC-mismatched tissue and the autoimmune response causing the endogenous β-cell destruction must be controlled [66].

Allogeneic islet transplantation invokes a number of host defense mechanisms that can contribute to graft damage and destruction. Cellular injury at the transplant site can cause the release of coagulation-activating tissue factor (TF), damage- and pattern-associated molecular patterns (DAMPs and PAMPs) such as HMGB1 and hyaluronan, as well as pro-inflammatory cytokines and chemokines such as IL-6, G-CSF, KC, RANTES, MCP-1 and MIP-1α [67-69]. These signs of tissue damage can trigger pattern recognition receptors (PRRs) such as Toll-Like Receptors that are highly upregulated on innate immune cells that trigger pro-inflammatory signaling cascades [67]. Initial cellular damage comes from multiple sources. Ischemic injury and hypoxia are both immediate concerns to isolated pancreata once removed from the donor [70, 71]. Studies have shown cold ischemia time of donor pancreata should ideally be kept under eight hours to maximize islet viability and functionality, though the limited supply of donors at times can force this standard to be loosened [72, 73]. The islet isolation process itself will also invariably damage the tissue through the disruption of the endogenous islet vasculature and must

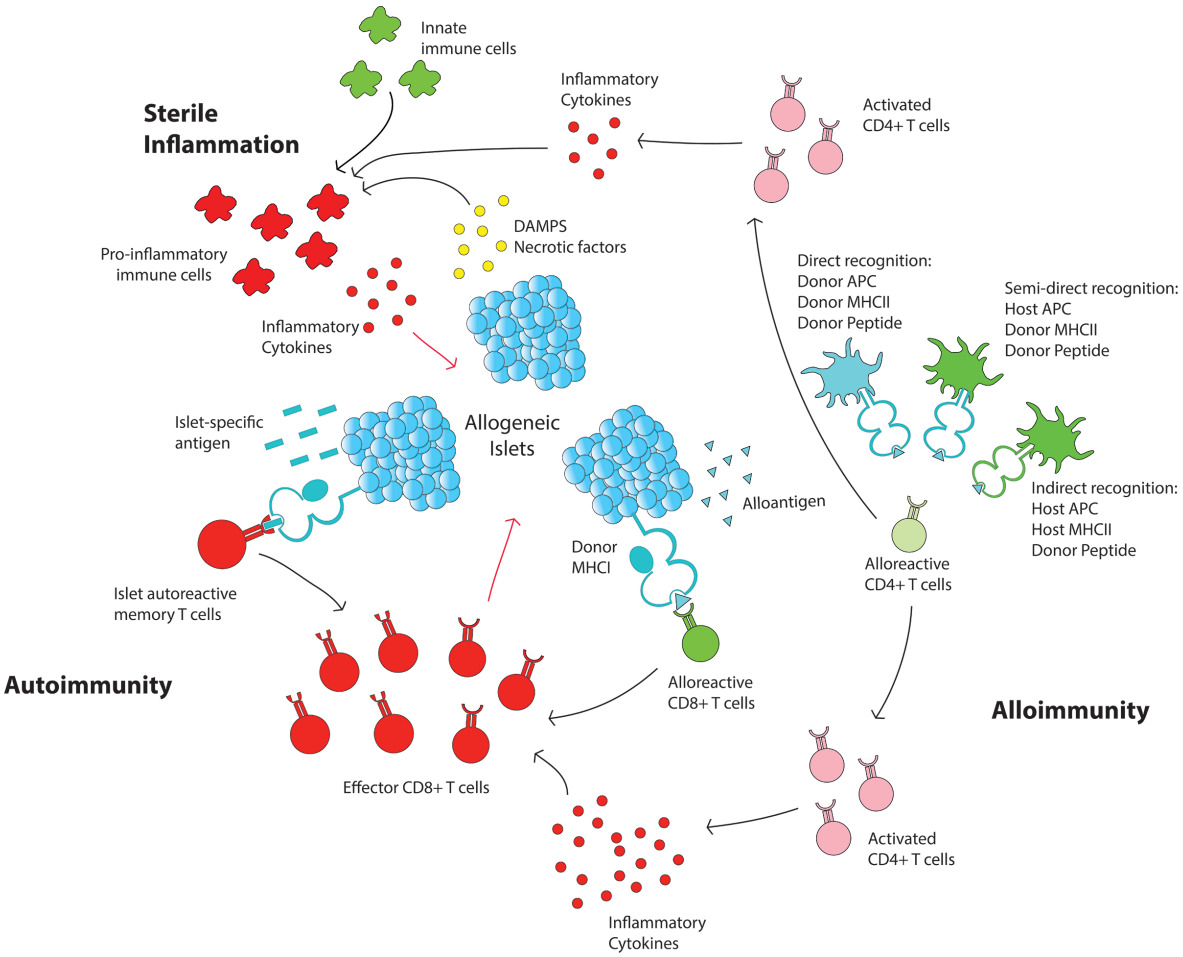

Figure 2.1 Immune recognition of transplanted islets
Transplanted islets are recognized non-specifically and specifically by the host immune system. Tissue damage to islets caused through the isolation process and the ensuing surgical procedure cause the release of damage-associated molecular patterns (DAMPS), which are sensed by innate immune cell lineages like neutrophils and macrophages, which secrete inflammatory cytokines in response. Alloimmunity is mediated by the recognition of foreign antigen by host immune cells. Foreign antigen can be detected by CD8+ T cells through an MHCI complex expressed on the surface of the islets, or by CD4+ T cells through the MHCII complex on professional APCs. Activated CD4+ T cells will secrete inflammatory factors that can either exacerbate the innate immune cells responsible for sterile inflammation or further activate CD8+ T cells, which directly damage the graft. Finally, in patients suffering from Type-1 Diabetes, the underlying autoimmunity means that memory T cells that are autoreactive against islets will likely exist and can quickly proliferate into an effector population.

be optimized to maximize the number of viable cells transplanted [74]. The removal of isolated islets from their native environment results in sustained hypoxic conditions and subsequent damage to the cells, which can be somewhat mitigated by maintaining the islets at low temperatures to slow necrosis and apoptosis [68]. The release of tissue damage factors into the host environment upon transplantation will recruit locally pro-inflammatory immune cells that are responsible for early graft damage prior to T cell recognition of the graft.

The allogeneic immune response is mediated by recognition of non-self antigens through interaction between major histocompatibility complexes (MHC) on APCs and host T cell receptors (TCRs) [75]. Both CD4+ T cells interacting with MHCII and CD8+ T cells interacting with MHCI play important roles in rejection of islet allografts [76]. There are three primary pathways of antigen recognition responsible for graft rejection: direct, semi-direct, and indirect. In direct antigen recognition, donor APCs that arrive at the graft as passenger leukocytes activate host T cells through interaction with a mismatched MHC [77]. Within the endogenous T cell repertoire, an estimated 1-10% of T cells can be activated by the mismatched MHC [78]. In the semidirect pathway, donor MHC-peptide complexes are transferred to host APCs. This process of MHC acquisition, also known as MHC cross-dressing, occurs due to the formation of exosomes that are released from the graft by donor APCs, traffic to the lymph nodes, and are subsequently internalized by host APCs [79, 80]. In the indirect pathway, host APCs present the alloantigen to host T cells, independent of the MHC mismatch. The indirect pathway becomes the dominant rejection pathway over time as donor APCs do not persist long term. Indirect pathway recognition is typically associated with chronic rejection mechanisms, though some studies have shown that acute rejection can still be mediated by indirect alloantigen presentation in the absence of direct pathways [81, 82].

Recurrent autoimmunity towards transplanted cells is a hurdle that distinguishes islet transplantation from other organ transplantation procedures. The initial immune response against the endogenous pancreatic β cell population generates memory T cells and islet autoantibodies that can be reactivated upon introduction of new islet tissue. T cell depletion by standard pharmacological immunosuppressive protocols can induce the expression of homeostatic T cell proliferation cytokines IL-7 and IL-15, leading to enrichment of memory islet-specific antigen T cell clones and representing a possible immunological mechanism for islet rejection even under maintenance immune suppression [83]. Islet-antigen specific clonal expansion of T cells can be induced through direct or indirect pathways [66, 84].

2.5 Clinical Immunosuppressive Strategies

Dr. James Shapiro from the University of Alberta established the modern guidelines for intraportal islet transplantation and post-operative care with what is now referred to as the Edmonton Protocol. In his landmark study published in the New England Journal of Medicine in 2006, Dr. Shapiro and his team transplanted 7 diabetic patients with allogeneic islets and treated them with a novel glucocorticoid-free immune suppression regimen [3, 85]. Glucocorticoids, which are a traditional component of anti-rejection dosing regimens in solid organ transplantation, cannot be used for islet transplantation due to their well known diabetogenic effects targeting pancreatic β-cells [86]. The Edmonton protocol pioneered by Dr. Shapiro was effective at retaining graft function and all 7 patients retained insulin independence after a year, representing a great step forward in preserving transplanted islet functionality.

In the initial Edmonton Protocol study, patients were given courses of sirolimus, tacrolimus, and daclizumab. All three immunosuppressants are delivered systemically and are

mechanistically aimed at halting T cell activation and proliferation. Sirolimus, which targets mTOR signaling, and tacrolimus, a calceneurin inhibitor, target IL-2 production, while Daclizumab is a monoclonal antibody that blocks the IL-2 receptor on T cells. Sirolimus and tacrolimus are routinely used to improve engraftment for allogeneic hematopoietic stem cell transplants in addition to other solid organ transplants [87]. While not as overtly toxic as glucocorticoids, dosages of sirolimus and tacrolimus for maintenance immune suppression are limited by their toxic effects on islets, as both have been shown to cause reversible loss of glucose sensitivity and insulin secretion [17, 88]. The anti-CD52 antibody alemtuzumab can be used to induce immune suppression through the depletion of multiple immune cell types including T cells, macrophages, and NK cells [89-91]. T cell depleting antibodies anti-CD3 and anti-thymocyteglobulin (ATG) have also been used part of potent induction immunosuppression (PII) protocols that may help combat both the allo- and auto-immune response mediated through T cells [92]. Agents blocking non-specific immunity have also been included in immunosuppression protocols with demonstrated clinical efficacy. Etanercept and anakinra decrease early inflammatory events by functioning as anti-TNF and anti-IL1 blockers respectively [93-95]. While current immune suppression protocols are sufficient to protect a minimal mass of islets through the first year post-infusion, the drop of insulin independence at 5 years to 25-50% suggests that even maintenance immune suppression is not sufficient to protect the graft from ongoing immune recognition and activation [9]. Thus, the pursuit of a true tolerance induction protocol, where a state of non-responsiveness to graft antigen can be achieved without the need for maintenance immune suppression, is necessary to make islet transplantation viable as an alternative strategy to T1DM treatment.

2.6 Cell therapies for transplantation tolerance

The traditional administration of immunosuppressive drugs is hampered by off target effects that can produce toxic effects to the liver and kidneys, in addition to the graft itself. Immune therapies where cells are used as "living drugs" have been pitched as a promising alternative. Cell-mediated therapies are potentially advantageous compared to chemical or pharmacological effects due to location-specific effects, ability to migrate to specific sites through direct transplantation or chemokine attraction, and specificity of targets. Technologies to robustly sort immune cell lineages, expand tolerogenic cells, and reinfuse them into the patient have exploded in the last several years.

Tregs are among the most potent options for cell-mediated immune suppression and can directly inhibit T-cell mediated rejection in both transplantation and autoimmune settings [96-99]. Most clinical trials using Tregs to induce tolerance rely on the purification and expansion of Tregs from blood [99, 100]. Current expansion protocols can expand isolated Treg populations 100-1000 fold through TCR activation and addition of T cell proliferative cytokines such as IL-2. Tregs have both active and passive mechanisms for suppression [101]. Tregs are able to secrete immunosuppressive factors such as IL-10 and TGF-β1 that can downregulate T cell activation. Tregs also express the coinhibitory cell marker CTLA-4, which can block costimulatory molecules CD80 and CD86 on APCs. Tregs do not produce IL-2 but express high amounts of the high-affinity IL-2 receptor CD25, which can result in Treg-mediated IL-2 depletion, depriving proliferating T cells from a local source of activation. Until recently, the numbers of Tregs required to invoke a tolerogenic effect has often been prohibitive for clinical success [99]. It is estimated that over 33% of the graft-localized T cell population must be regulatory in order to generate a graft-tolerogenic environment [102]. One future solution

involves using antigen-specific Tregs that have much more potent immune effects when compared to polyclonal Tregs. This insight has spawned significant interest in the development of expansion protocols targeting specific TCRs [103-105]. There have also been attempts to convert naïve T cells into *in vitro* Tregs through induced expression of Foxp3 by TCR activation with TGF-β1, but questions remain concerning the stability of these cells due to the methylation status of the *FOXP3* gene possibly leading to eventual loss of phenotype [106].

Mesenchymal stem cells (MSCs) are a lineage of multipotent stromal stem cells derived from bone marrow. As part of the hematopoietic stem cell (HSC) niche, MSCs are a crucial component of maintaining a local microenvironment supportive of hematopoiesis [107]. As part of their natural function to maintain quiescence of HSCs, MSCs demonstrate immunosuppressive capabilities including secretion of anti-inflammatory factors like TGF-β1, IL-10 and IDO. Their immune suppressive capabilities have been proven in a number of model systems including graft-versus-host disease, autoimmunity, and allogeneic transplant [108-110]. Importantly, MSCs are naturally recruited to sites of tissue damage and inflammation and require signaling by inflammatory cytokines like IFN-γ and TNF-α to activate their immunosuppressive phenotypes [111]. Thus, exogenously administering MSCs shows the most therapeutic benefit when the graft is expressing high amounts of inflammatory markers. Co-transplantation of donor MSCs with intraportally infused allogeneic islets in a non-human primate study supplemented with two subsequent intravenous administrations of MSCs resulted in enhanced islet function one month post-transplant and could reverse rejection episodes [112].

Dendritic cells are the primary professional APCs responsible for activating T cells, making them an appealing target for controlling the adaptive immune response [113]. Dendritic cells control T cell fate by presenting antigen through MHCII in the presence of different

combinations co-stimulatory signals and cytokines. During normal homeostasis, especially in tolerogenic tissue environments, T cell interact with DCs under sub-immunogenic conditions, which can result in a number of different cell fates that lead to non-activity towards the antigen, including T-cell anergy, AICD, or the development of a regulatory T cell phenotype [114, 115]. DCs can be conditioned *ex-vivo* with a number of different factors that either target the downregulation of co-stimulatory molecules required for T cell activation or prevent the secretion of inflammatory-polarizing cytokines like IL-12p70 to prevent immune activation [116]. Isolated DCs can also be selectively pulsed with antigens that are being targeted for tolerance [117]. Most of the major immunosuppressive drugs such as sirolimus as well as natural factors like IL-10, and Vitamin D have been successfully used to condition *ex vivo* DCs towards tolerance-inducing immune cell phenotypes [118-120].

2.7 Biomaterials to enhance islet transplantation

Biomaterials can be a useful tool to enhance the success of cell transplantation by attempting to either recapitulate native tissue signals or introduce novel characteristics that can enhance survival of the graft. In islet transplantation specifically, biomaterials can be deployed for three distinct functional strategies: cell integration, immune isolation and immune modulation. Cell integration approaches seek to enhance the engraftment of the transplanted islets into the host tissue by restoring vascularization, oxygen tension, innervation and other endogenous characteristics of the native organ environment [1]. Immune isolation approaches aim to directly inhibit cell-cell contact between the transplanted islets and the host immune response using a barrier surface, leading to a "silenced" immune phenotype [121]. Finally, immunomodulatory

biomaterials attempt to control the natural interaction between the material and leukocytes to polarize the local or systemic immune environment towards tolerogenic phenotypes [6].

The choice of biomaterial will depend on the priorities for the given transplant strategy, summarized in **Table 2.1** [122]. Given their derivation from biologic sources, natural polymers often show superior characteristics for cell adhesion and migration and are naturally degradable by human enzymes. Disadvantages of natural polymers include possible immunogenicity and batch-to-batch variability from the material purification process and lack of control over the mechanical properties. Synthetic polymers are extremely versatile and can be readily altered by controlling copolymer ratios and identities. Disadvantages of synthetic polymers can include the need to be modified to include supplementary cell adhesion sites and their degradation into acidic waste products, potentially damaging local tissue.

2.7.1 Tissue Integration and Engraftment

For tissue integration approaches, cells are typically incorporated into a porous scaffold that is designed to promote rapid neovascularization and tissue ingrowth to restore the physiological conditions necessary to meet islet metabolic demands. Since the general strategy of open-pore scaffolds is to allow for free tissue reintegration, scaffolds are typically designed to be biodegradable and with a highly interconnected pore structure. Growth factors or oxygen generating materials can also be included within the scaffold to enhance endogenous tissue repair pathways that can speed tissue engraftment [123, 124]. Depending on the desired pore size, scaffolds can be prefabricated and subsequently seeded with cells or polymerized after the introduction of cells.

Purpose	Application	Material	Reference
Tissue infiltration and host engraftment	Macroporous Scaffold	Poly(lactide-co-glycolide)	Blomeier et al. 2006; Gibly et al. 2011
		Polydimethylsiloxane	Pedraza et al. 2013
		Silk	Hamilton et al. 2017; Mao et al. 2017
	Pro-angiogenic degradable scaffold	Collagen/Chitosan	Dent et al. 2011; McBane et al. 2013
		Fibrin/Thrombin	Najjar et al. 2015; Baidal et al. 2017
Immune isolation	Macroencapsulation hydrogel	Alginate	Dufrane et al. 2010; Marchioli et al. 2015
		Poly(ethylene-glycol)	Phelps et al. 2015; Rios et al. 2016
		Poly(vinyl-alcohol)	Inoue et al. 1992; Qi et al. 2012
	Microencapsulation surface coating	Alginate	Lanza et al. 1991; Sun et al. 1992
		Heparin	Cabric et al. 2007
		Poly(ethylene-glycol)	Tomei et al. 2014

Table 2.1 Summary of biomaterial approaches to enhance islet transplantation

In order to retain as much of the native signaling as possible, many groups have used decellularized organs as a natural scaffold environment for cell transplantation [125, 126]. The removal of cells from an isolated organ leaves behind the native ECM matrix that can be reseeded with regenerating cells. Decellularization agents include chemical agents such as acids and bases, detergents, and hyper/hypotonic solutions, biological agents like trypsin or nucleases, or physical agents such as temperature and pressure [125]. In comparison to other organs, decellularization of pancreas matrix has been relatively unexplored. Several studies have worked out decellularization protocols and shown that the pancreatic acellular matrix (APM) can generally support survival and insulin secretion of islets or β-cell lines [127-129]. As a clinical option, the use of decellularized pancreas as a matrix suffers from the requirement of an additional pancreas for use in transplant, though recent work has showed that discarded human pancreata unusable for transplant can be successfully repopulated with pancreatic β-cell lines [127]. As an alternative to human derived APM, other groups have suggested the use of xenogeneic pancreata, given the approval of several other scaffold types produced from bovine or porcine sources by the FDA for use in humans [127].

Synthetic polymer is likely to be the easiest choice for the translation of scaffolds into the clinic, due to superior control of the material synthesis process and the completely artificial fabrication process that is in compliance with GMP standards [130]. Porous scaffolds can be fabricated by fusing polymer microspheres into a continuous matrix around a leachable particulate using a number of different methods including solvent casting or gas foaming [131, 132]. The pore size and porosity of the scaffold can thus be carefully controlled to optimize cell seeding and tissue ingrowth through the choice of particulate, commonly salt or gelatin. Synthetic scaffolds can also be functionalized with extracellular matrix proteins in order to

recapitulate some of the native ECM signaling, which has improved engraftment [133, 134]. PLG scaffolds transplanted into the epididymal fat pad have been shown to immediately restore normoglycemia in the streptozotocin-induced mouse model with 200 islets and can support a minimal mass transplant of 75 syngeneic mouse islets capable of reversing diabetes within 12 days, which is 10% of the normal graft size necessary for intraportal transplant [35].

In situ scaffolds produced from natural polymers can be produced from non-whole organ sources. As a primary component of blood clotting, fibrin derived from plasma are used to produce *in situ* three-dimensional biodegradable scaffold structures [135-137]. As previously mentioned, current clinical trials being conduced at the University of Miami transplanting islets into the human omentum utilize a fibrin clot scaffold [32]. Transplanted islets are mixed with an autologous plasma solution containing fibrin and clotted against the omentum using thrombin. Other potential natural polymer scaffold components that have been tested include collagen and chitosan, which can form hydrogels after incubation under cell-friendly buffer conditions at 37°C. Collagen and chitosan hydrogels have been synthesized co-encapsulating islets with vascularization promoting cell types such as circulating progenitor cells (CPCs) and circulating angiogenic cells (CACs) [138]. The Shapiro group has pioneered an endogenous “scaffold” production methodology by implanting a catheter subcutaneously to induce a foreign body response, resulting in local neovascularization and the formation of a “collagen scaffold” around the space that remains once the catheter is removed, effectively creating an *in vivo* scaffold that can serve as an space for islet transplantation for a macroencapsulation device [139].

Synthetic hydrogels comprised of poly(ethylene-glycol) (PEG) have become a popular choice to provide a scaffold environment for engraftment of islets, due in part to success from 3-D *in vitro* cultures. The PEG matrix is extremely easy to functionalize with extracellular

membrane proteins and adhesion molecules that enhance survival of entrapped islets [140, 141]. Growth factors such as VEGF have also been successfully incorporated into the PEG hydrogel to improve local vascularization around the graft [142].

2.7.2 Cell Encapsulation

A successful encapsulation strategy would completely eliminate the need for immunosuppression by preventing leukocyte detection and cell-cell contacts with the graft that initiate the host rejection response [121, 143]. There are two general strategies for encapsulation: microencapsulation and macroencapsulation. Microencapsulation describes a process where each individual islet is completely coated with the encapsulation material. The coating needs to be porous enough to allow for the diffusion of oxygen, nutrients, and blood glucose from the host tissue and insulin from the islet across the surface membrane while blocking leukocyte infiltration and inflammatory cytokines. ECM components can be introduced into the surface coating to prevent anoikis through loss of native ECM connections [144, 145]. Coating individual islet clusters theoretically maximizes access of resources to each islet, though it can be difficult to control coating homogeneity. The abrogation of donor-immune recognition potentially opens the door for a wider array of readily available insulin-producing donor material to be utilized, including human embryonic stem cell derived pancreatic progenitor cells or xenogeneic tissue [146]. In practice, immune isolation with encapsulation is rarely achievable. The porosity of the surface coating required to allow for diffusion of factors across the membrane ultimately will allow for inflammatory cytokines secreted from graft-adjacent leukocytes to diffuse into the membrane [147, 148]. Immunogenic antigens and chemokines produced by the islets also are too small to be trapped by the encapsulation material and can

cause activation of local immune lineages that lead to pericapsular fibrotic overgrowth, which will deprive cells of necessary nutrients and cause graft failure [149].

There are a number of different materials that have been used to encapsulate islets. Alginate isolated from seaweed is a popular choice due to its low immunogenic profile, stability, permeability, and ability to gel at physiological islet conditions [148, 150]. Alginate is cross-linked with the addition of divalent cations and can be blended with other polymers like poly-L-lysine or PEG to modulate mechanical properties [151, 152]. A common issue however prescribed to the use of alginate is the induction of a foreign body response by local macrophages that ultimately leads to fibrosis surrounding the transplanted capsules, which eventually leads to cell death from poor integration [153]. The foreign body response to alginate can be decreased by chemical modifications of the alginate backbone with triazole groups, as identified in a hydrogel library screen [154]. Alginate capsule thickness is also typically 0.3 mm to 1 mm, dramatically increasing the transplant volume of an average 150 μm islet [121]. This size increase has a knock-on effect of requiring the usage of the peritoneal space as the site of transplant, which has poor engraftment qualities and can make post-transplant retrieval of islets difficult [155]. Alginate microencapsulation approaches used in clinical human trials have yet to show improvement on graft survival compared to traditional immune suppression [156]. A number of synthetic materials have also been used for microencapsulation. PEG is a popular choice of microencapsulation due to its low adsorption of proteins. The Hubbell group first demonstrated a successful microencapsulation and subsequent functionality of porcine islet by PEG diacrylate [157]. Subsequent efforts to microencapsulate using PEG have focused on the ability evenly coat the islet surface with minimal and even thickness through a variety of processes including conformal coating and PEGylation [158-160].

Macroencapsulation makes use of a larger containment unit where a semi-permeable membrane encloses the total volume of transplanted cells. Most macroencapsulation devices are designed to be self-contained and removable such that non-functional cells can easily be replaced. Several companies have engineered encapsulation devices that have reached various levels of clinical trials including the Theracyte Encapsulation device, Viacyte Encaptra system, and βAir Artificial Pancreas [146, 161-163]. Islets ideally will be arrayed within the chamber to promote equal access to the diffusive membrane and vasculature. Advances in membrane engineering have allowed for the production of encapsulating films made from materials like PCL with thicknesses as low as 10 μm, allowing for response times to blood glucose changes close to nonencapsulated islets [164]. These devices can be supplemented with backup-oxygen generating systems to prolong transplanted cell survival and solve the issue of chronic hypoxia [165]. While the intent of macroencapsulation devices is to prevent leukocyte infiltration into the chamber, surrounding neovascularization is necessary to meet the metabolic needs of the transplanted islets, which can be induced by prevascularizing the transplant site or introducing additional outer membrane layers with pore sizes encouraging tissue ingrowth [166]. The non-degradable nature of macroencapsulation devices can lead to a foreign body response and fibrosis leading to device failure and significant work has gone into designing biocompatible surfaces for encapsulation [167]. Recent work in our lab has shown a non-degradable PEG macroencapsulating hydrogel can be produced to support islet engraftment [168].

2.7.3 Immune Intervention

The natural interaction between biomaterial and immune system can be exploited to promote local or systemic tolerogenic responses to transplanted islets. Just as immune privileged or

evasive tissue microenvironments are enriched with immunosuppressive cytokines and cell types that can permit the long-term survival of foreign antigen, biomaterials can be engineered with similar characteristics to control immunogenicity.

Co-localization of immune cells with the transplant graft can increase the efficacy of cell-based immune intervention. The Bromberg group demonstrated in 2009 that induction of allogeneic tolerance using *ex vivo* expanded Tregs required the Tregs to migrate sequentially from the graft to the draining lymph node [169]. While the typical clinical administration of cell therapies is performed intravenously, delivering cells through co-transplantation straight to the site of immune activation greatly increases initial local concentrations and may increase therapeutic efficacy. In our own lab's work, we have shown that mice transplanted with scaffolds seeded with islets and antigen-specific Tregs were able to achieve long term tolerance and had a significant graft survival extension over mice who received a tail-vein injection of the same number of Tregs [170]. Following this logic, co-transplantation of MSCs with islets on a scaffold would provide an easy method of colocalization to improve immunomodulatory efficacy and avoid potential issues of MSC migration and homing to irrelevant sites [171].

Synthetic polymers also offer the opportunity to tether local immunosuppressive ligands that can induce tolerance. The same immunosuppressive cell surface markers that are used by tolerogenic immune cell lineages can be purified and tethered either directly to the surface of cells or to the biomaterials themselves. TGF-β1, which has distinct properties in a surface bound versus soluble state, has been attached to the cell surface of Tregs through functionalization with PEG, which could easily be adapted to decorate a microencapsulation or scaffold surface [172, 173]. FasL, which has been identified as a major mediator of immune privileged

microenvironments and has been shown to induce tolerance when chemically linked to the surface of allogeneic islets, is another potential target [174].

Biomaterials can be synthesized to incorporate and release drugs and protein factors to modulate the local environment. Systemic delivery of factors can often result in low local concentrations, dosing limitations due to off-target effect, and short *in vivo* half-lives requiring multiple administrations. Biomaterial encapsulation and incorporation can offer the ability to create unique control release profiles and deliver factors in a targeted, controlled, and sustained fashion. PLG is a common biomaterial for factor release and can be used to encapsulate biologic factors due to relatively gentle synthesis conditions. Biomaterial scaffolds have been designed with factors incorporated to modulate the local environment [124, 175].

Biomaterial particles can be used to deliver subimmunogenic doses of antigen to induce tolerance. Recent work has found that the phagocytosis and processing of antigen loaded particles can create a tolerogenic response by activating the same pathways APCs use to prevent the activation of an immune response against apoptotic cells [176]. First tested in autoimmune models where a single disease-inducing peptide was delivered to induce tolerance, subsequent studies found similar success loading peptides derived from allogeneic donor tissue lysate in mouse transplant models [177, 178]. Particle-based antigen delivery may also offer a cure for the recurrent autoimmunity in T1DM patients.

Chapter 3:

Transforming growth factor-beta 1 delivery from microporous scaffolds decreases inflammation post-implant and enhances function of transplanted islets

3.1 Context

In this chapter, we offer the first demonstration that soluble factor release from the porous PLG scaffold can be used to control and delay immune rejection of allogeneic islet transplantation. While porous scaffolds have a long history in tissue engineering as facilitators of cell engraftment, much less work has been done attempting to control the local immune environment using these materials. While previous attempts in our lab used either cell transplantation or ECDI-treated splenocytes to induce tolerance to transplanted islets, it was not known whether immunosuppressive factors could be incorporated directly into the scaffold to influence infiltrating leukocytes. Given the long history of delivering factors from biomaterials, we were inspired to leverage our existing material technologies to try and release an immunosuppressive factor that could prevent or delay graft destruction by infiltrating leukocytes. A previous paper from our lab had pioneered a layered PLG scaffold design, where protein was first encapsulated into particles that were subsequently pressed into a solid layer between two porous outer layers. Due to the variable loading introduced by the protein encapsulation step, we decided to simplify the production process and mix lyophilized protein with particles prior to foaming to create the central layer.

We identified TGF-β1 as an appealing factor due to its well-characterized immunosuppressive properties. In particular, we were intrigued by the association between TGF-β1 and Tregs, given TGF-β1's crucial role as both an immunosuppressive effector mechanism of Tregs and driver of *Foxp3* expression in the *in vitro* induction of Tregs. Having previously published a study where *in vitro* induced Tregs localized to the scaffold were able to induce long-term tolerance in an autoimmune diabetic mouse model, we attempted to determine if we could achieve similar results in an alloimmune model using TGF-β1 incorporated directly into

the scaffold. We thus set out to test how local TGF-β1 release affected the local immune microenvironment and whether those alterations resulted in observable changes to graft survival. The development of a localized factor delivery strategy from our existing biomaterial platform would be an important building block towards directly addressing the immune response as a major hurdle for the success of clinical islet transplantation.

I completed this project in collaboration with Dr. R. Michael Gower. Together we designed and executed the experiments and analyzed data for this manuscript, receiving considerable experimental help from an undergraduate researcher Jesse Zhang. Dr. Xiaomin Zhang performed all of the surgeries involving islet transplantation.

3.2 Abstract

Biomaterial scaffolds are central to many regenerative strategies as they create a space for infiltration of host tissue and provide a platform to deliver growth factors and progenitor cells. However, biomaterial implantation results in an unavoidable inflammatory response, which can impair tissue regeneration and promote loss or dysfunction of transplanted cells. We investigated localized TGF-β1 delivery to modulate this immunological environment around scaffolds and transplanted cells. TGF-β1 was delivered from layered scaffolds, with protein entrapped within an inner layer and outer layers designed for cell seeding and host tissue integration. Scaffolds were implanted into the epididymal fat pad, a site frequently used for cell transplantation. Expression of cytokines TNF-α, IL-12, and MCP-1 were decreased by at least 40% for scaffolds releasing TGF-β1 relative to control scaffolds. This decrease in inflammatory cytokine production corresponded to a 60% decrease in leukocyte infiltration. Transplantation of islets into diabetic mice on TGF-β1 scaffolds significantly improved the ability of syngeneic islets to control blood glucose levels within the first week of transplant and delayed rejection of allogeneic islets. Together, these studies emphasize the ability of localized TGF-β1 delivery to modulate the immune response to biomaterial implants and enhance cell function in cell-based therapies.

3.3 Introduction

Cell transplantation holds tremendous potential for regenerative strategies such as those focused on the heart [179], liver [180], nervous system [181], and diabetes [182]; however, cell survival following transplantation and long-term function pose significant hurdles for these therapies. To address these issues, biomaterial scaffolds designed to enhance cell survival, engraftment, and function at the implant site have been the focus of intense investigation [183-185]. Biomaterials have been modified with biological signals, such as extracellular matrix proteins to modulate cell adhesion and migration, or inductive factors to stimulate cell survival, proliferation, or differentiation. The ultimate goal of these modifications is to create an environment within the implant site that will promote engraftment and long-term function of the transplanted cells.

Despite biological cues presented by the scaffold, tissue damage due to surgery and implantation evokes inflammation that will drastically alter the immune environment within the implant and can adversely affect the short- and long-term survival and function of transplanted cells. Tissue resident macrophages detect tissue damage through pattern recognition receptors leading to the release of inflammatory proteins such as tumor necrosis factor-alpha (TNF-α), interlukin-1beta (IL-1β), and chemokines that recruit neutrophils [186]. TNF-α, IL-1β, and IL-17, released by neutrophils, induce expression of monocyte chemotactic protein-1 (MCP-1) by tissue resident cells including fibroblasts, endothelial cells, and smooth muscle cells, leading to the recruitment of monocytes, dendritic cells (DCs), and natural killer (NK) cells. Neutrophils and NK cells can release reactive oxygen species, enzymes, and cytolytic factors that can damage endogenous and transplanted cells, irrespective of whether transplanted cells are autologous or allogeneic; however, if the transplanted cells are allogeneic, DCs will activate T

and B cells known to play critical roles in transplant rejection [187-192]. In this way, simply implanting allogeneic tissue initiates an inflammatory cascade that leads to its destruction. Thus, an ability to reduce local inflammation and promote non-activated or tolerogenic immune cell phenotypes during and immediately after implant has the potential to enhance both autologous and allogeneic cell-based regenerative therapies.

In this study, we investigated poly-lactide-co-glycolide (PLG) scaffolds designed to release recombinant transforming growth factor-beta1 (TGF-β1) in order to modulate the local immune environment. Localized delivery of immunomodulatory factors is emerging as a strategy for controlling the immune environment within the implant site. TGF-β1 has a substantial role in innate immunity, regulating the recruitment, activation, and function of neutrophils, macrophages, and NK cells [193]. Furthermore, TGF-β1 antagonizes antigen presentation and maturation of DCs [194, 195] and promotes the differentiation of naïve CD4+ T cells into regulatory T cells (Tregs) [196]. Thus, we hypothesized that TGF-β1 release from biomaterial scaffolds could decrease inflammation within the implant, enhance function of syngeneic cell transplants and delay immune rejection of allogeneic cells. This hypothesis was investigated using PLG scaffolds that support islet transplantation into the epididymal fat pad of diabetic mice [33, 35, 124, 132, 133], a model that allows for non-invasive monitoring of cell viability and function by measurement of blood glucose levels. Major objectives were to quantify the effect of TGF-β1 delivery on the inflammatory environment within the implant site and correlate these effects with the ability of the transplanted islets to establish and maintain euglycemia in diabetic animals.

3.4 Materials and Methods

3.4.1 Scaffold fabrication

Protein-loaded poly(lactide-co-glycolide) (PLG) scaffolds were fabricated using a previously described gas foaming and particulate leaching process [197], with a modified design containing a non-porous center layer for protein loading. PLG (75:25 mol ratio d,l-lactide to glycolide, 0.76 dL/g) (Lakeshore Biomaterials) was dissolved in dichloromethane to make either a 2% or 6% (w/w) solution, which was then emulsified in 1% poly(vinyl alcohol) to create microspheres. The microspheres were collected by centrifugation, washed with deionized water, and lyophilized overnight. The non-porous center layer for TGF-β1 scaffolds was made by reconstituting 2 mg of 2% PLG microspheres in sterile deionized water containing 1mg of mannitol (Sigma) and recombinant murine TGF-β1 (Cell Signaling Technology). The mixture was lyophilized and compressed into a 3 mm diameter disk with a height of 100 μm using a manual KBr pellet hand press (Pike Technologies). Center layers for the control scaffolds were made using the same procedures while omitting protein from the lyophilized mixture. The composite scaffold was constructed by sandwiching the protein-containing non-porous layer between two porous layers containing 6% PLG microspheres and NaCl particles 250–425 μm diameter combined in a 1:30 ratio. The three layers were pressed together in a 5 mm steel die at 1500 pounds per square inch using a Carver press into a 5 mm diameter disk with a height of 2 mm. The scaffold was then gas-foamed after equilibration to 800 psi under CO_2 gas in a custom-made pressure vessel. Salt particles were removed from the foamed scaffolds by immersion in 10 mL deionized water for one hour.

3.4.2 In vitro TGF-β1 release assay

Scaffolds were leached in 10 mL of water containing 1% BSA (fraction V, protease free, Millipore) for 1 hour to remove salt porogen and were then transferred into 1mL of EBSS (Life Technologies) containing 1% BSA, penicillin, and streptomycin and incubated at 37^{o}C for 28 days with gentle agitation. At 1, 3, 7, 14, 21, and 28 days scaffolds were placed in fresh EBSS and the old EBSS was frozen. At the end of the experiment TGF-β1 was measured using a TGF-β1 DuoSet® ELISA Kit (R&D Systems) as per the manufacturer's instructions.

3.4.3 Scaffold implantation

Prior to implant, scaffolds were disinfected in 70% ethanol and then washed twice in sterile phosphate buffered saline (PBS; Life Technologies). For non-transplant studies, mice received scaffold implants into both epididymal fat pads for a total of two per mouse. Scaffold implantation was performed as previously described [198]. Prior to implant, recipient mice were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg) and xylazine (5 mg/kg), and the abdomen was shaved and prepped in a sterile fashion. Following a lower abdominal midline incision, scaffolds were wrapped in the epididymal fat and returned to the intraperitoneal cavity. The abdominal wall was then closed with a running stitch, and the skin was closed with wound clips.

3.4.4 Flow cytometry

The following antibodies were purchased from Biolegend: anti-CD45 clone 30-F11; anti-CD8a clone 53-6.7; anti-Ly6G clone 1A8; anti-F4/80 clone BM8; anti-NK1.1 clone PK136, anti-CD19 clone 6D5, anti-I-A/I-E (MHCII) clone M5/114.15.2, and anti-CD16/32 clone 93. The

following antibodies were purchased from eBioscience: anti-CD11b clone M1/70, anti-CD11c clone N418, and anti-Foxp3 clone FJK-16s. Anti-CD4 clone RM4-5 was purchased from BD Biosciences.

Following euthanization, scaffolds were harvested and immediately washed in ice cold Hanks Balanced Salt Solution (HBSS; Life Technologies). Excess tissue was trimmed such that only the immediate scaffold environment and integrated tissue were analyzed. Scaffolds were minced and incubated in collagenase (Roche) at 37^{o}C for 20 min. The solution was then passed through a 70 μm filter, washed in PBS, and suspended in PBS containing anti-CD16/32 and LIVE/DEAD blue fixable dye (Life Technologies). Antibodies against extracellular antigens were then added. After extracellular antibody incubation, cells were washed to remove unbound antibody, fixed in fixation buffer (Biolegend) and analyzed on an LSRFortessa flow cytometer (BD Biosciences). The entire cellular infiltrate isolated from the scaffold and integrated tissue was analyzed by flow cytometry so the total number of immune cells could be reported. Data was analyzed in FlowJo software (Treestar). Isotype controls were used to set gates for immunophenotyping. Foxp3 was detected using eBioscience's Foxp3/Transcription factor staining buffer set.

The gating scheme for flow cytometry data is depicted in **Figure 3.1**. Cellular events were gated using forward scatter and side scatter. Viable leukocytes were then identified by CD45 expression and low signal from viability stain, while cell aggregates

3.4.5 Animals and induction of diabetes

Male C57BL/6 mice (Jackson Labs) between 8 and 12 weeks of age were used for syngeneic islet transplants. In allogeneic transplants, male BALB/c (Jackson Labs) and C57BL/6 mice

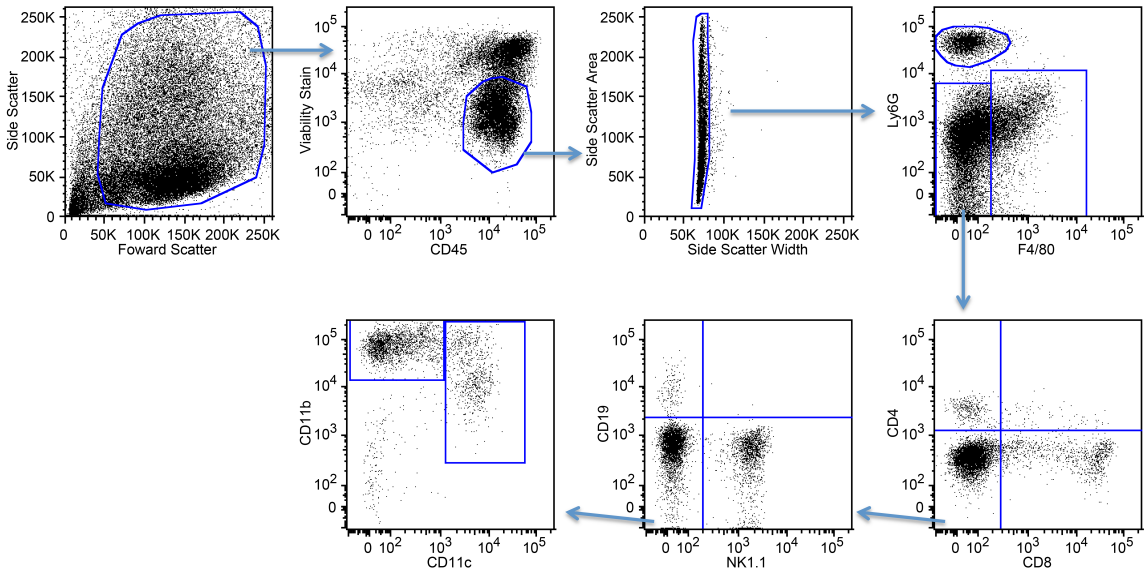

Figure 3.1: Gating scheme for flow cytometry of leukocytes in PLG scaffolds.

between 8 and 12 weeks of age were used as islet donors and transplant recipients, respectively. Clinical diabetes was induced by intraperitoneal injection of 190 mg/kg of streptozotocin (Sigma). Diabetes was confirmed by blood glucose measurements greater than 300 mg/dL on two consecutive days prior to transplantation. The Northwestern University Animal Care and Use Committee approved all studies.

3.4.6 Islet isolation, scaffold seeding, and transplantation

Islet isolation and scaffold seeding were performed as previously described [19]. Briefly, islets were isolated from donor pancreata by a mechanically enhanced enzymatic digestion using collagenase (type XI; Sigma). After filtration through a mesh screen, the filtrate was applied to a discontinuous ficoll gradient (Sigma). Islets were handpicked from the gradient, washed, and counted. Scaffolds were immersed in 70% ethanol and then washed in serum-containing media. Each scaffold was seeded with 250 manually counted islets in a minimal volume of media by applying them to a single side of the scaffold and allowing them to settle onto the microporous structure. Examination of the tissue culture media following removal of the scaffolds demonstrated that greater than 98% of the islets were retained within the scaffolds. Prior to transplant, recipient mice were anesthetized with an intraperitoneal injection of ketamine (10 mg/kg) and xylazine (5 mg/kg), and the abdomen was shaved and prepped in a sterile fashion. For each recipient, following a lower abdominal midline incision, an islet-seeded scaffold was wrapped in the right epididymal fat pad and returned to the intraperitoneal cavity. The abdominal wall was then closed with a running stitch, and the skin was closed with wound clips.

3.4.7 Assessment of graft function

To assess graft function, non-fasting blood glucose measurements were taken between 12:00 and 17:00 after transplantation. Graft rejection was confirmed by two consecutive measurements of blood glucose levels more than 250 mg/dL.

3.4.8 Statistics

Multiple groups were compared using one-way ANOVA with the Tukey post-hoc test. Comparisons between two groups were made with an unpaired *t* test. Comparisons between two groups overtime were made using a two-way ANOVA with Bonferroni's multiple comparison test. The log-rank test was used to compare Kaplan-Meyer survival curves depicting islet survival. The specific test and information on the number of animals and experiments are specified in each figure legend. All analysis was carried out using GraphPad Prism. In all figures, error bars denote SEM.

3.5 Results

3.5.1 Scaffold structure and protein release

A layered scaffold design was implemented to facilitate incorporation of protein, while retaining an interconnected porous structure suitable for cell transplantation [35]. The scaffold consisted of a thin, non-porous center layer of PLG sandwiched between two porous outer layers of PLG **(Fig. 3.2A).** Fusion of the microspheres within the composite structure by gas foaming resulted in an effective union of the adjacent layers that withstood salt leaching and surgical implantation. Importantly, the outer layers have a porous structure that allows for effective islet transplantation, and the inner layer can be separately designed for sustained protein release.

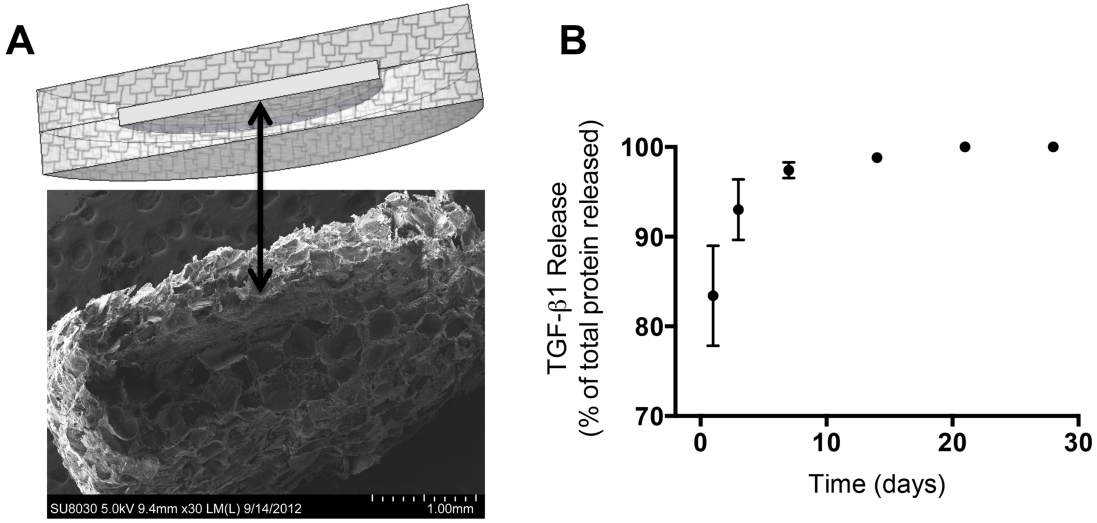

Figure 3.2: Scaffold microstructure and protein release profile.
(A) Diagram and scanning electron microscope image of a layered scaffold. Black arrow indicates the protein-containing center layer. **(B)** *In vitro* release profile from five layered scaffolds containing 2 μg of TGF-β1 as measured by ELISA.

We initially characterized the release profile of the TGF-β1 from scaffolds *in vitro* (**Fig. 3.2B)** as a prelude to investigating the immune response *in vivo*. Leaching of salt to remove the porogen resulted in loss of 13% of the TGF-β1 (data not shown). Of the remaining protein, 83% was released in the first day, with an additional 10% of the TGF- β1 released between days 1 and 3. The amount of TGF-β1 released after day 3 accounted for 7% of the total protein loaded into the center layer of the scaffold.

3.5.2 Characterization of leukocyte infiltration into TGF-β1 scaffolds

Initial *in vivo* studies investigated the leukocyte populations that infiltrate and reside within TGF-β1 loaded scaffolds following implantation. Flow cytometric analysis indicated that the number of leukocytes (identified by CD45 expression) within the scaffold 7 days after implantation was decreased in a dose dependent manner in response to TGF-β1 delivery. Scaffolds loaded with 0.2 μg and 2 μg of TGF-β1 contained 35% and 60% fewer leukocytes respectively, compared to scaffolds without TGF-β1 **(Fig. 3.3A)**. Numbers of CD45 cells isolated from 2 μg TGF-β1 scaffolds and control scaffolds were similar for 7 and 14 days after implantation (**Fig. 3.4)**.

We next investigated specific immune lineages residing in the scaffold after 7 days. Eight leukocyte populations were identified within the scaffolds, which were F4/80 macrophages, Ly6G neutrophils, CD11c DCs, CD11b monocytes, CD4 T cells, CD8 T cells, CD19 B cells, and NK1.1 NK cells **(Fig. 3.3B)**. Significant decreases in the numbers of all leukocyte populations studied were observed at the 2 μg dose relative to the empty scaffold control, with the 0.2 μg dose exhibiting average values intermediate between the control and 2 μg dose. Notably F4/80 macrophages and NK1.1 NK cells exhibited a reduction of more than 69% and 74%,

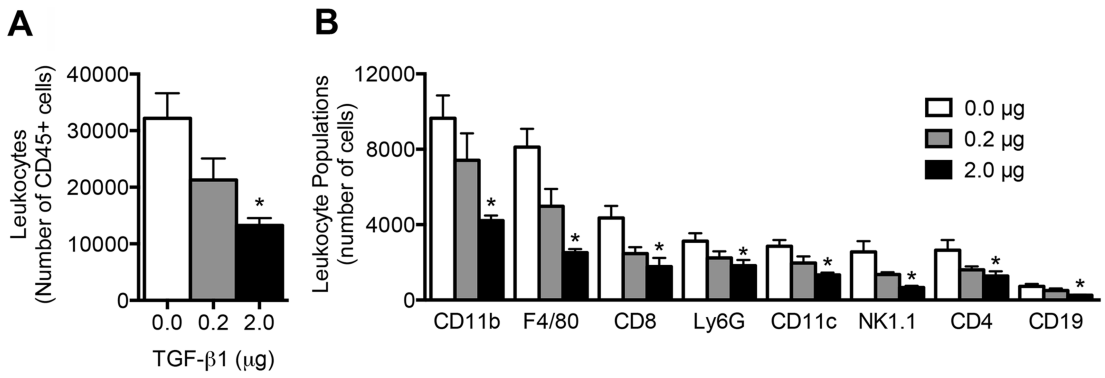

Figure 3.3: Leukocyte infiltration into TGF-β1 scaffolds.
(A) Number of leukocytes isolated from TGF-β1 scaffolds seven days after implant as measured by flow cytometry. **(B)** Prevalence of leukocyte populations within TGF-β1 scaffolds. Data is from 8 scaffolds from 4 mice per condition receiving bilateral scaffold implants into the epididymal fat pads. * indicates $P < 0.05$ versus 0 μg. Statistics determined by one-way ANOVA with Tukey's Multiple Comparison Test.

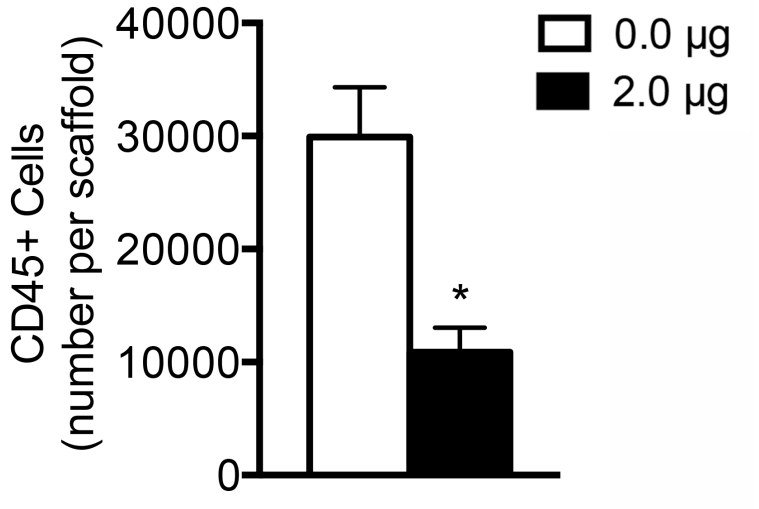

Figure 3.4: CD45 leukocyte infiltration at day 14.
Number of leukocytes isolated from scaffolds containing 0 µg (white bars) or 2 µg (black bars) of TGF-β1. Scaffolds were implanted for 14 days. Data is from 10 scaffolds from 5 mice per condition receiving bilateral scaffold implants into the epididymal fat pads. * indicates $P < 0.05$ versus 0 µg control. Statistics determined by an unpaired t-test.

respectively, within the scaffold for the 2 μg dose relative to the no protein control. Despite the known role of TGF-β1 in generating Tregs through expression of Foxp3, there was a decrease in the number of Tregs in TGF-β1 scaffolds compared to the control scaffolds (**Fig. 3.5**); however, there was an increase in the expression level of Foxp3 amongst Tregs in scaffolds loaded with 2 μg of TGF-β1.

3.5.3 Leukocyte activity within TGF-β1 scaffolds

The activation status of antigen presenting cells (APCs) within the scaffold was investigated by measurement of MHCII expression by flow cytometry; activated cells are expected to express higher levels of MHCII (**Fig. 3.6A**). F4/80 cells exhibited decreased MHCII surface expression within scaffolds loaded with 0.2 μg or 2 μg TGF-β1 scaffolds (20% and 30%, respectively) relative to control scaffolds. CD11c cells exhibited a 15% decrease in MHCII expression within scaffolds loaded with 2 μg of TGF-β1 compared to control scaffolds. In contrast, MHCII expression was not affected on CD11b Ly6G cells, which do not typically express MHCII.

The significant decrease in leukocyte numbers and MHCII expression on APCs led to the investigation of local cytokine and chemokine expression (**Fig. 3.6B**). At day 3, the NK cell chemokine CXCL10 and monocyte/macrophage chemokine MCP-1 were in highest abundance in scaffolds without TGF-β1. These factors exhibited a 53% and 47% decrease with TGF-β1 delivery, respectively. The T cell chemokine CCL5 was also decreased by 67%. TGF-β1 delivery decreased inflammatory cytokines TNF-α and IL-12 by 65% and 61%, respectively, while IL-1β exhibited a decreasing trend. Interestingly, expression of the anti-inflammatory cytokine IL-10 was also significantly decreased following TGF-β1 delivery. IFN-γ expression, if present, was below the level of detection (31 pg/mL).

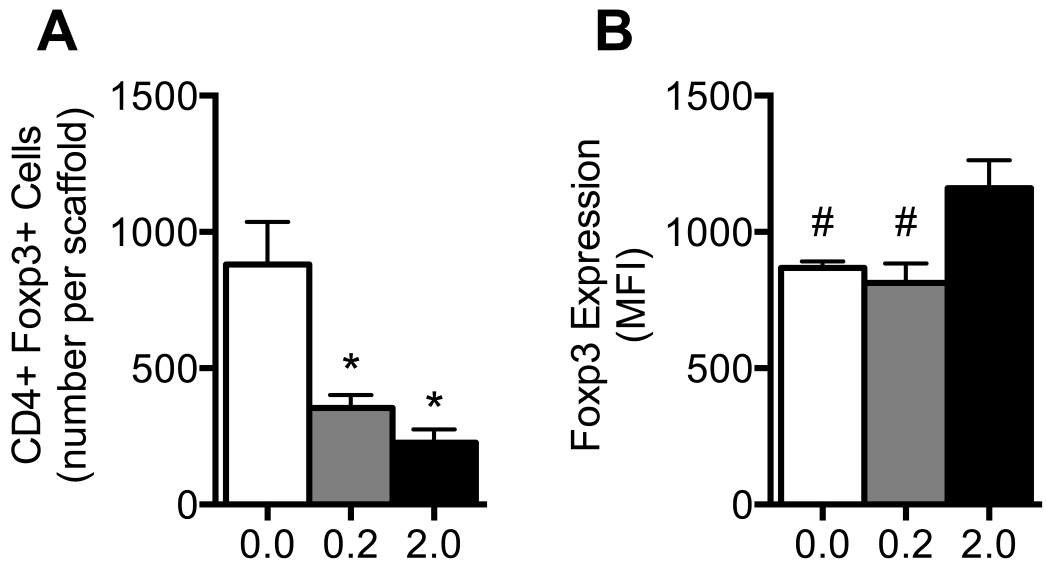

Figure 3.5: CD4 Foxp3 T cells in TGF-β1 scaffolds.
(A) Number of CD4 Foxp3 T cells and **(B)** Foxp3 expression isolated from scaffolds containing 0 μg (white bars), 0.2 μg (grey bars) or 2 μg (black bars) of TGF-β1. Scaffolds were implanted for seven days. The entire cellular suspension isolated from the scaffold was analyzed. Data is from 8 scaffolds isolated from 4 mice receiving bilateral scaffold implants into the epididymal fat pads. * Indicates $P < 0.05$ versus 0 μg. # Indicates $P < 0.05$ versus 2 μg. Statistics determined by one-way ANOVA with Tukey's multiple comparisons test.

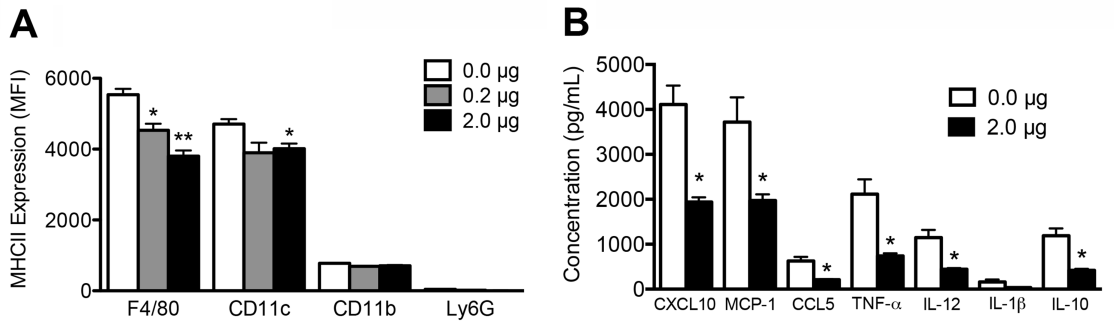

Figure 3.6: MHCII and cytokine expression within TGF-β1 scaffolds.
(A) MHCII expression on leukocytes within scaffolds containing a dose range of TGF-β1 collected seven days after implant. Data is from 8 scaffolds per condition isolated from 4 mice receiving bilateral scaffold implants into the epididymal fat pads. * indicates $P < 0.05$ versus 0 µg. ** indicates $P < 0.05$ versus 0.2 µg. Statistics determined by one-way ANOVA with Tukey's Multiple Comparison Test. **(B)** Cytokines detected by ELISA in scaffold homogenate collected three days after implant. Data is from 10 scaffolds isolated from 5 mice per condition receiving bilateral scaffold implants into the epididymal fat pads. * indicates $P < 0.05$ versus 0.0 µg. Statistics determined by unpaired t-test.

3.5.4 Syngeneic islet transplant on TGF-β1 releasing scaffolds

The ability of localized TGF-β1 delivery to enhance islet function was initially investigated by seeding syngeneic islets isolated from healthy C57BL/6 mice onto scaffolds containing 2 μg of TGF-β1 that were then implanted into male C57BL/6 mice rendered diabetic by streptozotocin injection. Blood glucose was monitored daily to assess islet function (**Fig. 3.7A**). Islets transplanted on scaffolds containing 0 or 2 μg of TGF-β1 maintained similar blood glucose levels for the first three days after transplant. However, between days 4 and 6, islets transplanted on TGF-β1 scaffolds maintained significantly lower blood glucose levels. Following day 6, daily blood glucose levels for the two groups were not different for the remainder of the study. Tissue infiltration into the scaffold and vascularization were studied at day 7 post-transplant. Hematoxylin and eosin stained sections indicated greater host tissue integration around the central layer of the scaffold in the presence of TGF-β1 (**Fig. 3.8**); however, we did not observe differences in CD31+ staining of endothelial cells between the two groups (**Fig. 3.9**).

The ability of the engrafted islets to clear glucose from the circulation was investigated using an intraperitoneal glucose tolerance test that was performed at day 70. At this time point, no significant difference in the area under the curve was observed between the two groups (**Fig. 3.7B**). Finally, at day 80, the fat pads containing islets were removed. Within 4 days, all mice in both groups reverted to hyperglycemia (**Fig. 3.7A**), indicating that euglycemia was due to islet transplantation and not regeneration of endogenous islets.

3.5.5 Allogeneic islet transplant on TGF-β1 releasing scaffolds

The protection of allogeneic islets from immune rejection by localized delivery of TGF-β1 delivery was subsequently investigated. Islets isolated from healthy Balb/C mice were seeded

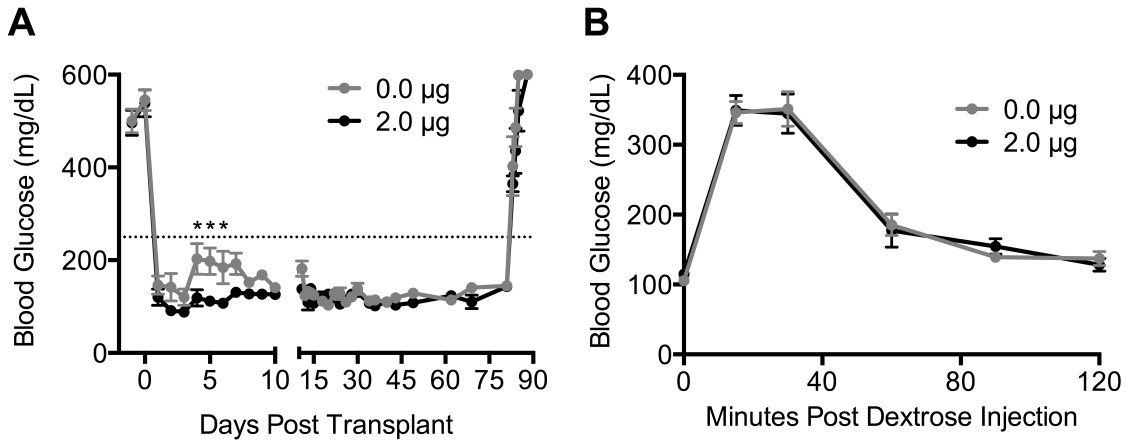

Figure 3.7: Syngeneic islet function on TGF-β1 scaffolds.
(A) Blood glucose versus days post transplant for diabetic mice receiving syngeneic islet transplants on TGF-β1 scaffolds. Fat pads containing islets were removed at day 80. Dotted line indicates a blood glucose level of 250 mg/dL, and two consecutive readings above 250 would indicate graft failure. Data is from 8 scaffolds from 8 mice per condition receiving one scaffold implant into the right epididymal fat pad. * indicates $P < 0.05$ versus 0 µg on same day. Statistics determined by a two-way ANOVA with Bonferroni's multiple comparisons test. **(B)** Blood glucose versus time following an intraperitoneal dextrose injection. Glucose tolerance test was performed on day 70.

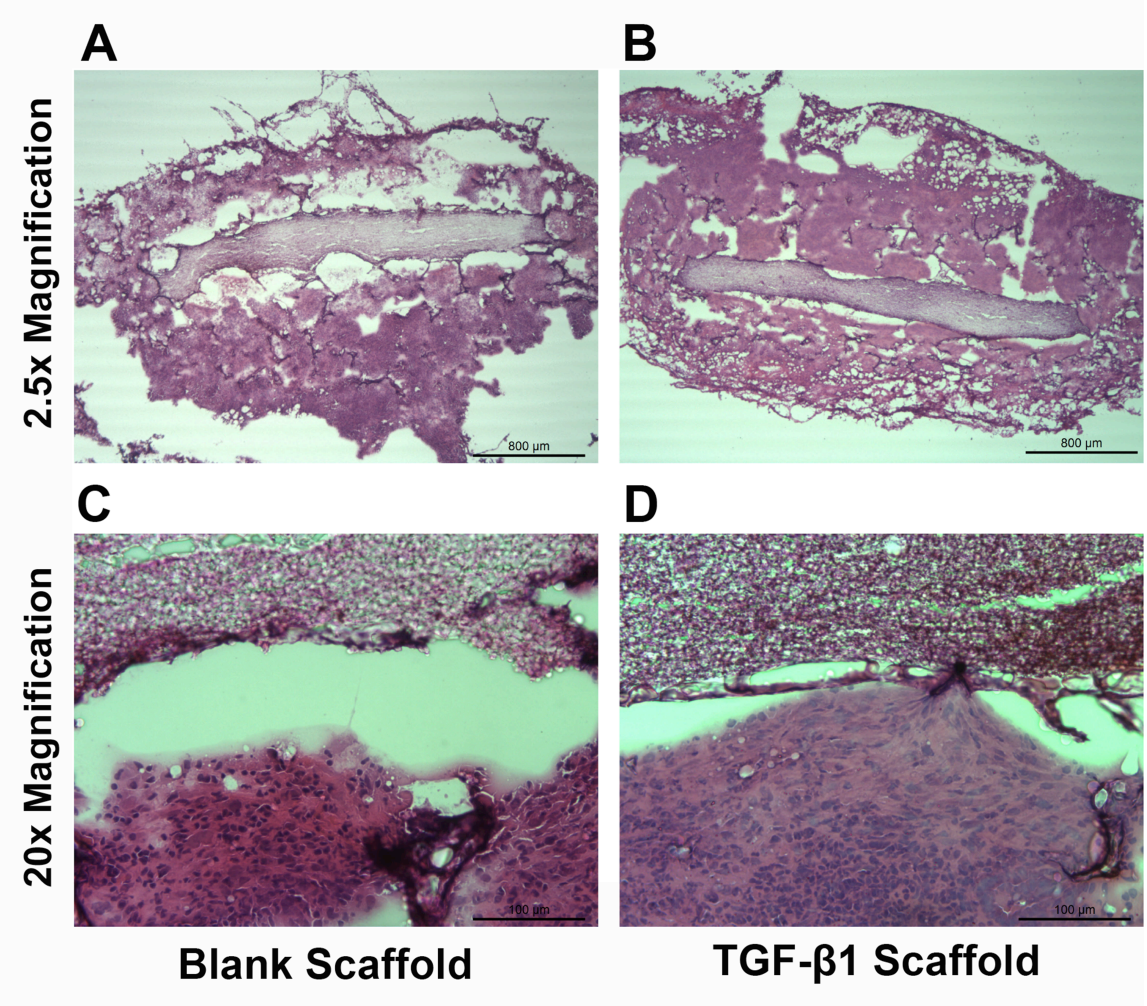

Figure 3.8: Histological analysis of syngeneic islet grafts on TGF-β1 scaffolds.
H&E staining of histological sections of islet grafts transplanted on scaffolds containing **(A and C)** 0 µg and **(B and D)** 2 µg of TGF-β1 at day seven after transplant at 2.5x magnification **(A and B)** and 20x magnification **(C and D)**. Images representative of sections from 4 mice per condition.

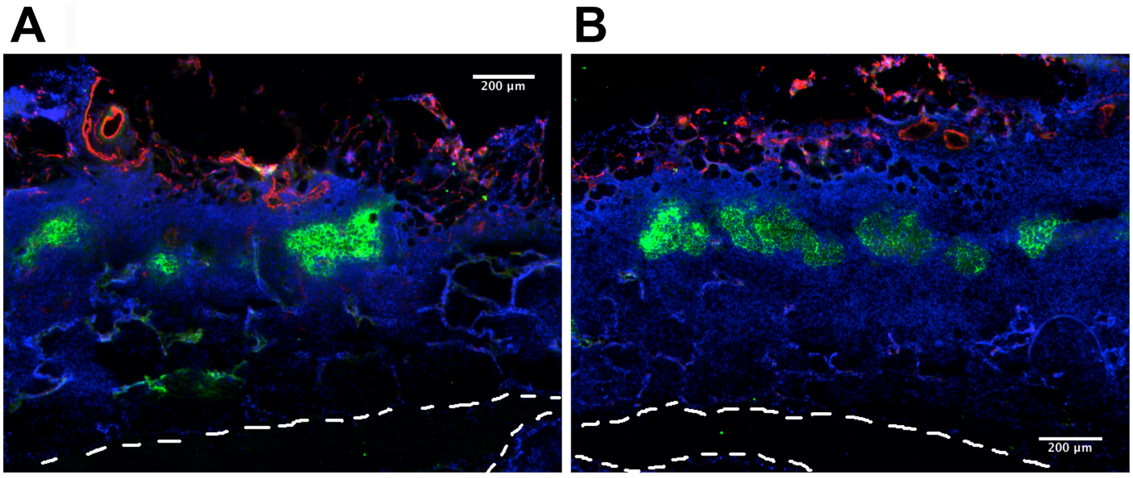

Figure 3.9: Immunofluorescence imaging of vasculature within syngeneic islet grafts. Immunofluorescent detection of CD31 (red), insulin (green), and nuclei (blue) within histological sections of islet grafts transplanted on scaffolds containing **(A)** 0 µg and **(B)** 2 µg of TGF-β1 at day seven after transplant. Boundary of scaffold center layer indicated by white dotted line. Images representative of sections from 4 mice per condition.

onto TGF-β1 or empty control scaffolds and transplanted into diabetic C57BL/6 mice. Two consecutive blood glucose measurements above 250 mg/dL indicated graft rejection. Kaplan-Meyer survival analysis of the two groups indicated that islets transplanted on TGF-β1 scaffolds functioned significantly longer than those transplanted on empty controls (19 versus 12 days, respectively) (**Fig 3.10**).

3.5.6 Leukocyte infiltration into allogeneic islet transplants

The mechanism for extended allogeneic islet survival with TGF-β1 scaffolds was investigated by performing flow cytometry on allografts 7 days after transplant, a time that immediately preceded graft failure for the control scaffolds (**Fig. 3.10**). In the presence of TGF-β1, grafts exhibited 30% less CD45 cells compared to grafts without TGF-β1 (**Fig. 3.11A**). Flow cytometry indicated that the numbers of F4/80 macrophages, CD11c DCs, and NK1.1 NK cells were significantly decreased by 70%, 45%, and 45%, respectively, in grafts delivering TGF-β1 relative to control (**Fig. 3.11B**). However, both CD4 and CD8 T cells numbers were unaffected by TGF-β1 delivery. As observed previously in the blank implantations, the overall number of Tregs within the scaffold decreased with TGF-β1 delivery (**Fig. 3.12**). Additionally, we did not see an increase in the expression level of Foxp3 in the CD4 Foxp3 population.

The spatial distributions of key leukocyte populations were investigated within allogeneic islet transplants through immunofluorescence imaging of histological sections for control scaffolds and scaffolds releasing TGF-β1. In the absence of TGF-β1, F4/80 (**Fig. 3.13A**) and NK1.1 (**Fig. 3.13B**) signal was detected throughout the scaffold and around the islets. In contrast, in the presence of TGF-β1, F4/80 (**Fig. 3.13D**) and NK1.1 (**Fig. 3.13E**) signals were primarily localized to the exterior surface of the scaffold. However, TGF-β1 delivery failed

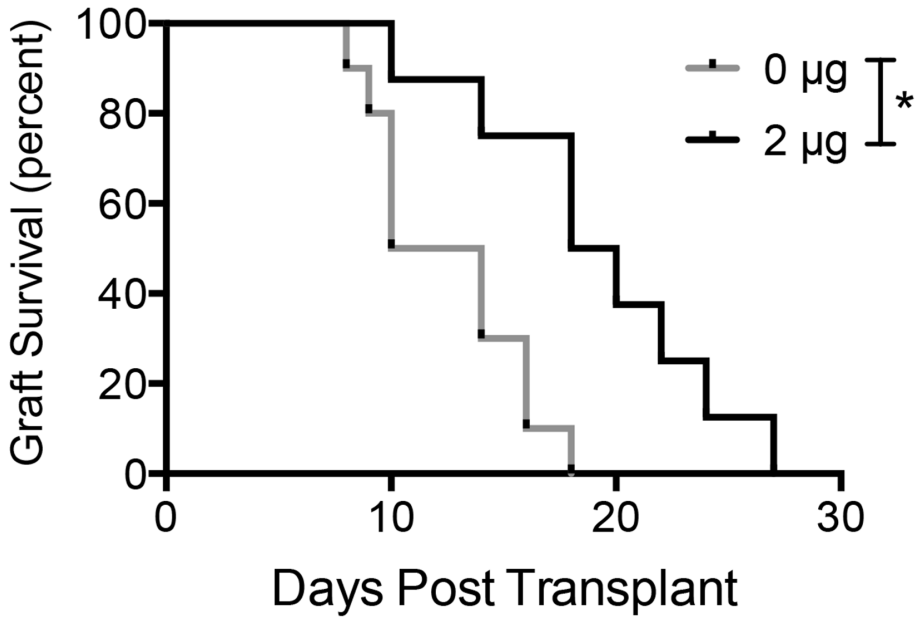

Figure 3.10: Allogeneic islet function on TGF-β1 scaffolds.
Kaplan-Meyer survival curve of islet grafts versus time. Two consecutive blood glucose measurements above 250 mg/dL indicated graft failure. Data is from 8-10 mice receiving one scaffold implant into the right epididymal fat pad. * indicates $P < 0.01$. Statistics determined by log-rank test.

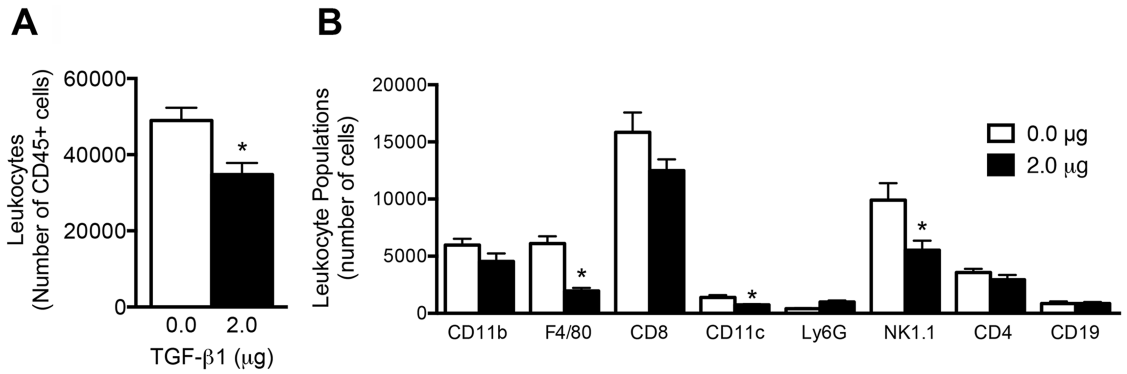

Figure 3.11: Leukocyte infiltration into allogeneic islet grafts implanted on TGF-β1 scaffolds.
(A) Total number of CD45 positive cells isolated from islet allografts seven days after transplant. **(B)** Leukocyte populations within the islet allograft seven days after transplant. Data is from 7-9 scaffolds from 7-9 mice per condition receiving one scaffold implant into the right epididymal fat pad. * indicates $P < 0.05$. Statistics determined by unpaired t-test.

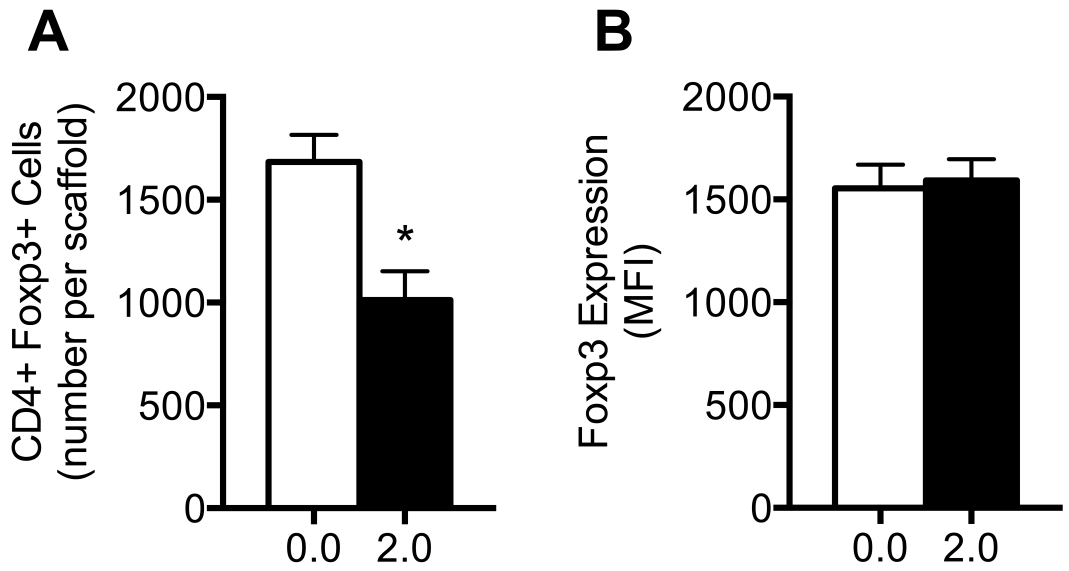

Figure 3.12: CD4 Foxp3 T cells in allogeneic islet grafts implanted on TGF-β1 scaffolds. **(A)** Number of CD4 Foxp3 T cells and **(B)** Foxp3 expression isolated from islet allografts on day 7 post-transplant. Allografts were transplanted on scaffolds containing 0 (white bars) or 2 μg (black bars) of TGF-β1. Data is from 7-9 scaffolds isolated from 7-9 mice receiving one scaffold implant into the right epididymal fat pad. * Indicates $P < 0.05$ versus 0 μg. Statistics determined by an unpaired t-test.

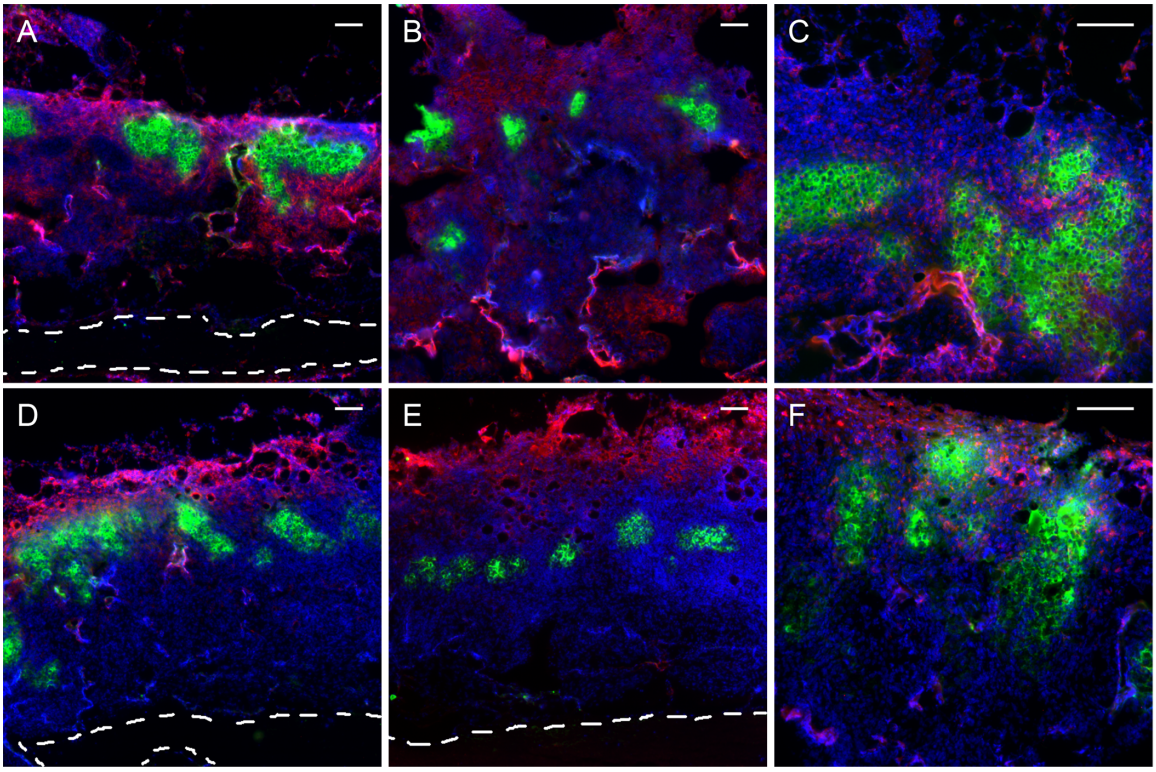

Figure 3.13: Immunofluorescence imaging of leukocyte infiltration into islet allografts Immunofluorescent detection of insulin (green), nuclei (blue) and **(A,D)** F4/80, **(B,E)** NK1.1, or **(C,F)** CD8 (red) within histological sections of islet grafts transplanted on scaffolds containing 0 µg (**A-C**) and 2 µg (**D-F**) of TGF-β1 at day seven. Scale bars located in the top right of each image indicate 100µm. Images representative of sections from 4 mice per condition.

to modulate CD8 T cell infiltration into the scaffold and amongst the islets (**Fig. 3.13C versus Fig. 3.13F**).

3.6 Discussion

This report investigated the delivery of TGF-β1 as a means to modulate inflammation following surgical implantation of PLG scaffolds. TGF-β1 delivery from scaffolds has been used to promote bone and cartilage regeneration *in vitro* [199-202] and *in vivo* [203-205]. Herein, we demonstrate that scaffold-based delivery of TGF-β1 suppresses the local inflammatory response. TGF-β1 was delivered from layered scaffolds, where protein was entrapped within a solid PLG-mannitol inner layer and surrounded by a porous PLG outer layer designed for cell seeding and tissue engraftment. This scaffold design maintained TGF-β1 bioactivity and provided short-term release that was able to decrease leukocyte infiltration and inflammatory cytokine production within the implant. Functionally, the TGF-β1 scaffolds promoted better blood glucose control of syngeneic islets immediately after engraftment, and delayed rejection of allogeneic islets in diabetic mice. These findings are significant because inflammation following cell transplantation contributes to transplanted cell dysfunction or death. TGF-β1 loaded scaffolds, in the absence of transplanted cells, reduced the infiltration of immune cells after implantation. All eight innate and adaptive immune cell types we identified by flow cytometry showed reduced populations within isolated TGF-β1 scaffolds.

The decrease in infiltration of these cell types was dose-dependent, with the most significant decreases observed with delivery of 2 μg TGF-β1. Though TGF-β1 can serve as either a pro- or anti-inflammatory stimulus depending on the local microenvironment [206], our model indicated TGF-β1 delivery has primarily immunosuppressive effects. While some reports have described

TGF-β1's role as a monocyte and macrophage chemoattractant [207, 208], we observed significant decreases in these same myeloid lineage cells that suggests protein delivery blocks migration of these populations or inhibits their local proliferation. TGF-β1 is also known to promote immature and tolerogenic phenotypes on APCs [194, 209-211], which is consistent with the observed decreases in MHCII expression on APC populations. Despite the *in vitro* release assay data indicating that the majority of TGF-β1 was released within the initial 3 days, leukocyte infiltration was reduced for up to 14 days (**Fig. 3.4)**. Thus, short-term release of TGF-β1 has relatively long-term effects on leukocyte infiltration into the implant site.

TGF-β1 loaded scaffolds reduced expression of inflammatory cytokines within the adipose tissue 3 days after implant, which likely contributed to the reduced leukocyte infiltration at 7 days. Macrophages are the most abundant leukocyte in the adipose tissue [212] and their adhesion to biomaterials can induce activation [213, 214]. Activated macrophages release inflammatory mediators (e.g., TNF-α and IL-1β) that induce adipocytes and stromal vascular cells to release chemokines that recruit additional leukocytes. Indeed, the most abundant factors measured in the surrounding tissue three days after scaffold implantation were the chemokines MCP-1 and CXCL10, which were each decreased by 50% with delivery of TGF-β1. MCP-1 is recognized by CCR2, which is highly expressed on inflammatory monocytes that differentiate into macrophages and DCs after they exit the blood stream [215]. CXCL10 is a chemokine produced by monocytes, macrophages, and fibroblasts upon inflammatory stimulation that attracts T cells, B cells, and NK cells, which contribute to the rejection of allogeneic tissue [216]. High levels of both MCP-1 and CXCL10 have been found to correlate with graft failure and rejection [216, 217]. We also noted reductions in IL-12, TNF-α, and CCL5, which are inflammatory cytokines that potentiate transplant rejection through activation of APCs,

polarization of naïve T cells towards a graft-destructive T_h1 effector phenotype, and migration of T cells to sites of inflammation [218, 219]. IL-10 expression was decreased in the presence of TGF-β1 delivery, likely due to the lower levels of inflammatory cytokines. Elevated expression of inflammatory cytokines has been reported to increase IL-10 expression by many leukocytes and other cell types and is thought to be a protective mechanism to control aberrant inflammation and damage to the host tissue [220].

Immediately after transplant, syngeneic islets on TGF-β1 releasing scaffolds were more effective at controlling blood glucose levels relative to islets on control scaffolds. While both scaffold types effectively reversed diabetes, we observed significantly lower blood glucose levels on days 4-6 post-surgery in mice receiving TGF-β1 releasing scaffolds. Since TGF-β1 has been reported to decrease islet insulin secretion [221] and vascularization of the scaffold at day 7 was not observed (**Fig. 3.6**), which could be indicative of TGF-β1 induced angiogenesis [222], we suggest that improved islet functionality is due to a more immunologically permissive microenvironment created by TGF-β1 delivery. Syngeneic or autologous cells can be damaged by nonspecific innate immunity [223]. Isolation and culture-related cell injury, ischemic reperfusion, or a foreign body response can contribute to a local inflammatory environment that damages the auto- or isograft during the initial engraftment period [224, 225]. Reduction of inflammatory cells and cytokines with TGF-β1 delivery within the peri-islet environment may prevent host-induced damage and enable more efficient engraftment that leads to superior function at early times. Importantly, maintenance of long-term euglycemia by syngeneic islets, as evidenced by daily blood glucose measurements and a glucose tolerance test conducted at day 70, confirmed that elevated local concentration of TGF-β1 at the time of implant did not deter

islet graft viability or functionality, which is a frequent concern when testing immunosuppressive agents.

TGF-β1 delivery from the scaffolds delayed the rejection of transplanted allogeneic islets. Transplantation of allogeneic cells enhanced the severity of the local immune response, which was observed by the 67% increase in leukocyte infiltration compared to scaffold implantation alone **(Fig 3.3A versus Fig 3.11A)**. The overall effect of TGF-β1 release on leukocyte infiltration was dampened in the allotransplants, where a 30% reduction in leukocytes was observed compared to 60% in scaffolds implanted without cells. There was a profound shift in the composition of infiltrating leukocytes towards lymphocytes, in particular CD8 T cells and NK cells, underscoring their role in allograft rejection. Significant decreases in the peri-islet innate immune cell populations (F4/80 macrophages, NK1.1 NK cells, CD11c DCs) were observed with TGF-β1 loaded scaffolds at day 7, which indicate innate leukocyte populations were still affected by protein release. These cell populations play significant roles in allograft rejection, thus delaying their arrival likely contributed to the delay in rejection. Macrophages act as both recruiters of inflammatory cell types and effectors of T cell-mediated graft destruction through the release of cytokines and reactive oxidative species [224, 226, 227]. DCs are the primary APC stimulating T cell proliferation and are a common therapeutic target to prevent allograft rejection [228]. Recent work has shown monocytes specifically recognize allogeneic tissue and preferentially differentiate into activated DCs that upregulate inflammatory cytokines compared to syngeneic tissue [229]. Finally, while NK cells may be required for induction of allograft tolerance through inhibition of T cell proliferation and destruction of graft-derived APCs [230], they also demonstrate the ability to eliminate allogeneic cells lacking self MHC-I molecules [231].

While TGF-β1 delivery delayed innate cell infiltration into allogeneic islet-containing scaffolds, CD4 or CD8 T cell populations did not show significant differences at day 7. Since T cells are the primary mediators of allograft rejection [81], we suggest that the 1-week extension in allograft survival seen with TGF-β1 delivery may be due to a delay in the full activation of infiltrating T cells. TGF-β1 can have suppressive effects on the priming of T cells [195, 196], and its release from the scaffold may provide a short-term stimulus that influences T cell activation. Additionally, considering the ability of CD4 T cells to express cytokines that can polarize macrophages toward inflammatory phenotypes [232], the decrease in overall macrophage population caused by the TGF-β1 scaffolds may have dampened a key effector mechanism of the CD4 T-cell response. While TGF-β1 is known to induce Foxp3 expression in CD4 T cells and may assist in the generation of peripheral Treg populations [196], we did not observe an increase in the induction of Tregs in the scaffolds in either the blank or allogeneic transplantation models (**Figs. 3.5A and 3.12)**). There was also no detectable increase in Foxp3 expression in Tregs present in scaffolds transplanted with islets. Thus, it is unlikely that improvements in graft function were due to a TGF-β1 induced enhancement of local Treg populations. Thus, we believe the delay in rejection of allogeneic islets transplanted on TGF-β1 loaded scaffolds may have been caused by the decreased initial nonspecific damage from innate immune cell populations and a delay in the fully activated T cell response in the graft environment. Even though transient TGF-β1 release did not result in long-term allograft survival, we hypothesize that the profound reduction in inflammation demonstrated in this study could be used in combination with a system for long-term delivery or tolerance induction, such as nanoparticle alloantigen delivery to non-activated APCs [177].

3.7 Conclusions

Scaffolds designed to release recombinant TGF-β1 elicited less inflammation after implant by decreasing local cytokine concentrations and leukocyte infiltration. This immunomodulated scaffold transplant environment supported better islet function and longer islet survival in syngeneic and allogeneic models of islet transplant. Analysis of leukocyte infiltration into allogeneic grafts revealed significant decreases in innate immune cell lineages, suggesting that extended allograft survival on TGF-β1 scaffolds could be linked to reductions in nonspecific tissue damage and a delay in the full T cell response. This approach for locally controlling inflammation after biomaterial implantation may enhance systemic strategies for tolerance induction in order to promote engraftment and long-term function of allogeneic transplants.

3.8 Acknowledgements

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Text and figures from this chapter were taken from Liu JM, Zhang J, Zhang X, Hlavaty KA, Ricci CF, Leonard JN, Shea LD, Gower RM. Transforming growth factor-beta 1 delivery from microporous scaffolds decreases inflammation post-implant and enhances function of transplanted islets. Biomaterials 2016; 80: 11-19.

Chapter 4:

Localized IL-33 scaffold delivery into adipose tissue expands local regulatory immune cell populations to extend allogeneic cell transplant survival

4.1 Context

The previous chapter successfully demonstrated that the biomaterial scaffold itself has utility beyond simply encouraging islet engraftment and could be used to release soluble factors to delay graft rejection by controlling leukocyte infiltration into the physical environment of transplanted cells without the help of a systemic immune suppressant. In looking closer at the mechanism of action, it was evident that TGF-β1 appeared to be working primarily by general depletion of leukocytes instead of expanding immunosuppressive lineages. In contrast to our initial hypothesis, TGF-β1 release did not expand local populations of Tregs, suggesting that there might be limited long-term effects once TGF-β1 was completely leached out of the scaffold. In considering why no increase was detected, we began to consider what cell populations existed within the scaffold that would be available to be polarized by the released factors. We thus aimed to identify a novel factor that could specifically target resident immune cell populations and polarize them towards graft-accepting phenotypes.

While the surgical procedure of implanting a biomaterial scaffold into adipose tissue is likely to induce some level of inflammation, it stands to reason that a portion of immune cells present within the adipose/scaffold environment might belong to resident populations from the original adipose tissue environment. Like all organs in the body, adipose tissue depots have immune cells responsible for fighting infectious agents in addition to maintaining organ-specific homeostasis. In healthy individuals, the adipose immune cell compartment is highly polarized towards an anti-inflammatory state. In this context, IL-33 has been shown to be a crucial factor for the maintenance of these populations, particularly amongst visceral adipose tissue (VAT) Foxp3+ Tregs and adipose-tissue macrophages. Given these general immune cell lineages are often identified as major contributors to the control of graft survival, we sought to determine whether

IL-33 responsive immune cell populations present in the scaffold could be further amplified to enforce an anti-inflammatory and ultimately tolerogenic local environment for transplanted islets. IL-33 also made for a compelling target due to its recent usage as a monotherapy to induce transplant tolerance. Systemically administered IL-33 has shown success in extending survival in allogeneic cardiac and skin transplant models as well as preventing graft-versus-host disease but has never been characterized for compatibility in an islet transplant model. Delivering IL-33 locally from the scaffold also offered the opportunity to test how concentrating the effects of the cytokine to an area with large populations of putatively responsive cell types might affect putative tolerogenic effects when compared to a more diffuse administration method.

I designed and completed the majority of the experiments and analyzed the data described in this chapter. I received experimental assistance from a M.S. student Shelby Joe at Michigan. For the graft survival studies, I again received help from Dr. Xiaomin Zhang, who performed the surgeries and monitored the animals post-operation at Northwestern University.

4.2 Abstract

The development of novel immunomodulatory strategies that might decrease the need for systemic immune suppression would greatly enable the utility of cell based therapies. Cell transplantation on biomaterial scaffolds offers a unique opportunity to engineer a site to locally polarize immunogenic antigen generation. IL-33 is a novel cytokine that has demonstrated beneficial immunomodulatory effects in certain transplant models and plays a role in maintaining anti-inflammatory immune phenotypes in adipose tissue. Herein, we investigated the localized delivery of IL-33 to determine its feasibility for use as an immunomodulatory agent. IL-33 delivery from poly(lactide-co-glycolide) (PLG) scaffolds implanted into the epididymal fat specifically expanded the Foxp3+ population of CD4+ T cells in both blank scaffold implants and scaffolds seeded with allogeneic islets. In allogeneic islet transplantation, we found IL-33 delivery results in a local upregulation of graft-protective T cells where 80% of the local CD4+ population is Foxp3+ and overall numbers of graft destructive CD8+ T cells are decreased, resulting in a prolonged graft survival. Interestingly, local IL-33 also delayed islet engraftment by primarily inducing a local upregulation of Th2 cytokines, including IL-4 and IL-5, leading to expansion of ST2+ Type 2 innate lymphoid cells (ILC2s) and Siglec F+ eosinophils. These results suggest that local IL-33 delivery from biomaterial scaffolds can be used to expand Tregs enriched in adipose tissue and reduce graft-destructive T cell populations but may also promote innate immune cell populations that can delay cell engraftment.

4.3 Introduction

The vast majority of human allogeneic islet transplantations for the treatment of Type 1 Diabetes Mellitus (T1DM) have been performed by infusion of donor islets into the hepatic portal vein. While these transplants have shown promise in restoring insulin independence, it is widely acknowledged that the hepatic vasculature is not an ideal transplant site due to initial loss of graft mass caused by the instant blood mediated inflammatory reaction (IBMIR), suboptimal engraftment related to poor local oxygenation and tissue revascularization, and the difficulties controlling the immune response to donor cells with systemic immunosuppressants that may also have islet toxicity [2, 24]. In searching for alternative transplant sites, the omentum, a major adipose tissue depot, has been identified as a promising target due to its large transplant area, similarity in portal drainage to the native pancreas environment, and ease of surgical accessibility [32, 137, 233]. Omental transplants can be modeled in mice using the perigonadal fat pad (epididymal fat pad in male mice) [33]. As a non-immunoprivileged site, the usage of adipose tissue as a novel transplant site requires an immune intervention strategy to protect the graft from the host-immune system. While CD4+ and CD8+ T cells are a primary mediator of transplant rejection, innate immunity can also contribute significantly to graft survival [234]. Adipose tissue constitutes a unique immune microenvironment that is maintained in homeostasis between an inflammatory and anti-inflammatory state by many of the same effector cells that mediate transplant acceptance and rejection [57].

Current clinical trials for human omental islet transplantation use biologic scaffolds to support islet transplantation [32]. While the aforementioned studies used scaffolds produced from autologous plasma and thrombin, a wide variety of materials including synthetic polymers like PLG can be used to create scaffolds with tunable mechanical and structural properties [1].

Scaffolds can facilitate integration into native tissue and represent a customizable environment that can be designed to incorporate additional intervention strategies to modulate the local environment, such as co-transplantation of synergistic cell types, surface coupling of modifying extracellular matrix proteins and other ligands, and the incorporation of releasable factors [35, 124, 133, 170].

Biomaterial scaffolds have been developed to provide a sustained, localized delivery of proteins, and the local delivery of cytokines has been applied to modulate infiltrating immune cell types that ultimately mediate graft failure and rejection at a primary site of interaction [235]. We have reported PLG scaffolds to support islet transplantation into the epididymal fat pad that also release TGF-β1 extend islet allograft survival through local depletion of leukocytes [235]. In choosing adipose tissue as a site for transplantation, identifying site-specific factors could lead to novel therapeutic targets. IL-33 has become a topic of interest in adipose tissue immunology, where it has been shown to interact with a number of different locally enriched cell types expressing the IL-33 receptor ST2, including CD4+ Th2 and regulatory T cells (Tregs), macrophages, and innate lymphoid group 2 cells (ILC2s) [236-239]. IL-33 is an IL-1 family cytokine that been shown to have both pro- and anti-inflammatory properties depending on the immunological context [240, 241]. In the realm of allogeneic transplant studies, IL-33 has been shown to have graft-protective effect in murine cardiac and skin transplant models [242-244].

In this article, we sought to use our PLG scaffold platform to release IL-33 to target the polarization of local immunoregulatory cell populations. Initial studies investigate the impact of IL-33 on the local tissue, with a focus on the expansion of ST2+ Tregs. We characterize the effects of local release of IL-33 on immune cells localized within an adipose tissue-biomaterial scaffold environment and explore the ramifications for future use of IL-33 as a tolerogenic factor

for allogeneic islet transplant. The ability to provide localized immune interventions may lessen the need for systemic immune suppression and its associated complications and side effects.

4.4 Materials and Methods

4.4.1 PLG scaffold production

2% or 6% W/V 75:25 poly-lactide-co-glycolide (Lakeshore Biomaterials) was dissolved in dichloromethane (DCM) and homogenized in 1% poly-vinyl alcohol using a Polytron 3100 homogenizer to create PLG microspheres. Particles were mixed for 3 hours to evaporate the organic solvent then washed with water to remove excess PVA. After washing, the microspheres were frozen in liquid nitrogen and lyophilized.

Control and IL-33 loaded layered scaffolds were created as described previously with modifications. Control inner layers were created by mixing 2 mg 2% PLG with 1mg BSA and 1 mg mannitol in a total volume of 100 μL water. The mixture was frozen in liquid nitrogen, lyophilized, and pressed into a disc (3mm diameter, 100 μm thick) using a hand press. For IL-33 scaffolds, recombinant murine IL-33 (Biolegend) was added to the above mixture. Full scaffolds were formed by sandwiching the pressed inner layer between two outer layers comprised of 1.25 mg 6% PLG and 37.5 mg NaCL (weights +/- 5%). Scaffolds were compressed using a 5mm dye and Carver Press. Scaffolds were gas foamed overnight under 800 psi CO_2 and stored at -20°C.

4.4.2 Animals

Animals were obtained from Jackson Labs or Charles River Laboratories. 8-12 week old C57BL/6 males were used for scaffold recipients. 8-12 week old Balb/c or C57BL/6 males were

used for allogeneic and syngeneic islet isolations respectively. For syngeneic and allogeneic islet transplant studies, diabetes was induced by a single i.p. injection of 180 mg/kg body weight streptozotocin delivered after a 4-6 hour fast (Sigma Aldrich). Mice were considered diabetic and eligible for transplant after two consecutive non-fasting blood glucose measurements over 350mg/dL. All procedures were approved by the Northwestern University Animal Care and Use Committee (ACUC) or University of Michigan Unit for Laboratory Animal Medicine (ULAM).

4.4.3 IL-33 in vitro bioactivity assay

Naïve T cells were isolated from the spleens of 8-12 week old C57 males using the Miltenyi Biotec Naïve T cell isolation kit. Spleens were crushed between frosted glass slides and filtered through 70 μm cell strainers. Red blood cells were lysed using ACK buffer (Thermo Fisher Scientific). The cell pellet was incubated first with a biotinylated antibody cocktail containing antibodies against CD8a, CD11b, CD11c, CD19, CD25, CD45R (B220), CD49b, CD115, MHCII, TER119, and TCRγ/δ, then anti-biotin magnetic beads. The cells were then passed through a MACS LS column and untouched CD4+ naïve T cells were collected and resuspended in RPMI 1640 supplemented with 10% FBS and 1x Pen-Strep. Naive cells were incubated for 1 hour at 37° C before being transferred to wells surface coated with 3mg/mL anti-mouse CD3ε. $5*10^5$ CD4+ T cells were added to each well, supplemented with 2ug/mL anti-mouse CD28. Blank or IL-33 scaffolds were added to wells and cells were incubated 72 hours at 37 degrees. Supernatants were collected, spun down to remove cells and debris, and frozen at -80°C. Samples were sent to the University of Michigan ELISA core for analysis of IL-13.

4.4.4 Scaffold implants

Prior to implantation, scaffolds were leached in 10mL of milliQ water per scaffold for 1 hour. Water was changed after 30 minutes. Scaffolds were disinfected in 70% ethanol for 1 minute, then washed twice in media supplemented with 10% FBS. Mice were anesthetized using isoflurane (2% flow rate). The abdomen of each mouse was shaved and prepared in a sterile fashion with 3 successive administrations of Betadine and ethanol. The intraperitoneal space was exposed by a lower abdominal midline excision, the epididymal fat pad was exposed, and scaffolds were wrapped securely and returned to the cavity. The abdominal wall was closed with a running stitch using a 5-0 suture and the skin was secured with wound clips. Mice received 1 post-operative subcutaneous injection of Carprofen (5mg/kg) 24 after implant and surgery sites were monitored until termination of the study or for 10 days until clips were removed.

4.4.5 Islet isolation and transplantation

Islets were isolated from mice as described previously. Briefly, pancreata from euthanized mice were inflated with 0.51 mg/mL collagenase XI (Sigma) via bile duct cannulation and digested for 15 minutes in a 37˚C water bath with periodic agitation. After filtration through a mesh screen, islets were separated from acinar tissue using a density gradient (Biochrom or Histopaque). Islets were picked from the gradient interface and washed thoroughly before being transplanted immediately. Islets were counted and seeded onto scaffolds using a customized glass pipette tool.

4.4.6 Flow cytometry

Mice were euthanized by cervical dislocation under isoflurane-induced anesthesia. Tissue

was dissociated into a single cell suspension. Tissues were harvested immediately and stored in HBSS on ice. Spleen samples were mechanically disrupted by agitation between frosted glass slides. The resulting tissue homogenate was filtered through a 70 μm cell strainer and washed with MACS (PBS supplemented with 2mM EDTA and 0.5% BSA). For scaffold implants and adipose tissue, enzymatic digestion was used to create a single cell suspension. Tissues were weighed and placed into petri dish with 0.5mL 10mg/mL Collagenase Type II (Sigma Aldrich) 2.5 mL of digestion buffer (HBSS with Calcium Chloride and Magnesium Chloride [Thermo Fisher] supplemented with 0.5% Bovine Serum Albumin). Tissue was finely shredded and transferred to a 15mL conical tube. Dish was washed with 2 mL digestion buffer and added to tissue homogenate to bring final concentration of collagenase to 1 mg/mL. Tissue was incubated in a 37° C water bath for 30 minutes with gentle shaking every 5 minutes. 100 μL of 0.5 M EDTA was added to each tube to a final concentration of 10 mM and incubated for an additional 5 minutes at 37° C. Tissue homogenate was strained through a 70 μm filter and washed with MACS. The resulting cell pellets were then incubated with 1 mL ACK buffer on ice to lyse the red blood cells and washed with MACS. In preparation for staining with Live/Dead fixable stain, cells were washed with PBS.

Live Dead Fixable Violet stain (Thermo Fisher Scientific) was used for removal of dead cells from analysis. The Foxp3/Transcription Factor Staining Buffer (Ebioscience) was used for cells requiring intracellular staining. The following conjugated antibodies (clone) were purchased for analysis from Biolegend, Ebioscience, or Biorad: CD3ε (145-2c11), CD4 (RM4-5), CD5 (53-7.3), CD8a (53-6.7), CD11b (M1/70) CD11c (N418), CD25 (PC61), CD45 (30-F11), CD45R/B220R (RA3-6B2), CD127 (A7R34), CD301 (ER-MP23), F4/80 (BM8), FcεRIα (MAR 1), Foxp3 (FJK-16s), Ly6C (HK1.4), Ly6G (1A8), NK1.1 (PK136), Siglec F (1RNM44N), ST2

(RMST2-2). Isotype antibodies were used to establish gating.

Samples were analyzed on the DAKO Cyan 5 ADP. In order to derive absolute cell numbers in each scaffold, 50 μL of Absolute Countbright Beads (Thermo Fisher Scientific) were added to each sample and gated in each sample to use as an internal control. Approximately 10,000 beads were counted per sample and overall cell numbers were adjusted based on the expected numbers of beads specified by each lot.

4.4.7 Gene expression

RNA isolation was prepared from scaffold implants using the Qiagen RNeasy Kit with modifications for fatty tissue. Scaffold implants were extracted from mice and immediately snap frozen in isopentane on dry ice for storage at -80°C. Frozen grafts were homogenized in 1mL Trizol (Ambion) using a rotor-stator homogenizer at 10,000 rpm. Homogenates were incubated for at least 5 minutes at room temperature then centrifuged for 10 minutes 12,000 G at room temperature. Supernatant between a top layer of adipocytes and pellet of insoluble material was transferred to a new tube. 200 μL of chloroform was added to the Trizol supernatant and the resulting mixture was shaken for 15 seconds, incubated at room temperature for 2-3 minutes, then centrifuged for 15 minutes 12000G 4°C. The upper clear organic phase was then transferred to a new tube, mixed with an equal volume of 70% ethanol, and pipetted onto an RNeasy mini spin column. After washing the column, an on-column DNase digest was performed by applying 80uL Rnase free DNAse (Qiagen) to the filter and incubating for 15 minutes at room temperature. After washing with ethanol, RNA was eluted using 50 uL of nuclease free water. RNA concentration and purity was assessed by Nanodrop 2000.

Two-step RT-PCR was used to assess changes in gene expression. RNA was converted to cDNA using the iScript cDNA conversion kit. Qiagen Sybr Green PCR master mix was used for PCR. Primers were designed using Mouse Primer Depot [245]. Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method. *Hrpt1* was used as the housekeeping gene for normalization. Primers used for analysis are listed in Table 4.1.

4.4.8 ELISA and Luminex analysis

Cell culture and scaffold wash supernatants were measured for cytokine concentration using R&D ELISA Duoset kits. Supernatants were collected and stored at -80°C before analysis. Tissue homogenate protein levels were assayed using the Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel – Premixed 32 Plex (Millipore). To prepare samples for analysis, tissues were homogenized in ice-cold PBS supplemented with 1x protease inhibitor cocktail (Pierce) using a rotor-stator homogenizer. Homogenates were spun down at 10,000g for 15 minutes at 4°C and the clarified supernatant was separated from the upper layer of fat and insoluble pellet. Protein concentration was determined by BCA (Pierce). All samples were diluted to 1mg/mL before analysis.

4.4.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism (La Jolla, Ca). Graphs depict mean and standard error of the mean (SEM). Unpaired student t-test was used to calculate statistical significance unless otherwise indicated.

Il10 For	ATCGATTTCTCCCCTGTGAA
Il10 Rev	TGTCAAATTCATTCATGGCCT
Foxp3 For	TGGCAGAGAGGTATTGAGGG
Foxp3 Rev	CTCGTCTGAAGGCAGAGTCA
Il2 For	AACTCCCCAGGATGCTCAC
Il2 Rev	CGCAGAGGTCCAAGTTCATC
Ifn-γ For	ACAGCAAGGCGAAAAAGGAT
Ifn-γ Rev	TGAGCTCATTGAATGCTTGG
Tnf-α For	CCACCACGCTCTTCTGTCTAC
Tnf-α Rev	AGGGTCTGGGCCATAGAACT
St2 For	CGTGTCCAACAATTGACCTG
St2 Rev	CAAGTAGGACCTGTGTGCCC
Ccl2 For	CCTGCTGTTCACAGTTGCC
Ccl2 Rev	ATTGGGATCATCTTGCTGGT
Arg1 For	AGAGATTATCGGAGCGCCTT
Arg1 Rev	TTTTTCCAGCAGACCAGCTT
Nos2 For	TGAAGAAAACCCCTTGTGCT
Nos2 Rev	TTCTGTGCTGTCCCAGTGAG
Il4 For	TGAACGAGGTCACAGGAGAA
Il4 Rev	CGAGCTCACTCTCTGTGGTG
Il13 For	TGTGTCTCTCCCTCTGACCC
Il13 Rev	CACACTCCATACCATGCTGC
Il6 For	TGATGCACTTGCAGAAAACA
Il6 Rev	ACCAGAGGAAATTTTCAATAGGC
Hprt1 For	TCCTCCTCAGACCGCTTTT
Hprt1 Rev	CATAACCTGGTTCATCATCGC

Table 4.1 qRT-PCR primers

4.5 Results

4.5.1 IL-33 incorporated into PLG scaffold design retains in vitro bioactivity

We first tested the inclusion of carrier protein into the inner layer of our protein-incorporating scaffold to increase protein loading and recovery. *In vitro* protein release from scaffolds loaded with 2 μg of IL-33 showed an average total recovery of 1000 ng of IL-33 with BSA incorporation compared to 200 ng using the previous BSA-free design, validating our inclusion of a carrier protein (**Fig. 4.1A**). BSA incorporation also decreased the amount of IL-33 lost during the initial leaching steps. The scaffold shows a bolus release of protein where the majority of protein is recovered within a day of incubation. *In vitro* bioactivity of IL-33 scaffolds was determined by the induction of the IL-13 from activated naïve T cells [246]. Incubation with IL-33 scaffolds significantly increased production of IL-13 from naive T cells undergoing anti-CD3/28 activation (**Fig 4.1B**).

4.5.2 Implantation of PLG scaffold alters local composition of innate immune cells

We initially investigated the impact of implantation of control scaffolds on the local immune cells in adipose tissue, which has a unique microenvironment that controls metabolic processes. In normal adipose tissue and day 7 post-implant scaffolds, close to 70% of recovered immune cells express CD11b+ and belong to the myeloid lineage (**Fig. 4.2A**). The distinct population of F4/80 Hi CD11b+ macrophages previously identified as a resident population in normal adipose tissue is reduced in scaffolds recovered 7 days post-implant [59]. In characterizing resident macrophage phenotype, 70% of tissue resident F4/80 Hi CD11b+ macrophages in adipose tissue had a surface expression of CD11c- CD301+ associated with the anti-inflammatory alternatively activated phenotype compared to 20% expressing the CD11c+ CD301- inflammatory

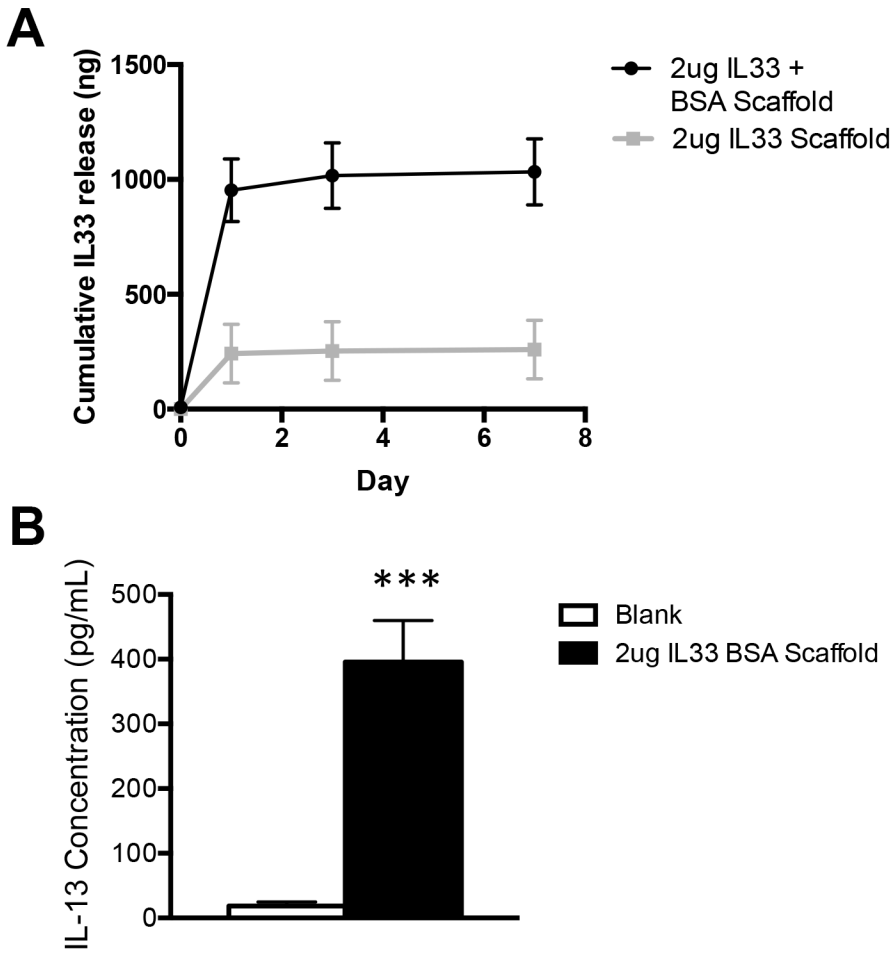

Figure 4.1 IL-33 scaffold characterization
(A) *In vitro* release profile collected at Day 1, 3, and 7 from layered scaffolds loaded with 2 μg IL33 with or without 1mg of BSA incorporated as a carrier protein **(B)** Supernatant concentration of IL-13 detected by ELISA from naïve T cells activated by plate bound anti-CD3. N = 4 scaffolds per condition ***p < .001. Statistics determined by unpaired t test.

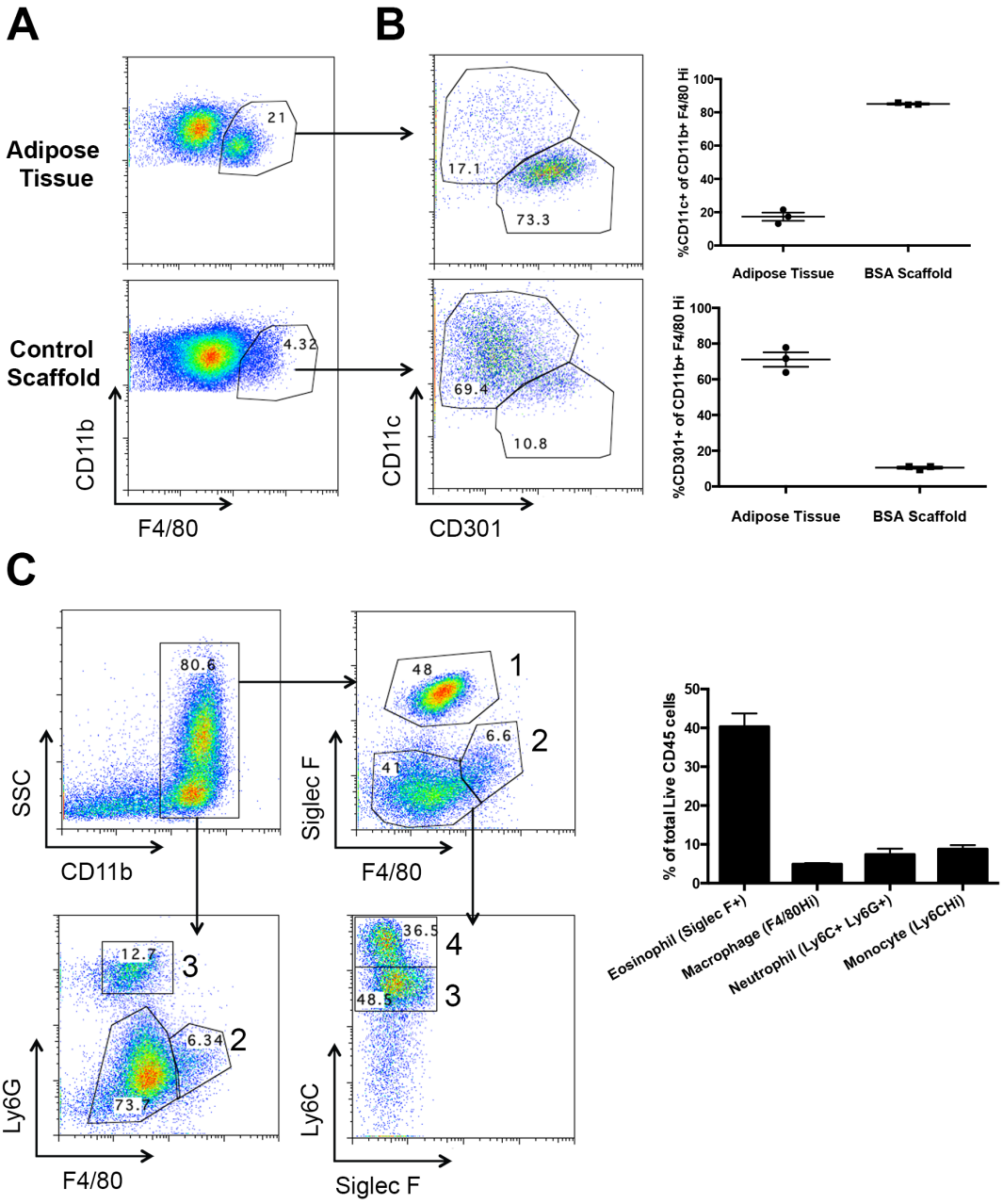

Figure 4.2 Innate immune cell environment of PLG scaffold implants.
(A) Representative flow cytometry plots comparing F4/80 expression on live CD45+ CD11b+ cells between unimplanted adipose tissue and PLG scaffolds seven days after implant **(B)** Representative flow plots and quantification of CD11c+ and CD301+ subsets of F4/80 hi cells. **(C)** Gating scheme and quantification of subsets of CD11b+ cells isolated from PLG scaffolds including eosinophils (CD11b+ F4/80 Int Siglec F+), macrophages (Cd11b+ F4/80 hi Siglec F- Ly6G-), neutrophils (CD11b+ F4/80 intermediate Ly6G+ Ly6C Int), and monocytes (Cd11b+ F4/80 Int Ly6C Hi). Subsets are quantified as percentage of total live CD45+ cells isolated from the scaffold. N = 4 scaffolds per condition. Graphs depict mean ± SEM.

macrophage phenotype (**Fig. 4.2B**). In the scaffold, this ratio was reversed with roughly 70% expressing the inflammatory phenotype compared to 10% expressing the alternatively activated phenotype. Close to 40% of the identified CD45+ cells expressed a SSC Hi CD11b+ F4/80 Int Ly6G- Siglec F+ phenotype consistent with eosinophils (**Fig. 4.2C**). Other CD11b+ myeloid cell subtypes also residing within the scaffold include the aforementioned tissue resident macrophages (F4/80 Hi Ly6G- Siglec F-), neutrophils (F4/80- Ly6C+ Ly6G+ Siglec F-), and monocytes (F4/80- Ly6G- Siglec F- Ly6C Hi), though all populations were significantly lower than the aforementioned eosinophils.

4.5.3 Scaffold-mediated IL-33 delivery expands local CD4+ Foxp3+ Tregs

Localized delivery of IL-33 was subsequently investigated for its ability to expand Foxp3+ Tregs, in particular the ST2+ subpopulation naturally enriched in adipose tissue.

For *in vivo* studies, we increased the loading amount in the scaffold to 5 μg IL-33 to match the cumulative amounts of protein via i.p. injection administered in previously published allogeneic transplant models [242, 243] IL-33 release within the scaffold environment increased proportion of Foxp3+ cells within the total CD4+ population from 25% to 45% (**Fig. 4.3A**). Total populations of both ST2+ and ST2- Foxp3+ CD4+ cells both significantly increased in the IL-33 scaffold. Interestingly, the proportion of ST2+ Foxp3+ Tregs did not increase significantly relative to total Foxp3+ Tregs, suggesting expansion of Tregs may be occurring by a direct engagement between IL-33 and ST2+ Tregs in addition to an indirect IL-33 induced mechanism (**Fig. 4.3B**). We also analyzed splenic populations of Tregs to identify whether the effects of local IL-33 delivery could be detected systemically. We did not detect a significant change in the percentage of Foxp3+ Tregs present with the spleen nor an increase in ST2+ expression

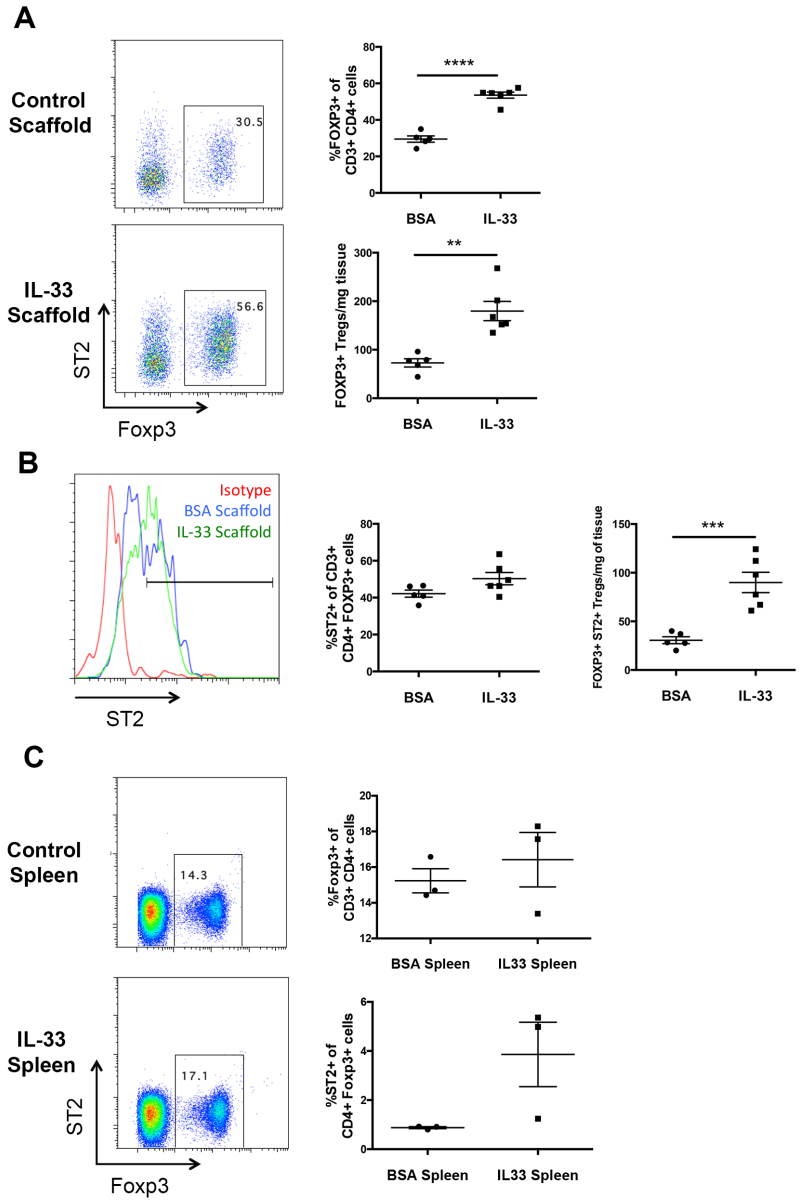

Figure 4.3 IL-33 expands local CD4+ Foxp3+ Tregs in blank scaffold implant.
(A) Representative flow cytometry plots and quantification of percentages and overall numbers of live CD3+ CD4+ Foxp3+ Tregs isolated from control and IL IL33 scaffolds seven days after implant. Frequency of ST2+ Foxp3+ CD4+ T cells 7 days after implant as quantified by flow cytometry **(B)** Representative flow cytometry plot and quantification of percentages and overall numbers of ST2+ Foxp3+ CD4+ T cells 7 days after implant **(C)** Representative flow cytometry plots and quantification of percentages of CD4+ Foxp3+ cells and ST2+ Tregs isolated from spleens of animals receiving control or IL-33 scaffolds. Overall numbers are normalized to weight of tissue immediately after isolation. Graphs depict mean ± SEM. N = 5-6 scaffolds per condition. $^{**}p < 0.01$ $^{***}p < 0.001$ $^{****}p < 0.0001$ Statistics determined by unpaired t-test.

amongst Tregs, indicating the effects of IL-33 were concentrated within the localized scaffold environment (**Fig. 4.3C**).

4.5.4 IL-33 decreases CD8+ cells and expands ST2+ Tregs with allogeneic islets

We next focused on the impact of IL-33 on the local adaptive immune response by tracking CD4+ and CD8+ populations in scaffolds transplanted with allogeneic islets.

Release of local IL-33 led to a significant decrease in the number of CD3+ CD8+ cells recovered from the allograft environment (**Fig. 4.4A**). Moreover, the ratio of CD4 to CD8 T cells increased from an average of 0.75 to 4 ($p<0.01$) (**Fig. 4.4B**). Taken together, localized IL-33 release decreases the CD8 T cell response to allogeneic cells within the scaffold at day 7. In the allograft model, the percentage of Foxp3+ cells in the total CD4+ population increased from 40% to 75% with the addition of IL-33 (**Fig. 4.4C**). Unlike the blank implantation model, IL-33 specifically expanded the ST2+ population of CD4+ Foxp3+ Tregs, increasing the percentage of ST2+ Tregs from 35% to 60% (**Fig. 4.4D**). A significant increase in ST2 expression was also detected in the ST2+ Treg population.

4.5.5 IL-33 delivery extends allograft survival

We then analyzed the effect of IL-33 delivery on long-term islet allograft survival. 250 islets isolated from Balb/c donors were seeded onto control or IL-33 scaffolds and transplanted into diabetic C57BL/6 recipients. Blood glucose was continuously monitored. We observed that islets transplanted on control scaffolds engraft rapidly and have similar survival times compared to the control scaffolds used in Chapter 3, demonstrating incorporation of BSA into the inner layer does not produce a local immune response that compromises local function of islets.

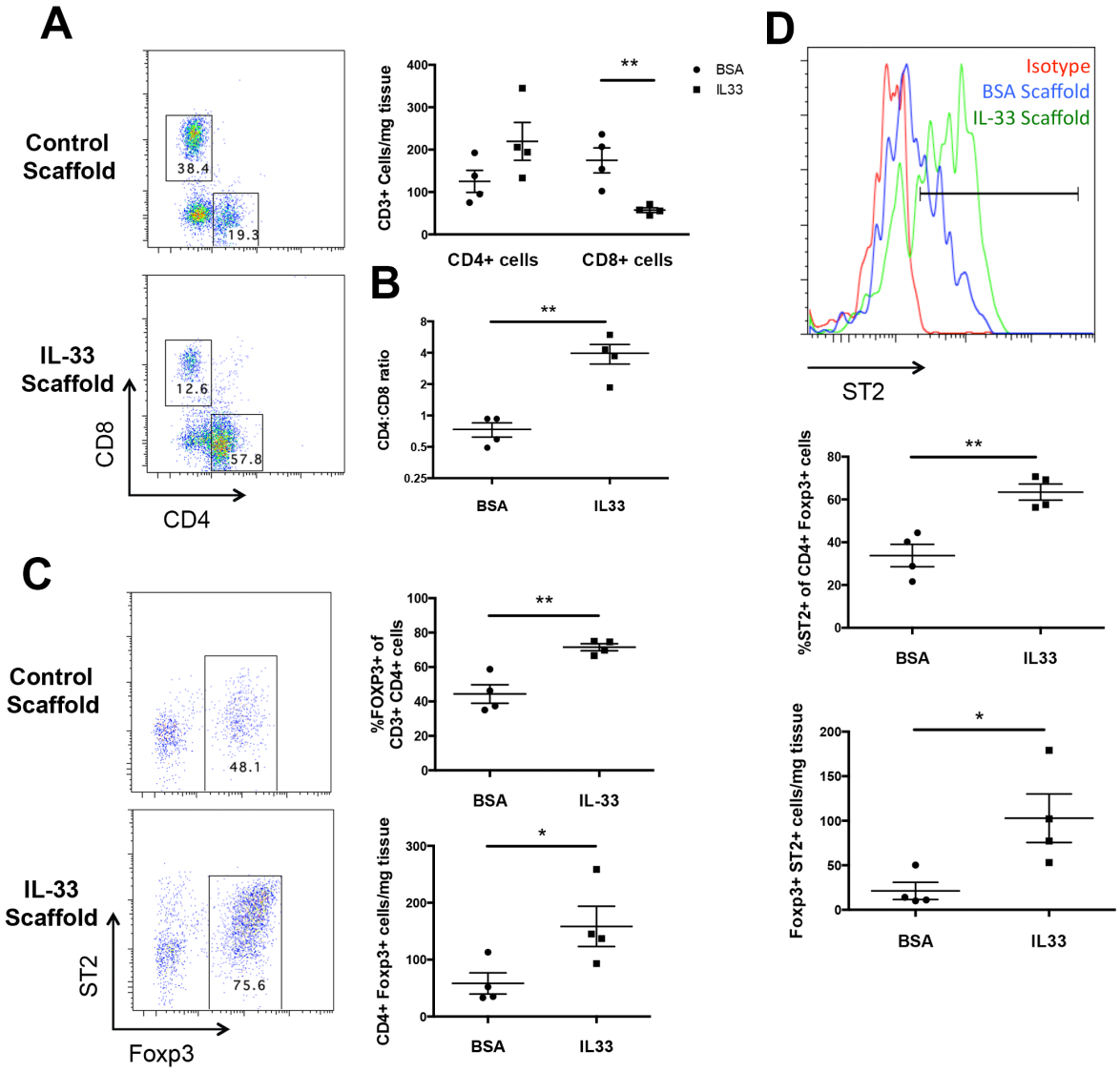

Figure 4.4 IL-33 decreases CD8+ T cells and expands ST2+ Tregs with allogeneic islets. **(A)** Representative flow cytometry plots and quantification of CD4+ and CD8+ populations of live CD3+ cells isolated from control and IL-33 scaffolds seven days after transplant with allogeneic islets **(B)** Ratio of overall CD4:CD8 cells isolated from scaffolds **(C)** Representative flow cytometry plots and quantification of percentages and overall numbers of live CD3+ CD4+ Foxp3+ Tregs isolated from allografts **(D)** Representative flow cytometry plot and quantification of percentages and overall numbers of ST2+ Foxp3+ CD4+ T cells 7 days after transplant. Overall numbers are normalized to weight of tissue immediately after isolation. Graphs depict mean ± SEM. N = 5-6 scaffolds per condition. *$p < 0.05$ **$p < 0.01$ Statistics determined by unpaired t-test.

Release of IL-33 delays early engraftment of islets compared to control scaffolds. The delay in engraftment appears to be intrinsic to IL-33 release given a similar delay was noted when using a syngeneic transplant model where C57BL/6 islets were used for transplant. However, all allogeneic IL-33 transplants recovered normoglycemia by Day 10, indicating high blood glucose was not due to graft failure. The median survival time of allogeneic grafts on IL-33 scaffolds increases median survival time from 14 to 33 days (**Fig. 4.5)**.

4.5.6 Scaffold delivery of IL-33 induces expression of a Type 2 immune response

Gene expression in the scaffold following localized IL-33 delivery indicated that IL-33 was responsible for inducing a local Th2 polarizing response. RNA was isolated from scaffolds excised three days after implantation, in order to better assess the direct impact of IL-33 release. Increased expression (100-300 fold) was detected for the cytokines IL-4, IL-5, and IL-13. (**Fig. 4.6A**). We also identified a similar level of upregulation for Ym1, which is associated with an alternatively activated phenotype in macrophages but also can act as an eosinophil chemotactic factor. Nos2 and Arg1 showed increased RNA expression levels of 10-15 fold (**Fig. 4.6B**). While Nos2 and Arg1 are normally used to distinguish M1 and M2 macrophages respectively, they are also expressed by a variety of other immune cell lineages that may be included in the total RNA isolated from the scaffold, including eosinophils and ILC2s. We did not detect any change in expression of IFN-γ or TNF-α, though an increase in IL-6 was observed (**Fig. 4.6C**). Consistent with an increase in Tregs, we detected an increase in Foxp3 expression (**Fig. 4.6D**). However, we did not detect changes in the expression of IL-10 or TGF-β1.

In order to follow the longer-term effects of the observed gene expression changes induced by IL-33, we performed a Luminex analysis on homogenized scaffold implants 7 days after

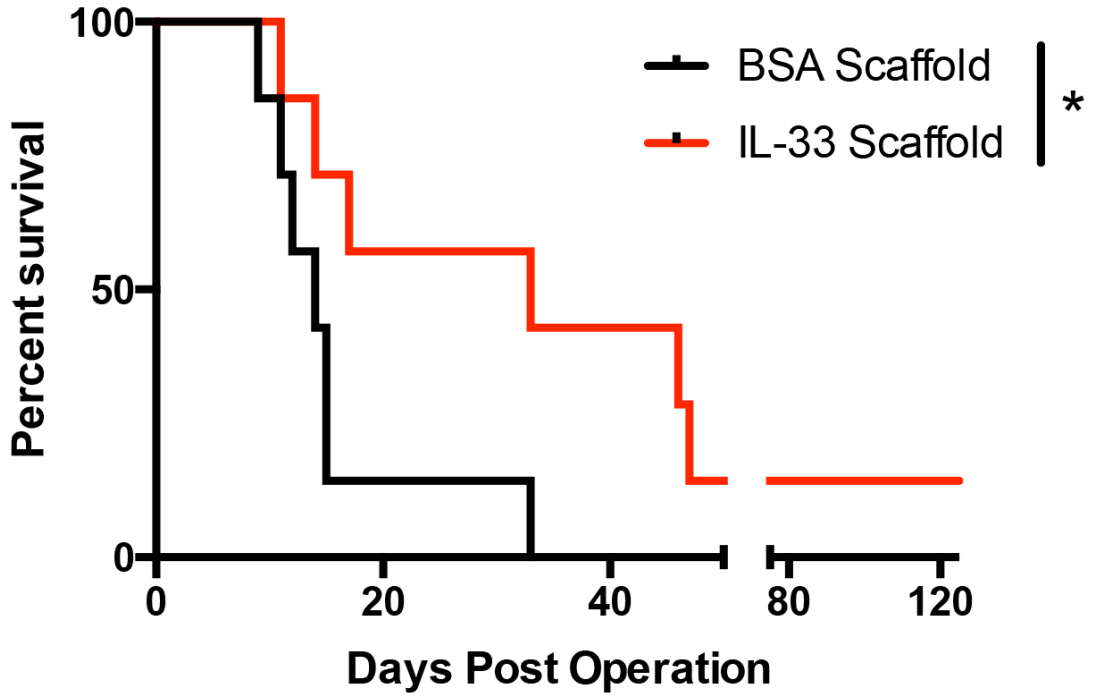

Figure 4.5 IL-33 extends islet allograft survival.
Kaplan-Meyer survival curve of islet allografts grafts over time. Two consecutive readings above 250mg/dL with no recovery of consecutive days of normoglycemia indicated graft failure. N = 7 mice per condition. * $p<0.05$. Statistics determined by log-rank test.

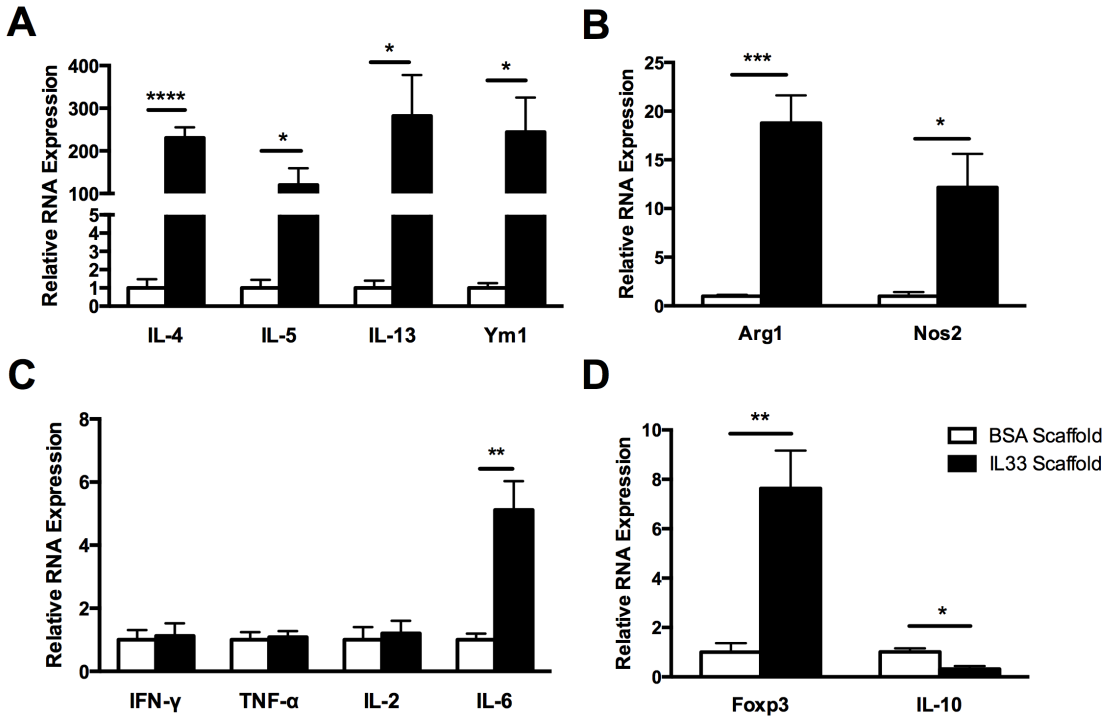

Figure 4.6 IL-33 induces RNA expression of Th2 cytokines 3 days after implant.
(A) Gene expression measured by two-step RT-PCR of IL-4, IL-5, IL-13, and Ym1 from RNA isolated from scaffolds harvested 3 days. **(B)** Gene expression of Arg1 and Nos2 **(C)** Gene expression of IFN-γ, TNF-α, IL-2, and IL-6. **(D)** Gene expression IL-10 and FOXP3. Expression was calculated using $2^{-\Delta\Delta Cq}$ method. Experimental genes were normalized to the housekeeping gene HPRT1. N = 4 scaffolds per condition. *p<0.05 ** p<0.01 ***p<0.001 ****p<0.0001 Statistics determined by unpaired t-test.

implant, assessing the cytokine milieu closer to the time of graft failure. Significant increases in IL-4 and IL-5 were detected in the IL-33 condition though neither IL-10 nor IL-13 were detectable within the local environment (**Fig. 4.7A**). While IL-6 was also significantly increased in the IL-33 scaffold, cytokines indicative of a Th1, Th17, or CD8 expanding response such as IFN-γ, TNF-*α*, IL-1β, IL-12p70, and IL-17 were under the detectable limit (**Fig. 4.7B)**. A significant decrease in T cell homeostasis cytokines IL-2 and IL-15 was noted in the IL-33 scaffold (**Fig. 4.7C)**. While a number of chemokines such as Eotaxin, KC, MCP-1 were expressed at detectable concentrations in both scaffold conditions, we noted IL-33 scaffolds showed a significant decrease in MIG and IP-10 expression, two chemokines typically induced by IFN-γ (**Fig. 4.7D**).

4.5.7 Scaffold delivery of IL-33 expands ST2+ ILC2 cells and eosinophils

We next assessed the innate immune cell populations present within the scaffold 7 days after implant following release of IL-33. Delivery of IL-33 tripled the number of live CD45+ cells recovered from the scaffold at Day 7. As previously noted, the control scaffold is already enriched for eosinophils. With the local delivery of IL-33, the eosinophil population increased from 40% to 70% of the total CD45+ population in the SVF (**Fig. 4.8A**). While the overall numbers of other myeloid lineages remained constant between BSA and IL-33 scaffolds, the number of eosinophils increased from 1500 to 10,000 cells/mg tissue. IL-33 has been shown to potently increase ST2+ ILC2s in adipose tissue. ILC2s are detected as lineage negative (CD11b, CD11c, CD45R/B220, Ly6G, Ly6C, CD3, CD4, CD5, FcεRIα, NK1.1) ST2+ CD25+ CD127+ (**Fig. 4.8B**). While a negligible population of ILC2s was found in the control, IL-33 delivery significantly expanded the population of ILC2s to 700 cells/mg of tissue, corresponding

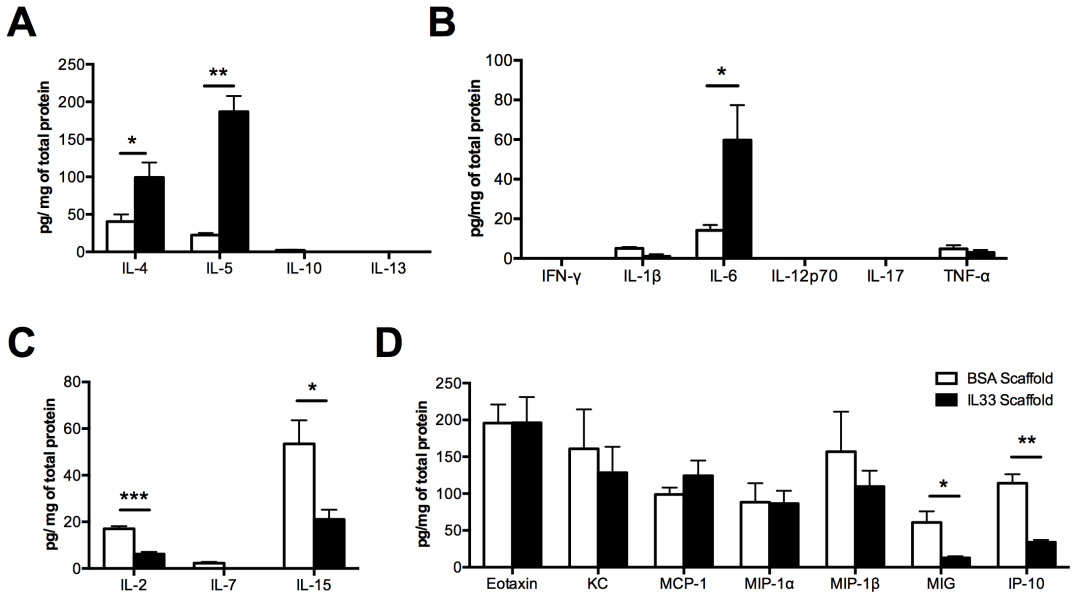

Figure 4.7 IL-33 delivery induces protein expression of Th2 cytokines 7 days after implant. Cytokines were detected by Luminex analysis of protein homogenates from scaffolds. **(A)** Th2-associated cytokines detected from the scaffold. **(B)** Th1 and Th17-associated cytokines detected from the scaffold. **(C)** T cell proliferation-associated cytokines detected from the scaffold. **(D)** Chemokines detected from the scaffold. 1mg of total protein was loaded for each sample in technical duplicates. Graphs depict mean ± SEM. N = 4 scaffolds per condition *$p<0.05$ **$p<0.01$. Statistics determined by unpaired t-test.

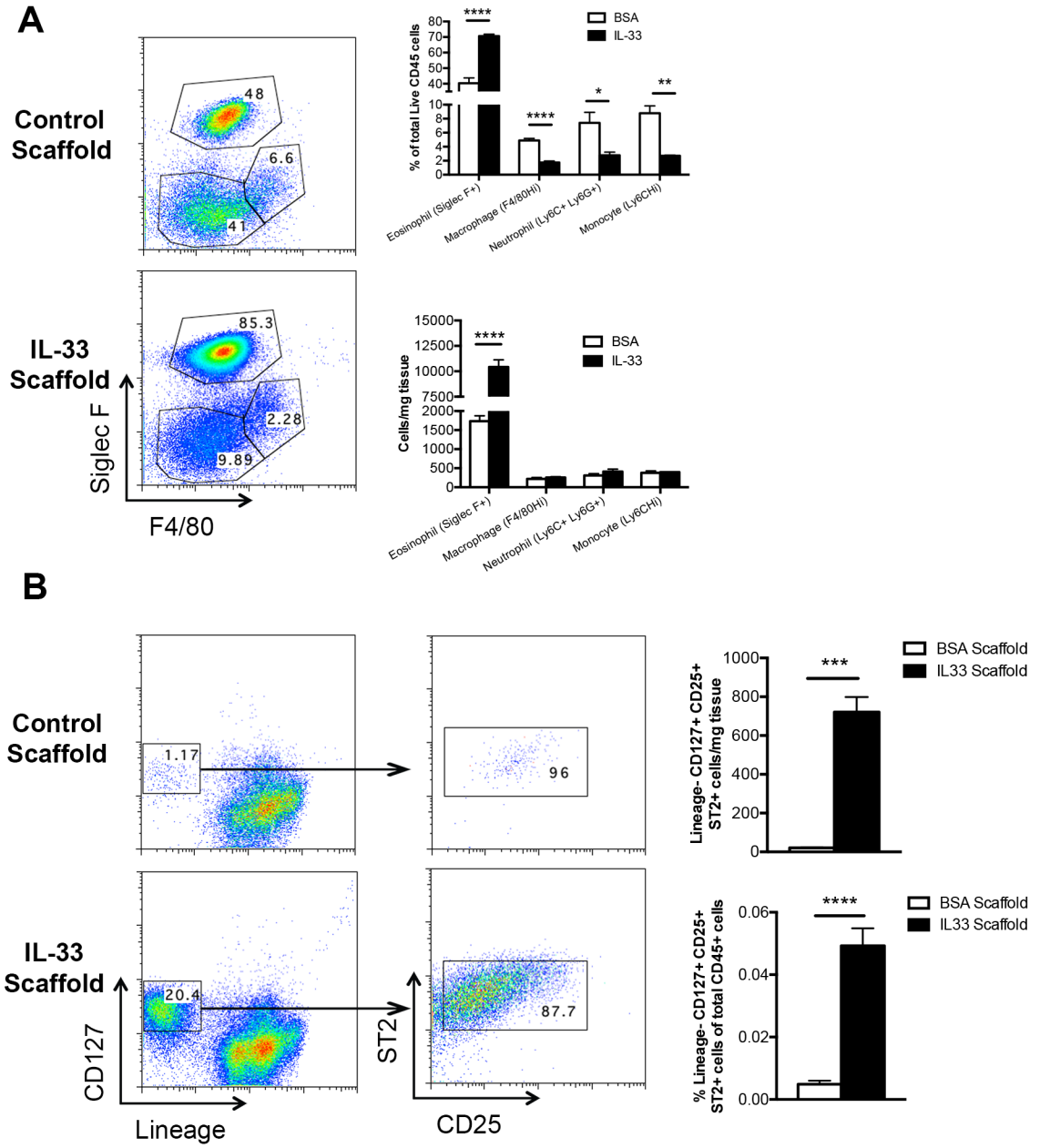

Figure 4.8 IL-33 delivery expands eosinophils and ILC2s
(A) Representative flow cytometry plots and quantification of percentage and overall number of innate cell subsets as defined in Figure 2, focusing on changes in the eosinophil population **(B)** Representative flow cytometry plots and quantification of percentage and overall number of ILC2s Overall numbers of Foxp3+ cells normalized to tissue weight Graphs depict mean ± SEM. N = 4 scaffolds per condition ***p<0.001 ****p<0.0001. Statistics determined by unpaired t-test.

to roughly 0.05% of the total CD45+ leukocytes in the scaffold. We did not detect any increase in the CD11b+ F4/80 hi population of macrophages present within the scaffold, nor did we detect significant changes in the polarization state away from a classically inflammatory CD11c+ phenotype.

4.6 Discussion

In this manuscript, we report the effects of the novel immunomodulatory cytokine IL-33 when delivered locally to the epididymal fat pad via release from a biomaterial scaffold. IL-33 is a pleiotropic cytokine with a number of putative targets among adipose-tissue resident cell lineages. Like most cytokines, IL-33 can be found to influence divergent effects depending on the combinations of factors that are being co-expressed [241, 247]. IL-33 monotherapy studies using systemic administration have generally found IL-33 to extend graft survival in allogeneic cell transplantation [242-244].

IL-33 expands CD4+ Foxp3+ Tregs both in a blank implant model as well as the allograft model. A number of groups have reported expansion of Foxp3+ Tregs through IL-33 administration, including specifically within the adipose tissue [62, 242, 248]. Our work confirms IL-33 delivery into the adipose tissue biomaterial scaffold environment robustly increases Foxp3+ cells present in the graft, with and without the presence of the allogeneic antigen. The ability to expand Tregs in the presence of alloantigen is crucial given the differences in T cell mediated responses when introducing allogeneic tissue, which can be seen through differences in the CD4+ and CD8+ populations between blank scaffolds and scaffolds containing allogeneic islets. Interestingly, the ST2+ population of Tregs that would theoretically be responsive to IL-33 was only significantly expanded amongst the total Foxp3+ pool with the

presence of allogeneic islets. This may suggest that IL-33 induced Treg proliferation is enhanced by TCR activation. IL-33 also has pro-inflammatory capabilities in certain disease models and can activate CD8+ cells, which are a key component of allograft rejection [249, 250]. We did not observe any additional activation of CD8+ cells with the addition of IL-33, which re-emphasizes the importance that the presence of other cytokines play in controlling immune responses within tissues.

While it is unclear from our experiment whether the Foxp3+ Tregs within the scaffold take an active role in immune suppression, IL-33 appears to polarize T cells towards non-destructive graft phenotypes. We did not observe an enhancement in gene expression of common immunosuppressive factors IL-10 and TGF-β1. In the case of IL-10, a decrease in both day 3 gene expression and day 7 protein expression was present, making immune suppression by soluble factor release unlikely. However, the fact that 70% of CD4+ T cells recovered from scaffolds transplanted with allogeneic islets expressed Foxp3+ is consistent with the notion that there likely was a decrease in the presence of Th1 CD4+ cells that are linked with acute cell-mediated rejection. While IL-10 and TGF-β1 were not upregulated even with the expansion of local Tregs, a known mechanism of Treg-mediated immune suppression is the ability to consume IL-2 through expression of the high-affinity IL-2 receptor subunit CD25 while not producing additional IL-2 to activate additional T cells. Recent work has shown *in vivo* Treg-associated IL-2 depletion can suppress expansion of CD8+ cells, which is consistent with the allogeneic experiments [251]. Though IL-33 has been shown in some cases to promote a Th1 response that would be graft destructive, we do not detect the presence of any inflammatory cytokines associated with a Th1 or Th17 response besides IL-6. This lack of Th1 response is consistent the low levels of IL12p70 in the scaffold, which has been implicated as a factor that induces Th1

when coexpressed with IL-33 and signifies IL-33 is not sufficient alone for inducing Th1 responses [252]. Luminex analysis detected significant populations of chemokines 7 days after implant that could be sources of monocyte recruitment into the scaffold. However, IL-33's effects seemed limited to decreasing the concentration of two IFN-γ induced chemokines CXCL9 and CXCL10.

Scaffold delivery of IL-33 induced a Th2 cytokine response that is consistent with previous literature [246, 247]. Gene expression analysis of localized IL-33 scaffold delivery confirms the induction of a type 2 immune response within the localized adipose scaffold environment. Day 3 RNA expression revealed close to 100 fold inductions of *IL4*, *IL5,* and *IL13*, all known to be induced by IL-33 [247]. Curiously, while IL-4 and IL-5 remain upregulated within the IL-33 scaffold 7 days post implant, IL-13 was undetectable. While we did not stain the remaining CD4+ cells for the Th2 transcription factor GATA3 (a marker of the Th2 lineage) for flow analysis, the elevated presence of Th2 cytokines IL-4 and IL-5 in the IL-33 scaffold suggests that a significant portion of the remaining 30% of CD4+ T cells would be polarized towards the Th2 lineage. While there have been some reports of Th2-Treg plasticity, it does not appear that there is any loss of Tregs due to IL-4/IL-5 upregulation by IL-33 as the percentage of Foxp3+ CD4 cells increased in both blank scaffold implantation and allogeneic islet transplantation and no decrease in Foxp3 expression was detected [253]. While Th2 cells are not inherently tolerogenic, several studies have suggested that shifting the T cell polarization from Th1 to Th2 can prolong allogeneic islet engraftment [254, 255]. However, alloreactive Th2 cells have also been shown to be sufficient to mediate islet graft rejection [256, 257]. While mechanisms exist for a Th2 mediated rejection of allografts, the prevention of a Th1 inflammatory response is still ultimately an important goal to extend allograft survival.

The primary cell population expanded by local IL-33 delivery was CD11b+ F4/80 Int Siglec F+ Side Scatter Hi eosinophils. Systemic IL-33 delivery has been shown to promote eosinophilia specifically due to the induction of Th2 cytokines [258]. Eosinophils are a primary immune cell lineage required to maintain immune homeostasis within adipose tissue through the release of the same type 2 cytokines that are responsible for allergy [247]. However, high local proliferation of eosinophils has been linked to transplant rejection in cardiac allograft models, typically mediated through IL-5 [259, 260]. It is worth noting that even without the addition of IL-33, there is a dominant population of eosinophils that make up close to half of the observed live CD45+ cells in the BSA scaffold. This underscores the importance of local immune context in deciphering the importance of singular factors. The enhanced presence of eosinophils in the control scaffold does not appear to have negative effects on engraftment and long-term survival of transplanted islets. This may be an indication that activation of eosinophils by IL-33 is required for any engraftment disruptive characteristics to be manifested.

We also looked at a number of other innate immune cell lineages that have been shown in the literature to be targets for modulation by IL-33, including ILC2s, adipose resident macrophages, and myeloid-derived suppressor cells (MDSCs). While only a few publications have focused on potential roles of ILC2s in transplant biology, their role in inducing a Th2 response has been well established in allergy and adipose homeostasis models [60, 261, 262]. As a cell type identified by expression of ST2, it is also available to respond directly to IL-33. We saw significant expansion of an ILC2 population that, while only comprising a small proportion of the total immune cell population, may contribute heavily to the local Th2 cytokine response [238, 261]. Adipose resident macrophages, characterized as CD11b+ F4/80 Hi, are important regulators of local inflammation. In lean mice, adipose tissue is enriched for alternatively activated CD301+ M2

macrophages over inflammatory CD11c+ M1 macrophages [263, 264]. In contrast to published literature, scaffold delivery of IL-33 appeared to have limited effects on the macrophage population in the scaffold. While the control scaffold environment is enriched for F4/80 hi macrophages possessing a classically inflammatory CD11c+ phenotype, IL-33 does not significantly alter the number of these cells in the scaffold. A number of studies using IL-33 to promote transplant tolerance have reported IL-33 mediated immune suppression is mediated by a MDSCs population [242, 243]. This heterogeneous population of cells is often characterized as CD11b+ Gr-1 intermediate but most accurately is defined by its namesake suppressive capacity. In the gene expression analysis of total RNA, we saw a similar rise in both Arg1 and Nos2, which are both upregulated in MDSCs [242]. However, while flow cytometry analysis of the scaffold identified a population of CD11b+ F4/80 Int Ly6C Int cells, most of these cells expressed Siglec F+ and were identified as eosinophils rather than MDSCs. It does not appear as though MDSCs are prevalent in the control scaffold either and thus this lack of IL-33 induced MDSCs may reflect that protein is being delivered to an area where the cell of interest are not located or recruited.

4.7 Conclusions

In summary, we used a novel biomaterial platform to locally deliver a pleiotropic cytokine IL-33 into a unique adipose tissue transplant environment to try and reconcile its studied effects in an allotransplant setting. We altered previous designs of the scaffold to include a carrier protein to maximize IL-33 loading. Due to IL-33's importance in adipose tissue immunity, we identified a number of immune cell lineages that were able to respond robustly to the delivered IL-33. IL-33 robustly increased the local Foxp3+ population with and without the presence of

allogeneic tissue, thus confirming it's ability to expand an important immunosuppressive phenotype. IL-33 also is able to decrease local CD8+ T cell proliferation in the allograft model. We then saw a significant increase in the allograft survival of islets, suggesting that there are long-term benefits to localized IL-33 delivery. However, we also found that IL-33 delayed islet engraftment in both allogeneic and syngeneic models, which may be explained by the induction of a type-2 cytokine response and concurrent expansion of eosinophils and ILC2s within the transplant environment. We suggest that IL-33 may be a compelling factor to deliver locally in combination with other factors that might be able to synergize with its ability to expand locally tolerogenic phenotypes such as Tregs while controlling its engraftment-delaying characteristics.

Chapter 5:

Comparison of Encapsulating and Microporous Hydrogels for Islet Transplantation in Rodent Models of Diabetes

5.1 Context

The previous two chapters are centered on modifying the existing PLG scaffold platform pioneered by the Shea lab for islet transplantation. However, in considering future goals of using the scaffold itself to modify the local environment, there are a number of other synthetic polymers with unique characteristics that might offer advantages over PLG. In particular, PEG has become an increasingly popular material for *in vivo* uses due to its low protein adsorption and purported non-immunogenic properties. Physically, PEG hydrogels can be synthesized into flexible structures with tissue-like mechanical properties compared to rigid PLG scaffolds, making them easier to handle for surgery. Depending on the method of cross-linking, PEG hydrogels can be produced to be non-degradable, allowing for recovery of the graft. PEG is also easily functionalized and is amenable to chemistries with bioactive molecules. Thus, in order to broaden our cell transplantation platform, we decided to design two different PEG scaffolds to support syngeneic islet transplantation. Through these studies, we sought to compare their properties to determine how scaffold architecture might affect transplant performance on a novel material. While the ultimate goal of this project would be to enhance clinically relevant models of islet transplantation, we first characterized the PEG scaffolds in syngeneic models to establish our lab's ability to successfully use a different material to engraft islets and understand how the material interacted with the local transplant environment.

The two scaffold architectures chosen reflect the two dominant and competing philosophies for using materials to enhance islet transplantation. The first design was an encapsulating gel fabricated by crosslinking a mixture of PEG precursor and islets with a non-degradable peptide linker. The resulting hydrogel has a mesh size that prohibits cell infiltration into the structure, thus theoretically creating an immune-isolating environment. Though the background section of

this dissertation details some of the long-term engraftment disadvantages of pursuing an encapsulation strategy, this design is nonetheless a popular choice for islet transplantation and serves as a useful comparison for alternate scaffold designs. The second design was a microporous scaffold reminiscent of the porous PLG scaffold design, fabricated prior to islet seeding by free-radical polymerization of a PEG-precursor solution saturated with salt as a leachable particulate. This design aimed to recreate the rapid tissue infiltration and engraftment of earlier PLG designs. While we hypothesized that islets transplanted on the microporous PEG scaffold should perform very similarly to the PLG scaffolds, we were interested in determining whether the physical properties of the PEG scaffold such as low substrate stiffness and the low protein adsorption on the surface might have a different effect than PLG scaffolds on infiltrating leukocytes. By fabricating hydrogels comprised of the same PEG-maleimide backbone, we were able to directly compare the effect of encapsulation versus porous design on islet engraftment and interactions with the local immune environment.

This project was a collaboration between myself and two other Ph.D. students, Peter Rios and Michael Skoumal. Michael provided much of the materials expertise and developed the methodology for synthesizing the different hydrogel architectures. At Northwestern, Peter performed the initial engraftment studies and blood glucose monitoring studies and completed the IPGTT studies for the microporous hydrogels, with the assistance of Dr. Xiaomin Zhang. At Michigan, I completed the islet transplants and IPGTT experiments for the encapsulated hydrogels with the assistance from Michael and was responsible for the planning and execution of the flow cytometry experiments.

5.2 Abstract

Islet transplantation as a therapeutic option for Type 1 diabetes mellitus is currently limited by poor engraftment in the hepatic portal vasculature. Biomaterials have been employed as a means to promote islet engraftment and function at extrahepatic sites. Two common strategies involved the use of encapsulation or microporous scaffolds that can either isolate or integrate islets with the host tissue respectively. Herein, we investigated the initial engraftment and function of islets *in vivo* using either an encapsulation or microporous scaffold approach through the fabrication of non-degradable polyethylene glycol (PEG)-based hydrogels. Transplantation of islets within encapsulating or microporous hydrogels restored normoglycemia in less than three weeks after transplantation, but with distinct blood glucose dynamics during the immediate post-transplantation period. Encapsulated islets remain separated from the host tissue, whereas the microporous scaffolds allowed for revascularization of the islets post-transplant. A glucose challenge test at one month post-transplant indicated that encapsulated islets had a delay in glucose-stimulated insulin secretion, whereas microporous hydrogels were able to restore normoglycemia in time consistent with native pancreata. The microporous hydrogel immune microenvironment had a distinct recruitment of neutrophils coinciding with the initiation of the day 7 - 14 hyperglycemic period. Collectively, these findings suggest that both encapsulation and microporous PEG scaffold designs allow for stable engraftment of islets but introduce architecture-specific challenges to early engraftment and glucose responsiveness.

5.3 Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that impacts 5-10% of diabetic patients and destroys pancreatic beta cells, rendering patients unable to regulate blood glucose levels [2]. Despite use of exogenous insulin and the development of insulin pumps, tight control of normal blood glucose levels and secondary complications remain a concern. Alternatively, islet transplantation into the hepatic portal vein has been used to restore endogenous insulin production and aid in maintenance of normoglycemia and prevention of severe hypoglycemic events [1, 10]. However, this procedure is not widely employed clinically due to poor survival and engraftment of transplanted islets in the hepatic vasculature. Upon injection into the hepatic portal vein, transplanted islets are subject to the instant blood inflammatory response (IBMIR), which can account for more than 60% of islet loss days post-transplant. IBMIR promotes a pro-inflammatory environment, which then leads to activation of adaptive immunity and additional injury to islets [1]. Challenges associated with delivery of islets into the liver have motivated the development of extrahepatic sites conducive to islet engraftment to prevent IBMIR-mediated destruction of islets and improve clinical outcomes [137].

Biomaterial-based strategies have been employed as a means to provide a controlled delivery system for isolated islets into extrahepatic sites. A variety of scaffold materials have been used to localize islets in extrahepatic locations, including the kidney capsule and omentum, and restore normoglycemia in diabetic rodent models [35, 131, 132, 168, 265]. The design of these scaffolds has implications for islet engraftment. The two most common scaffold designs used for housing islets involve usage of encapsulating or microporous biomaterials. Biomaterial approaches are being developed to ideally work in combination with improved immunosuppressive drugs and protocols to induce transplant tolerance [121, 266-268]. Encapsulating materials protect islets

from direct contact with host immune cells, yet permit efflux of insulin and exchange of metabolites. Mass transport limitations and inconsistent results in large animal models have limited usage of micro- or macro-encapsulating technologies [268-270]. Alternatively, microporous scaffolds can be used to seed islets and permit infiltration of host tissue and vasculature around transplanted cells, which minimizes challenges related to mass transport. In the context of islet transplantation, microporous PLG scaffolds have demonstrated efficacy *in vivo* in mouse and porcine models of diabetes [35, 124, 132, 170, 235]. However, microporous scaffolds with seeded cells are subject to infiltration by host immune cells. Strategies to utilize encapsulation, in combination with a porous architecture, may advance islet transplantation and have been examined in a recent study [168]. Evaluation of how these different scaffold designs impact islet engraftment is needed to develop the next-generation of biomaterials that can improve long-term transplantation outcomes.

In this report, we investigated both islet function and the host response as a function of the scaffold architecture. Towards this goal, we fabricated non-degradable polyethylene glycol (PEG)-maleimide hydrogels, using polydimethylsiloxane (PDMS) molds to easily and reproducibly create both encapsulating and microporous gels with similar dimensions. The use of a non-degradable design allows for easy-post surgical retrieval of the implant. We used a previously-explored nonporous design hydrogel representative of macroencapsulation strategies in addition to a non-encapsulated microporous scaffold created using particulate leaching designed to encourage tissue infiltration and islet engraftment [121, 132, 168]. Though two different crosslinking methods were used, both hydrogels consist of the same PEG-maleimide backbone, allowing our studies to focus on comparing the physical features of the two scaffold designs. Using a syngeneic transplant model in an extrahepatic site (i.e. epididymal fat pad) of

diabetic mice, we evaluated islet survival and function and characterized the innate immune response during the initial 30-day period following transplantation to identify how PEG hydrogel architecture affects engraftment properties. Correlating the hydrogel architecture to the innate immune response and ultimately graft function can reveal critical design parameters necessary to develop a translational system.

5.4 Materials and Methods

5.4.1 Encapsulating and Microporous Hydrogel Fabrication

Encapsulated hydrogels were formed by mixing PEG-maleimide (4-arm, 20kDa MW, JenKem Technology USA) and CGRGDS (CelTek Peptides) in HEPES Buffer (pH 7.2) to yield a final PEG concentration of 10% (weight/volume) and RGD concentration of 5 mM. The PEG-CGRDS solution was allowed to react via Michael-Type addition for 5 minutes at room temperature and then stored on ice. Next, the functionalized PEG precursor was added to sedimented islets in an Eppendorf tube (in approximately 6 μL of HBSS 1X media (Corning) supplemented with 10% FBS). The bottom of a disc-shaped PDMS mold (diameter = 5 mm, height = 1 mm) was covered with 3 uL of a non-degradable peptide crosslinker solution (GCYKNRGCYKNRCG, custom synthesis and purification by CelTek Peptides). The peptide contained tyrosine (Y) and asparagine (N) amino acids in the D-configuration to prevent cleavage from plasmin, which inhibits hydrogel degradation. The PEG precursor containing islets was added to the mold containing the YKNR solution and an additional 3 uL of YKNR was added on top for a final YKNR concentration of 9.6 mM. The hydrogel was incubated at 37°C for 30 minutes to allow the crosslinking reaction to reach completion. Final gel volume was approximately 25 μL.

Microporous PEG hydrogels were fabricated by dissolving 20 kDa 4-arm PEG-maleimide (JenKem Technology USA) in HEPES buffer for a final concentration of 20% (wt/vol). The photoinitiatior, Irgacure 2959 (BASF) was dissolved in N-vinylpyrrolidone at a concentration of 600 mg/mL and added to the PEG precursor solution for a final concentration of 1 wt%. NaCl was added to the PEG precursor to make a saturated solution. Forty milligrams of NaCl particles (average diameter = 250 um) were then added to a polydimethylsiloxane (PDMS) mold (diameter = 5 mm, height = 1 mm) and 10 uL the saturated PEG solution was added. After irradiation with UV light, salt was leached from the scaffolds in ultrapure water for two 10-minute washes. Final gel volume was approximately 25 μL.

5.4.2 Islet Isolation and Transplantation

For syngeneic studies, islets were isolated from healthy 10-12 week old male and female C57BL/6J mice (Jackson Laboratories) following standard islet isolation procedures. Male C57BL/6J recipient mice were between 14-18 weeks of age. Four days prior to islet transplantation, recipient mice were injected with 220mg/kg of streptozotocin (Sigma) to chemically induce irreversible diabetes. Nonfasting blood glucose levels were taken using a OneTouch Basic Glucose Monitor (Aviva) and only those mice with a measurement of 300 mg/dL or greater on consecutive days (day before and day of transplant) were used as recipients. Normoglycemia was denoted as <200 mg/dL in syngeneic studies. Hydrogel materials, encapsulating or microporous, contained 700 islet equivalents (IEQ). To load microporous hydrogels, islets were concentrated in 30 μL and applied to the top of a dehydrated hydrogel. Each mouse received one gel into the fat pad transplantation site. All studies were approved by

the Northwestern University Animal Care and Use Committee or the University of Michigan Unit for Laboratory Animal Medicine (ULAM).

5.4.3 Intraperitoneal Glucose Tolerance Test (IPGTT)

Intraperitoneal glucose tolerance tests (IPGTTs) were performed at 4 weeks post-transplantation to assess the ability of the hydrogel materials to respond to glucose challenges. A D-glucose solution (250 mg/mL in sterile PBS) was created for injection. After a 3-hour fast period, mice received an i.p. injection of 2 g/kg D-glucose. Blood glucose levels were measured at baseline (before injection), 15, 30, 60, 90, 120, and 150 minutes after injection.

5.4.4 Immunohistochemistry

Scaffolds were harvested from euthanized mice and immediately snap frozen in isopentane at on dry ice to preserve tissue architecture. Frozen scaffolds were embedded in Tissue-Tek O.C.T. supplemented with sucrose. 14 μm cryosections were prepared and stored at -20°C until staining. Representative sections were fixed in 4% PFA and blocked with 10% normal donkey serum and 0.1% Triton-X in PBS before staining with primary antibodies. Sections were stained with guinea pig polyclonal anti-swine insulin (Jackson Labs, 1:250) and Hoechst for nuclear counterstaining (Invitrogen, 1:2000). Dylight donkey anti-guinea pig 488 (Jackson Labs, 1:400) was used as a secondary antibody for visualization.

5.4.5 Flow Cytometry

Mice were euthanized by cervical dislocation under isoflurane-induced anesthesia. Tissue was dissociated into a single cell suspension. Tissues were harvested immediately and stored in

HBSS on ice. The resulting tissue homogenate was filtered through a 70 μm cell strainer and washed with MACS (PBS supplemented with 2mM EDTA and 0.5% BSA). For scaffold implants and adipose tissue, enzymatic digestion was used to create a single cell suspension. Tissues were weighed and placed into petri dish with 0.5mL 10mg/mL Collagenase Type II (Sigma Aldrich) 2.5 mL of digestion buffer (HBSS with Calcium Chloride and Magnesium Chloride [Thermo Fisher] supplemented with 0.5% Bovine Serum Albumin). Tissue was finely shredded and transferred to a 15mL conical tube. Dish was washed with 2mL digestion buffer and added to tissue homogenate to bring final concentration of collagenase to 1mg/mL. Tissue was incubated in a 37° C water bath for 30 minutes with gentle shaking every 5 minutes. 100 μL of 0.5 M EDTA was added to each tube to a final concentration of 10 mM and incubated for an additional 5 minutes at 37° C. Tissue homogenate was strained through a 70 μm filter and washed with MACS. The resulting cell pellets were then incubated with 1mL ACK buffer on ice to lyse the red blood cells and washed with MACS. In preparation for staining with Live/Dead fixable stain, cells were washed with PBS.

Live Dead Fixable Violet stain (Thermo Fisher Scientific) was used for removal of dead cells from analysis. The Foxp3/Transcription Factor Staining Buffer (Ebioscience) was used for cells requiring intracellular staining. The following antibodies (clone) were purchased for analysis from Biolegend or Ebioscience: CD45 (30-F11), Ly6G (1A8), F4/80 (BM8), Siglec F, and CD11b. Isotype antibodies were used to establish gating. Samples were analyzed on the DAKO Cyan 5 ADP.

5.5 Results

5.5.1 Encapsulating and Microporous Hydrogel Fabrication

Encapsulating hydrogels were formed by Michael-type addition at 37°C using a 4-arm PEG maleimide functionalized with cell adhesion peptide CGRGDS and crosslinked in a PDMS mold, using a non-degradable, three-cysteine-containing crosslinking peptide (GCYKNRGCYKNRCG) (**Fig 5.1A)**. Islets were added into the precursor solution prior to gelation and mixed well to achieve distribution through the gel (**Fig 5.1B)**. Dimensions of the encapsulating gel were 5 mm in diameter and approximately 1 mm in height. Islet viability with these gelation conditions was confirmed in a previous study (Rios et al., 2016). Microporous hydrogels were fabricated by mixing salt particles in functionalized PEG precursor containing cytocompatabile photoinitiator. Following polymerization using UV and leaching of the gel to remove the salt porogen, the gels retained an interconnected porous architecture (**Fig. 5.1 C,D**). The PEG% (w/v) of the microporous hydrogel was increased from 10% in the encapsulating formulation to 20%, as lower concentrations resulted in collapsible gels with insufficient integrity for islet seeding. The volume and dimensions of the microporous hydrogel were the same as the encapsulating hydrogels. Following swelling, encapsulating and microporous hydrogels were approximately 6.5 mm in diameter and 1.5 mm in height, a size still suitable for transplantation into the epididymal fat pad.

5.5.2 Syngeneic Islet Transplants in Encapsulating and Microporous Hydrogels

The engraftment and function of islets encapsulated in bulk hydrogels or seeded on microporous hydrogels was investigated by transplantation into the epididymal fat pad of

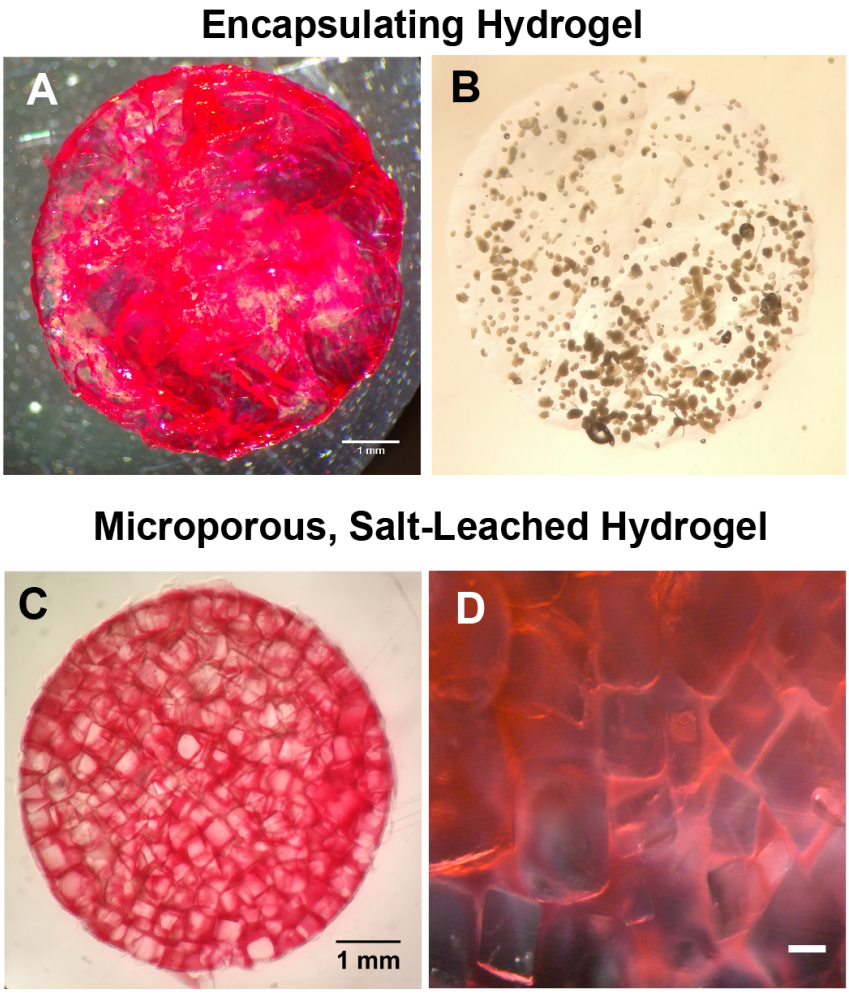

Figure 5.1 Encapsulating and microporous hydrogels for islet transplantation.
Light microscopy images of **(A)** a blank encapsulating PEG hydrogel produced by addition of a crosslinking peptide to a mixture of islets with PEG-precursor **(B)** an encapsulated gel loaded with 700 IEQ mouse islets **(C)** a microporous hydrogel fabricated by UV-crosslinking a PEG solution saturated with salt, which was subsequently leached for to leave pores for islet seeding **(D)** increased images of pores left after leaching Scale bar: **(A,C)** 1 mm **(D)** 200µm. Gels in A,C,D were stained with Sirius red for visualization.

streptozotocin-induced diabetic mice. Bulk, non-degradable encapsulating hydrogels with 700 islet equivalents (IEQ) reversed diabetes in recipient mice and achieved consecutive days of normoglycemia between day 12 and day 20 **(Fig. 5.2A)**. 4 out of 5 salt-leached, microporous hydrogels stably restored normoglycemia by day 15 post-transplant **(Fig. 5.2B).** The blood glucose dynamics of the pre-engraftment period varied between the two designs. In the encapsulated gel, blood glucose levels remained consistently elevated above 300 mg/dL and reached as high as 400mg/dL between Day 2 and 10 before stabilizing and approaching normoglycemia. In contrast, the microporous hydrogels attained near normoglycemic blood glucose levels for the first 6 days after transplant, and then rose above 200mg/dL between days 7 and 14 before reestablishing normoglycemia during day 14. In both conditions, removal of the hydrogel from the mouse resulted in a return to normoglycemia. Collectively, these syngeneic transplant studies confirm the utility of non-degradable, encapsulating and microporous PEG hydrogels to support islet function post-transplant in the fat pad transplant site.

5.5.3 Graft functional test

An intraperitoneal glucose tolerance test (IPGTT) was performed 30 days after transplantation to investigate glucose responsiveness of the different hydrogel architectures. The encapsulated hydrogels showed a delayed response to restoring normoglycemia in response to bolus glucose injection **(Fig. 5.3A)**. The blood glucose levels of recipient mice with encapsulating hydrogels peaked at 30 minutes post-injection of glucose, versus control mice, which peaked at 15 minutes post-injection. After 60 minutes, blood glucose levels of both groups of mice are decreasing towards normoglycemia, though AUC analysis confirmed a significant difference between the glucose resolution of the control mice and encapsulating hydrogels.

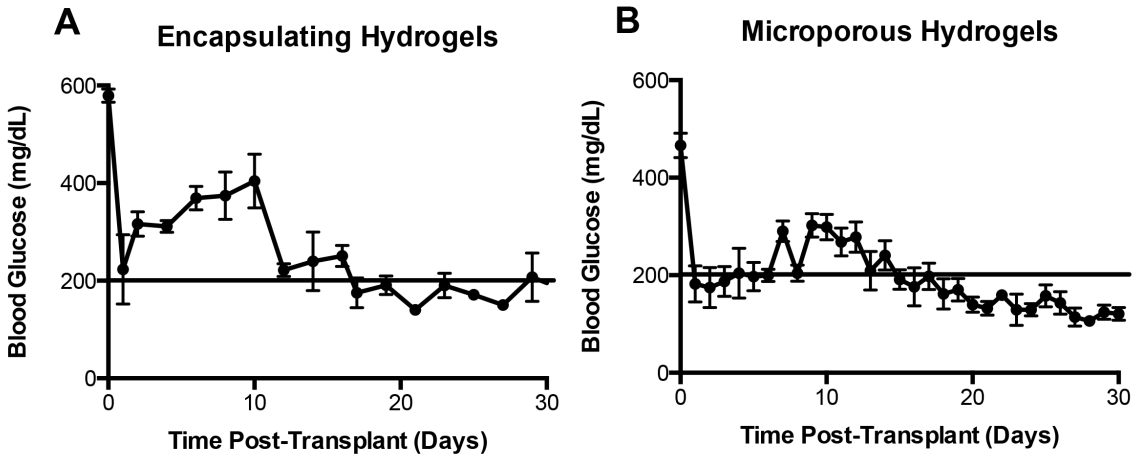

Figure 5.2 Blood glucose monitoring for syngeneic islet transplantation on PEG hydrogels. Blood glucose versus days post transplantation for diabetic C57BL/6 mice receiving 700 IEQ syngeneic islets via **(A)** encapsulated PEG hydrogel. N = 3 or **(B)** microporous PEG hydrogel. N = 5. Solid line indicates normoglycemic blood glucose level (200mg/dl). Graphs show mean ± SEM.

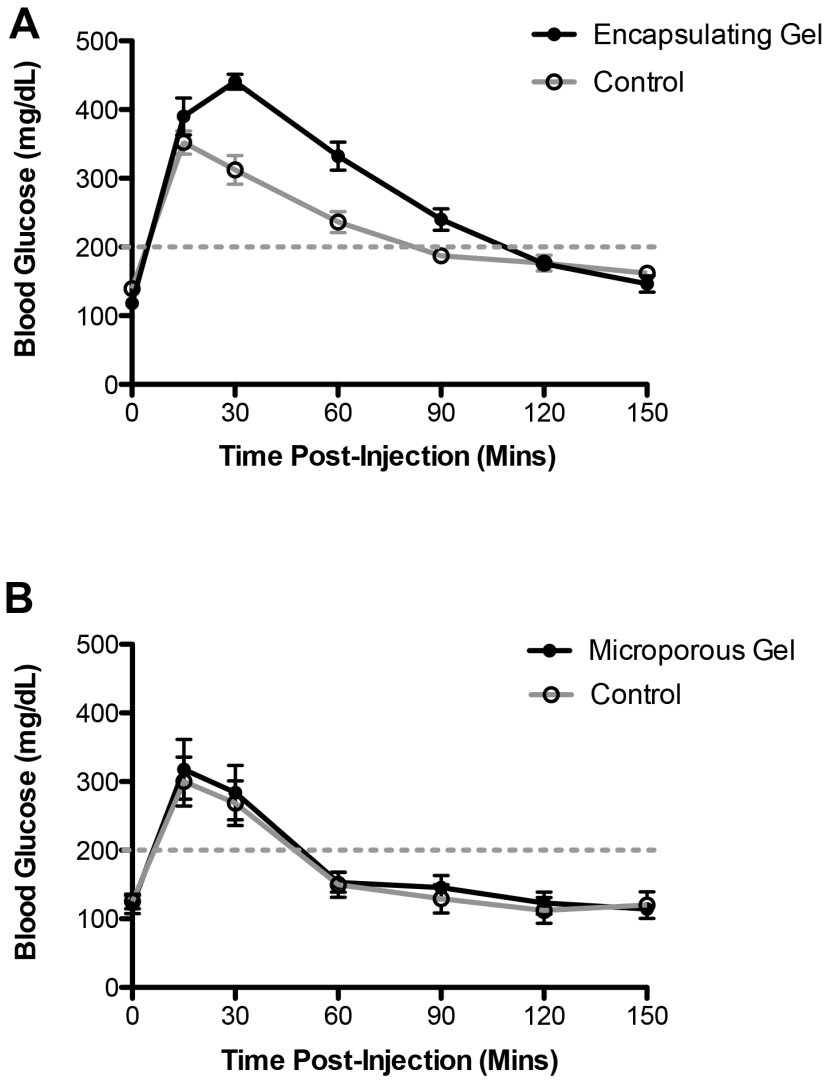

Figure 5.3 Graft responsiveness of hydrogels 1-month post-transplantation.
Blood glucose versus time post-i.p injection of glucose for mice 30 days after receiving 700 IEQ syngeneic islets via (A) encapsulated hydrogel. N = 5 or (B) microporous hydrogel. N = 4. Both conditions are compared to naive healthy C57BL/6 mice with endogenous islets as a control that received a simultaneous injection. N =9. Dotted line indicates normoglycemic blood glucose level (200mg/dL). Graphs show mean ± SEM.

Though blood glucose continued to decrease, mice with encapsulating gels remained hyperglycemic through 120 minutes. In contrast, microporous gels showed no significant difference in response to blood glucose **(Fig. 5.3B)**. The blood glucose levels of the microporous hydrogel group and control group peaked at 15 minutes post-injection of glucose. At 30 minutes, blood glucose levels mice decreased toward normoglycemia in both groups. At 60 minutes, the microporous gel and control group both achieved normoglycemic levels and their blood glucose remained normoglycemic for the remainder of timepoints. Area under the curve indicated no statistical significance at any timepoint. The glucose challenge results demonstrate microporous hydrogels support well engrafted transplanted islets can respond to glucose changes at a comparable rate to endogenous islets, while encapsulated islets show a delayed response.

5.5.4 Histological analysis of hydrogel explants

Transplanted hydrogels remained intact and well-secured in the highly vascular fat pad transplant site upon removal **(Fig 5.4A)**. Hydrogels were sectioned and stained with insulin and a Hoechst nuclear counterstain to confirm their functionality and engraftment post-encapsulation *in vivo*. Insulin-positive islets were retained and identified in implanted both encapsulating and microporous hydrogels after stable engraftment and restoration of normoglycemia **(Fig. 5.4B,C)**. These results indicate islets encapsulated in or seeded on PEG hydrogel materials were able to maintain their morphology, function, and engraft in the fat pad transplant site. The microporous gel showed penetration of the host tissue into the pores of the scaffold as expected. Sections of the non-encapsulated gel show no host tissue infiltration surrounding insulin positive cells.

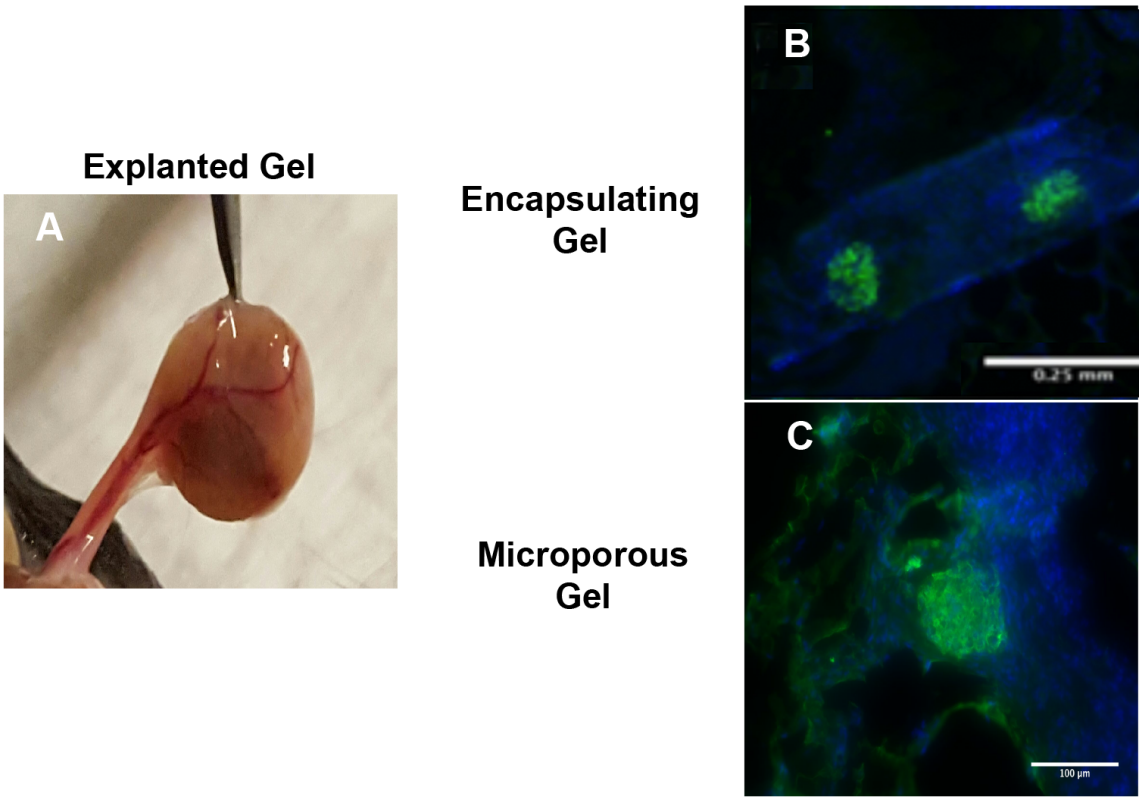

Figure 5.4 Insulin-Positive Islets Identified non-degradable hydrogels.
(A) Explanted microporous hydrogel 1 month post-transplant. **(B)** Insulin-positive islets identified 60 days post-implant from non-porous encapsulating gel. Image taken from Rios et al. 2016 using gels produced by same fabrication technique [168] Scale bar: 250 µm **(C)** Microporous hydrogels removed at Day 30 after stable engraftment was reached. Scale bar: 100 µm. Slides representative of 3 sections imaged per condition.

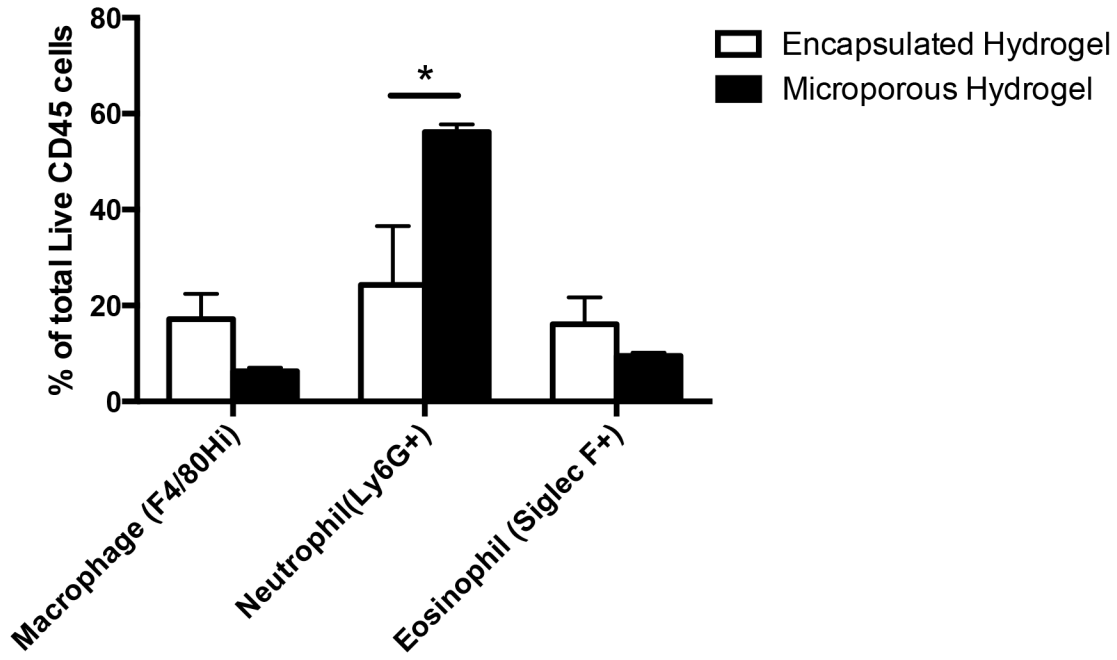

Figure 5.5 Innate immune cell populations in the PEG hydrogel environment. Percentages of innate cells identified from hydrogels extracted from hydrogels 7 days after implantation. Graph depicts mean ± SEM. N = 4 hydrogels per condition *<0.05. Statistics determined by unpaired T test.

5.5.5 Innate immune cell infiltration in encapsulating and microporous hydrogels

We investigated whether the innate immune response during the initial stages of engraftment changes with respect to hydrogel architecture. 7 days after implantation, the microporous PEG hydrogel shows an extremely large population of neutrophils (**Fig. 5.5**). An average of 56% of recovered leukocytes from microporous gels expressed a CD11b+ Ly6G+ F4/80- phenotype consistent with neutrophils compared to only 24% in the encapsulated graft. Our lab has observed neutrophils are typically only ~10% of total CD45+ cells recovered from porous PLG scaffolds and 1-2% in lean adipose tissue, consistent with published literature [63]. Though not statistically significant, we also observed encapsulated hydrogels showed decreased populations of eosinophils and macrophages (6% and 9% versus 16% and 17% in the microporous scaffold), which are both enriched innate immune cell phenotypes in adipose tissue.

5.6 Discussion

PEG is a versatile biomaterial to engineer scaffolds for cell transplantation, which could be formed into encapsulating and non-encapsulating formats that supported islet function [140, 157, 271, 272]. PEG is soluble with water and crosslinking can create a hydrogel in cytocompatabile conditions, allowing for both prefabricated and *in situ* gelation strategies. The porous PEG mesh allows for diffusion of oxygen and nutrients from the host environment. PEG hydrogels have mechanical properties similar to soft tissue, which can help limit immune polarization due to material rigidity [273, 274]. PEG can be readily functionalized, allowing for the introduction of ECM components into the hydrogel that can provide necessary support for islet adhesion [142, 265, 271]. Many hydrogels developed for cell encapsulation are formed with a degradable peptide linker that allow for the encapsulated tissue to be gradually integrated with the host

tissue. Herein, for islet encapsulation, non-degradable cross-linking peptides were employed, which cannot be degraded by endogenous proteolysis, thereby producing a non-degradable hydrogel. The non-degradable nature of this hydrogel ensures that the transplanted cells remain isolated from the host tissue, and helps with the ease of post-implant retrieval. We also fabricated PEG hydrogels into a microporous structure similar to what has been employed with PLG scaffolds. The non-degradable porous PEG hydrogels were formed by photo-crosslinking in the presence of a leachable particulate. Collectively, the PEG solution can thus be formed into encapsulating or non-encapsulating hydrogels using different crosslinking strategies, which were subsequently investigated for their effects on the engraftment and function of transplanted islets.

Both hydrogel designs ultimately established stable normoglycemia after transplantation, though the dynamics following the initial transplantation were dependent upon the encapsulating or non-encapsulating design. For encapsulating systems, the average blood glucose reading exceeded 300mg/dL and none of the transplanted achieved normoglycemia for the first 10 days before engrafting between days 12 and 17. Nondegradable biomaterials are more likely to initiate a foreign body response due to the inability of innate cells to phagocytose the material, leading to a "frustrated" phenotype that may lead to enhanced secretion of pro-inflammatory cytokines [225]. Furthermore, the presence of the encapsulating material provides a diffusion barrier that may contribute to elevated levels at early times post-transplantation. Alternatively, the microporous hydrogel design was expected to perform similarly to our previous studies with microporous PLG scaffolds, which typically engraft stably shortly after transplantation of an adequate islet mass [35]. Following transplantation on the microporous PEG hydrogels, 4 out of 5 mice were non-diabetic between days 2 and 6 post-transplant with three of the mice recording three consecutive blood glucose readings below 200mg/dL between days 3-5. However, between

days 7 and 14, all 5 mice experienced at least 5 blood glucose levels readings exceeding the normoglycemic standard of 200mg/dL (as high as 350mg/dL) before restabilizing. This transient increase may result from the foreign body response to the PEG hydrogel, addressed below. While PEG is often employed as a means to minimize protein adsorption, a foreign body response can still develop [167, 275].

IPGTT data demonstrates that scaffold designs allowing for integration with the host to respond quickly to changes in blood glucose levels. At day 30 post implantation, islets transplanted on microporous hydrogels restored normoglycemia after bolus glucose delivery with kinetics indistinguishable from healthy mice with endogenous islets. In contrast, islets encapsulated within the hydrogel produced a delayed recovery to normoglycemia. In the microporous gel, tissue ingrowth results in vascularization of engrafted islets, which would allow for rapid sensing of glucose and release of insulin. Conversely, the encapsulating hydrogel platform only allows for vessels growth around the hydrogel, with a distance between the islets and vasculature ranging from 100 μm to 500 μm [121]. This distance would produce a delayed response due to the transport of both the glucose into and the insulin from the graft. An intermediate strategy has employed a degradable hydrogel, with vascular ingrowth promoted through the sustained release of VEGF to improve vascularization locally, and ultimately their reconnection with the islets [5, 142, 265]. Nevertheless, preventing the direct revascularization of encapsulated islets provides a delay in responsiveness that was not present with non-encapsulated islets [276, 277].

The foreign body response induced by encapsulating and microporous hydrogel had differences in immune cell recruitment. For the microporous hydrogel, over 50% of CD45+ leukocytes recovered at day 7 after implantation were neutrophils. Previous work with porous

PLG scaffolds that engraft stably after transplantation had much lower percentages of neutrophils. The persistence of neutrophils within the hydrogel is unusual given circulating neutrophils typically undergo spontaneous apoptosis within 5 days in the absence of extracellular stimuli [275]. Since no cells were transplanted for the analysis of the foreign body response, the neutrophil recruitment would seem to result from the interaction between the host tissue and material. This neutrophil recruitment is consistent with a previous report that identified a similar neutrophil-based response to a sterile implanted biomaterial implanted in the intraperitoneal space, with the neutrophil response attributed to an inability to degrade the material [278]. Importantly, the elevated neutrophil levels relative to encapsulation or native adipose was not sufficient to cause graft failure, as all microporous hydrogel recipients eventually recovered to stable normoglycemia. Additionally, while neutrophils were greatly increased, we did not see an increase in the population of macrophages, which are typically the primary driver in a traditional foreign body response induced by biomaterials. This lends support to the well-established idea that PEG is a well-tolerated synthetic polymer for biomaterial implant [272]. Interestingly, the encapsulated nonporous hydrogel did not recruit a similar percentage of neutrophils despite being composed of the same PEG backbone material. This may suggest UV-induced crosslinking used in the pre-fabricated microporous hydrogel design leads to an unknown chemical modification to the scaffold surface that is not induced by the YKNR crosslinking. Alternately, while non-porous devices are typically associated with higher levels of leukocyte adhesion, the encapsulated hydrogel offer less non-degradable surface area to the host immune system, which offers a possible explanation if the inability to phagocytose the PEG background is the root cause of the immune response discrepancy.

5.7 Conclusions

In this study, we characterized the effect of nondegradable PEG hydrogel architecture on success of extrahepatic islet engraftment. We compared a nonporous encapsulation design preventing host infiltration against a porous scaffold encouraging tissue ingrowth. Both designs enabled stable engraftment by the third week post-transplantation though each experienced different destabilizing hyperglycemic events during the immediate post-engraftment period. While islets transplanted on porous scaffolds showed insulin release kinetics indistinguishable from endogenous islets, islets encapsulated in the nonporous design experienced a delay in glucose-stimulated insulin secretion in response to an exogenous glucose challenge. Our studies also revealed that despite using a porous scaffold design with a highly biocompatible material, an aberrant neutrophil-based immune response to the material was still provoked that might have temporarily affected graft function. This underscores the importance of investigating material design parameters such as degradability and alterations to the surface properties from the fabrication process that can lead to unforeseen material-tissue interactions. Our work establishes a PEG hydrogel fabrication platform that can be modified to imbue future designs with engraftment promoting characteristics.

Chapter 6:

Conclusions

6.1 Summary and Significance of Findings

The development of clinical islet transplantation is at an interesting inflection point. Since the advent of the Edmonton Protocol in 2001, intrahepatic islet transplantation has become an increasingly standardized procedure with enough demonstrated clinical benefit to pass a phase III clinical trial run through 8 different transplant centers [10]. It has been shown to be an effective clinical option for T1DM patients prone to SHEs that require a more responsive insulin delivery system than state-of-the-art external monitoring devices can provide at the moment. Islet transplantation is currently restricted in its usage due the poor long-term survival of the transplanted cells in the hepatic vasculature. Identification of an alternative extrahepatic islet transplantation site would solve many of the site-specific issues associated with the standard portal vein infusion of transplanted islets. The omentum has been identified as a promising future site for islet transplantation as it retains desirable characteristics of the portal vein such as surgical accessibility and portal drainage while eliminating some of the site-specific challenges to islet viability such as the IBMIR and nonideal vascularization and oxygen tension [30, 137, 233, 279]. Using a translatable site (the epididymal fat pad) in preclinical murine models, the Shea lab has pioneered the use of porous PLG scaffold constructs that allow for rapid engraftment and long-term survival of transplanted islets [35, 124, 132, 133, 235]. Control of the immune response to the transplanted cells still represents a largely unresolved issue, as systemic immunosuppression regimens (a) typically require maintenance administration and are unable to produce durable tolerance (b) show under 50% rates of success maintaining graft survival after 5 years and (c) have been shown to be toxic to islets, limiting dosages that can be administered. I thus recognized an opportunity to design immunomodulatory strategies in the immediate graft environment by taking advantage of the existing biomaterial platforms developed in the Shea lab.

When I joined the lab, a former MD/PhD student Jack Graham had just published a study investigating a co-transplantation strategy where *in vitro* induced Tregs were delivered with islets on PLG scaffolds into an autoimmune mouse model of diabetes [170]. His work demonstrated that localizing the initial transplant of Tregs to the scaffold site conveyed graft survival advantages that could not be recapitulated by an *i.v.* injection of the same number of Tregs. This study underscored the importance of directing immune interventions towards the actual site of immunogenicity and served as a jumping off point for the lab to begin thinking of novel strategies to control the local immune microenvironment.

I began my career in the lab working with a postdoc R. Michael Gower redesigning the PLG scaffold to incorporate and release immunosuppressive factors. Given the difficulties Jack and Michael had experienced with the induction of Tregs *ex vivo*, we reasoned that a more promising strategy moving forward might be to deliver immunosuppressive factors directly into the transplant environment. We selected TGF-β1 as a factor for delivery due to its long history as an immunosuppressive factor and for the possibility that it could endogenously produce local Tregs and recapitulate the effects shown in Jack's work [235]. We also changed the transplantation model from an autoimmune model using spontaneously diabetic NOD mice to a streptozotocin-induced diabetic C57/Bl6 model that could be transplanted with MHC-mismatched islets isolated from Balb/c donors, allowing us to simulate the alloimmune response that human islet transplant recipients face. We modified a previously used layered PLG scaffold design to incorporate TGF-β1 into an inner layer surrounded by the normal porous PLG matrix. Initial characterization of TGF-β1 release from scaffold implants in the absence of islets revealed that leukocyte infiltration was severely decreased across 8 different characterized immune lineages in a dose-dependent response. TGF-β1 delivery was also able to decrease surface expression of activation markers on

APCs and decreased inflammatory cytokine expression within the graft. TGF-β1 showed good compatibility with engraftment of syngeneic transplants and had the desired effect of extending allograft survival time. Analysis of the allograft again showed that a local depletion of leukocytes was achieved at Day 7, though the primary affected cell populations were neutrophils, macrophages and NK cells. Immunofluorescence staining revealed that TGF-β1 release limited localization of immune cells in the peri-islet spaces, particularly in tissue closer to the site of factor release. This study demonstrated that the PLG scaffold itself could be used as a factor delivery platform specifically to delay graft rejection through the modulation of the local immune microenvironment.

While TGF-β1 release showed promising results extending graft survival, its mechanism of action appeared generally immunosuppressive. Additionally, TGF-β1 did not induce an expansion of tolerogenic phenotypes, which would be required to maintain immune tolerance once the factor was depleted. With this in mind, I identified IL-33 as a potential therapeutic candidate based on its recently identified role in maintaining a local anti-inflammatory phenotype in adipose tissue and promising results using systemic delivery to extend cardiac and skin allotransplant survival [237, 241, 243, 244, 248]. Specifically, I was interested in determining whether an adipose-tissue specific population of Tregs expressing the IL-33 receptor ST2 could be expanded by its cognate ligand and used to control the alloimmune response. I altered the design of the PLG scaffold to include BSA as a carrier protein that enabled for increased protein loading into the scaffold. I then confirmed that within the blank implant model, IL-33 release robustly increased the percentage of Foxp3 expressing CD4+ T cells amongst the total CD4+ population. With the addition of allogeneic islets in the scaffold, IL-33 showed even more potent effects expanding Tregs, as I found that upwards of 70% of CD4+

cells in the allograft expressed Foxp3+. The allograft model also showed a large increase in ST2 expression amongst the Treg population, demonstrating TCR activation might enhance the Treg expanding effects of IL-33. Allograft survival data confirmed that IL-33 could enhance transplant survival, with over 50% of allograft transplants surviving over 30 days. Given IL-33 seemed to slow islet engraftment and the literature revealed a number of other potential local cell targets, I also investigated the effects of IL-33 beyond Treg expansion. Gene and protein expression analysis showed the graft environment was highly enriched in Th2 cytokines such as IL-4 and IL-5. Correspondingly, I also identified significant expansions of eosinophils and ILC2s within the graft. Both of these populations are responsive and contribute to a Th2 environment. Th2 cytokines have a controversial in transplant acceptance and rejection and appear to be a likely source of the delay in engraftment caused by IL-33. Despite the short-lived half-life of extracellular IL-33, local release from the scaffold showed long-term effects expanding ST2+ Tregs and inducing target gene expression, making it an intriguing future target for tolerance. This approach also demonstrates the value of interrogating the tissue-specific traits of transplant-site resident immune cells when choosing factors for localized delivery, given not all of the cell types responsible for adipose-tissue homeostasis appeared to benefit allograft survival.

My final contribution to the lab was to begin investigating the use of PEG as an alternative biomaterial to support islet transplantation. Despite our lab's long history of success using PLG for islet engraftment, PEG has a number of appealing properties as a material including its biocompatibility, compatibility with functionalization strategies, and adaptability to various gelation strategies. Previous work in the lab established that nondegradable PEG hydrogel with nonporous architecture preventing islet-tissue contact was able to restore normoglycemia in syngeneic islet transplants. Using the same material to construct a porous hydrogel design aimed

at encouraging tissue infiltration similar to our PLG scaffolds, I worked on characterizing islet engraftment and host response to the two distinct non-degradable hydrogel architectures representative of different biomaterial strategies to enhance islet graft survival. Both scaffold architectures were ultimately conducive to stable engraftment within three weeks of islet transplantation. The pre-engraftment blood glucose levels of the encapsulated hydrogel remained severely hyperglycemic for roughly 10 days while the microporous scaffold showed early normoglycemic readings before experiencing a temporary spike to moderate hyperglycemic levels one week after transplant. As expected, the microporous scaffold which was able directly engraft seeded islets with host tissue performed identically to endogenous islets from a healthy mouse in clearing a bolus injection of glucose, while the encapsulated gel experienced a slight delay, which could be linked to mass transport limitations across the barrier. Interestingly, despite the biocompatibility properties of PEG, the microporous scaffold showed an extreme recruitment of neutrophils into the scaffold environment. The amount of non-degradable surface area appears to play a role since the non-porous hydrogel that limited tissue infiltration did not seem to induce the same response.

The use of biomaterials for immune modulation is still relatively rare outside of experimental pre-clinical models. However, as many natural and synthetic materials gain FDA approval for *in vivo* usage and fabrication techniques become more standardized, it can be expected that more innovative material-based strategies will be pursued that can successfully interface with the body's natural immune response. Increased understanding of the mechanisms of transplant rejection and natural tolerance induction pathways will allow for the identification of novel targets to control. The ability to provide highly localized and controlled immune interventions through the natural interaction between host tissue and transplanted material may lessen the

future need for systemic immune suppression and make islet transplantation a feasible clinical option for a wider range of T1DM patients. Over the course of my dissertation research, I have demonstrated how the design of the biomaterial scaffold itself could be modified to exert control over infiltrating leukocytes that will hopefully serve as a stepping-stone to engineer more sophisticated and durable tolerance-induction strategies.

6.2 Future Directions

6.2.1 Extension of factor release from the scaffold

The work in Chapters 3 and 4 makes use of a layered PLG scaffold design loaded with protein in a central core. Based on *in vitro* release data, we expect the majority of protein to be released within a day of implantation. The protein-incorporating core of these scaffolds contains a mixture of blank 2% PLG microspheres, mannitol to help stabilize the lyophilization product, and the protein of interest. While this fabrication technique is simple and provides consistent loading, the *in vitro* release shows it does not protect protein from immediately being leached out of the scaffold. While this burst release profile showed efficacy modulating the local immune microenvironment immediately after transplantation and effects can extend beyond the first week of transplant, the eventual failure of the graft with these scaffolds can be linked to decreases in factor delivery over time, corresponding loss of immunomodulation, and failure to induce linked suppression and expansion of immunoregulatory cell populations.

Extending factor release could be achieved by a return to the original encapsulated protein designs. The first layered scaffolds for protein release designed in the Shea Lab were developed using PLG microspheres with encapsulated proteins [124]. For some proteins, inner layers fabricated from encapsulated particles showed bimodal release curves. However, the use of

encapsulated microspheres adds a considerable amount of variability to the scaffold production. Encapsulation of protein within PLG microspheres is a complicated process that requires a double emulsion methodology that adds a number of processing steps that can compromise protein bioactivity [280]. The efficiency of protein loading may be highly variable and is dependent on the properties of the protein. Additionally, the encapsulation process adds a number of processing steps that may decrease the bioactivity of the protein. Simpler methods have also been recently developed demonstrating that proteins can be electrostatically adsorbed to the PLG particle surface in a manner that prevents burst release and ensures high efficiency loading, which provide inspiration for design modifications to our PLG scaffold platform [281].

Another approach to extending factor release would be to use viral gene delivery. Our lab in the past has added lentiviral vectors to the scaffold in order to transduce the local cells to overexpress IL-10 to create a locally immunosuppressive site [198]. Compared to biomaterial factor release approaches, viral transduction offers a more sustained release profile depending on the promoter used and the efficiency of gene incorporation. It is unclear whether administration of lentiviral particles directly to the endogenous transplant site will be adaptable for the clinic or if *ex vivo* manipulations of cells will be an easier route of gene editing and engineered factor expression. While extracellular cytokines can affect a wide variety of cells, alternate approaches might be able to specifically target cells of interest. The Bacchetta lab has shown that lentiviral transduction of the *FOXP3* gene into pathogenic CD4+ cells can restore a regulatory phenotype, which could be a powerful approach for developing antigen-specific Tregs [282].

6.2.2. Surface modification of biomaterial scaffolds

The Shea lab has now designed immunomodulatory scaffolds that take advantage of co-

transplantation of immunosuppressive cells in addition to the work detailed in this dissertation delivering soluble cytokines. An additional approach that has yet to be fully explored is the surface modification of the scaffold to conjugate immunosuppressive ligands. Material surface properties play an important role in controlling immune cell phenotype, which is well chronicled through study of the FBR [167]. Though modification of innate material properties like surface charge and stiffness can affect immune activation, specific pathways within immune cells can also be targeted if the ligand-receptors pairs are known [153, 273].

Surface coupling of factors may be able to change their local effects. While we delivered TGF-β1 as a soluble factor, it has been observed that TGF-β1 coupled to a cell surface may have a distinct immunoregulatory mechanism associated with infectious tolerance [283]. TGF-β1 has been bound using a PEG linker to the surface of both a Treg cell membrane or an inert material surface [172]. In addition to possibly preserving TGF-β1 at the graft site longer post-transplant by preventing free diffusion of TGF-β1, surface coupling could be a more faithful recreation of the immunosuppressive mechanisms utilized by regulatory T cells and may help provide a more sustained presence of factors incorporated into our biomaterials.

FasL is a well-known apoptosis-inducing ligand that is a crucial component of immune privileged sites and interacts with the highly expressed FasR/CD95 on T cells to prevent activation. Surface immobilized FasL has been developed as a tolerance induction strategy for islet allografts [174, 284-286]. Initial studies were done by chemically modifying the surface of islets with FasL, leading to induced apoptosis of immune cells coming into contact with islets. While strategies that require additional modification of transplanted islets are not likely to be desirable for clinical use, the presentation of immobilized FasL can be easily replicated by binding it to the surface of a transplantable biomaterial. Our lab has collaborated with another

group to show surface conjugation of FasL to PLG scaffolds provides long-term immune protection allogeneic islets transplanted into the epididymal fat pad. Scaffolds produced with biotinylated PLG microspheres were bound to streptavidin-FasL constructs.

The ability to easily perform surface conjugation is what makes the PEG hydrogel formulations detailed in Chapter 5 an appealing platform going forward. The 4 PEG macromers that were used to create our hydrogel constructs are easily functionalized with peptides [287, 288]. The synthesis of non-degradable hydrogels could lead to sustained presentation of apoptosis or suppression-inducing ligands and cytokines in close proximity to the transplanted at cells, helping maintain long-term survival.

6.2.3 Identifying source of neutrophils in microporous PEG hydrogels

We observed that the majority (>50%) of leukocytes recovered from microporous PEG scaffolds 7 days after implantation expressed a CD11b+ F4/80- Ly6G+ phenotype consistent with neutrophils. This was an unexpected result given the normal percentage of neutrophils present within adipose tissue is closer to 1% and only 10-20% of the leukocytes recovered from the porous PLG scaffolds can be classified as neutrophils. Since the PEG microporous scaffolds were designed to mimic the overall physical structure of the PLG scaffolds, it was surprising to see such a discrepancy in the recruitment of neutrophils, which are typically associated with early stage inflammation, given both PEG and PLG are considered immunologically inert biomaterials. However, there are enough basic differences between the intrinsic physical properties of the two materials that could be identified as the cause of neutrophil recruitment. A time course experiment to examine neutrophil populations at an early time point (e.g. Day 3) compared to a late time point after normoglycemia is re-established (Day 14-21) would be

illuminating to determine the actual dynamics of the neutrophil response. The time course comparison would specifically be useful in comparing immune responses to PEG versus PLG as the neutrophil population at the early time points would allow us to distinguish whether the neutrophil discrepancy at Day 7 is due to an abnormally slow resolution of early neutrophil infiltration versus an increased initial recruitment of neutrophils specifically in the PEG scaffold. We have produced PEG microporous scaffolds using PEG-maleimide (PEG-mal) and PEG-acrylate (PEG-acryl), both of which induce the increased neutrophil populations at Day 7. It is unclear how the free radical polymerization used to crosslink PEG might alter the surface chemistries on the different functional groups. Recent work has shown that altering singular functional groups on alginate can mitigate the foreign body response, opening the possibility that an unforeseen change to the PEG backbone caused by the gelation process could ultimately be causing the aberrant neutrophil infiltration. We are currently in the process of fabricating microporous hydrogels from PEG-vinyl sulfone (PEG-vs) based on the recommendations of the Shikanov lab at University of Michigan, who've anecdotally observed a decreased foreign body response to hydrogels made using PEG-vs compared to PEG-mal or PEG-acryl. Despite the hydrophilicity of PEG being held up as a desirable quality for *in vivo* uses, it would be useful to screen scaffolds fabricated from other polymers with varying degrees of hydrophilicity to determine if the hydrophobicity of polymers like PLG might alter interactions between the material surface and infiltrating cells and be the cause of the large neutrophil population.

6.3 Perspectives on the future of clinical islet transplantation

The future of islet transplantation and whether it will be an option for larger subsets of T1DM patients ultimately rests on the ability to ensure long-term function of transplanted cells.

While current management strategies for T1DM using insulin therapy have clear drawbacks, the limited pool of donor islets makes it practically impossible to offer islet transplantation as a solution for patients that are not at high-risk for SHEs. Moreover, it is difficult to argue that the quality of life decrease from a maintenance immunosuppressive regimen required for graft survival is an improvement on the secondary complications of intensive insulin therapy.

In an ideal future, stem-cell derived beta-cells could be either MHC-matched or otherwise engineered to subvert the problem of the allogeneic immune response, in addition to vastly increasing the available supply of transplantable tissue. While recent research has shown promising results generating insulin-producing stem cell-derived cell clusters mimicking natural human islets, studies transplanting these cells are just beginning in non-human primates and thus it is unclear when exactly such technologies would be ready for expanded human trials. Moreover, while our understanding of T1DM disease pathology is incomplete, it stands to reason that the islet-specific T cell response that incites the disease itself will have a detrimental effect on any transplanted tissue resembling the biology of the islet, whether derived from stem cells or xenogeneic sources. Thus, whether aimed at halting the disease itself or solving the issue of donor-host incompatibility, some level of immune tolerance induction will be required to unlock islet transplantation's full potential. Development of a tolerogenic strategy to correct T1DM-related autoimmunity will likely go hand-in-hand with identifying the best strategy for inducing tolerance to transplanted cells.

Systemic immune suppression is acknowledged by the field to be a poor solution to ensure transplant survival, but its persistence in modern islet transplantation protocols speaks to the fact that a more clinically effective alternative to enhance graft survival does not exist at the moment. The human immune system is necessarily robust in its maintenance of homeostasis. While the

field has identified a number of mechanisms the body uses to distinguish self from non-self, we do not yet understand these processes in sufficient depth in order to truly "reprogram" a patient's immune system without severe intervention. The only existing clinical methodology of tolerance induction that has been successful in humans is mixed chimerism, where the patient's immune system is essentially reconstructed to incorporate both self and donor hematopoietic cells. For a procedure such as islet transplantation where much appeal comes from the minimal invasiveness, systemic immune suppression is ultimately a much more practical solution than an invasive procedure like total body irradiation or lymphocyte depletion coupled with bone marrow transplantation. However, the success of mixed chimerism for different solid organ transplant systems hints at the requirement for tolerance induction strategies to contain both central and peripheral tolerance components. The use of exogenously infused Tregs and other regulatory immune cell populations is likely to be a solution for the peripheral tolerance component moving into the future as technologies to both efficiently expand and genetically edit leukocytes improve. However, it remains an open question of whether deletion of reactive leukocytes can be achieved in a more targeted manner. The idea of delivering antigen under subimmunogenic conditions is not new, but pursuing such a strategy to anergize or decrease reactivity of potentially graft-destructive cells prior to transplant may decrease the need to use broad lymphocyte ablation strategies. It is also possible that general lymphodepletion will always be necessary to sufficiently alter the ratio of suppressive to reactive cell types present in the immune system in order to allow for co-existence of the foreign graft in the host. Ultimately, durable survival of the graft without the requirement of maintenance immune suppression might make the acceptance of temporarily invasive procedures more palatable.

As much work in the immune tolerance community has revealed, it is likely that a combination of immune interventions will be necessary to allow for host acceptance of islet grafts. The core idea forwarded in this dissertation of attempting to localize immunomodulatory effects to the site of immunogenicity ultimately is at the heart of most material-based approaches to enhance islet transplantation. With the field actively looking at using alternative transplant sites like the omentum that can accommodate engineered biomaterials, there is a clear opportunity to imbue transplantable materials with properties that allow them to interact with the local immune response. It is unclear if locally administered interventions alone will be sufficient to control the host immune response but there are clear benefits to targeting delivery of immune intervention to the immune cells being directly activated to attack transplanted cells.

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Vitae

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