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TRANSIENT DISPLAY OF CHIMERIC PROTEINS ON BIOLOGICAL SURFACES AS AN EFFECTIVE STRATEGY FOR MODULATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES

By

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B.Sc, Tribhuvan University

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A Dissertation Submitted to the Faculty of

the School of Medicine at the University of Louisville

in Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy in Microbiology and Immunology

Department of Microbiology and Immunology
University of Louisville
Louisville, Kentucky

May 2020

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April 8, 2020

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DEDICATION

To my parents who supported and inspired me to work hard to pursue my career

To my brother who adores and tells me to have patience and hope

To my dear wife who has been enduring me, caring for me and supporting me mentally, emotionally and physically and makes me a better person.

ACKNOWLEDGEMENT

Foremost, I would like to express my sincere gratitude to my mentor Dr. Haval Shirwan, for the opportunity and continuous support for my PhD research. His continuous motivation, guidance, enthusiasm and support made my research and dissertation possible. I am also truly grateful to my co-mentor Dr. Esma S. Yolcu for believing in me and providing the opportunity to work with her. Her guidance for quality research, motivation for hard work and exceptional support has definitely made this journey a success. I am very much thankful to my committee members Dr. Nejat K. Egilmez, Dr. Mariusz Ratajczak and Dr. Bing Li for their time, support and scientific insights that helped to progress this study. I am also truly grateful to all my past and present lab members Hong Zhao, Kyle Woodward, Hampartsoum Barsoumian, Helen Tan, Feng Zhang, Orlando Grimany, Ali Turan, Alper Togay, Christine Akimana, Lalit Batra, Tariq Malik, Lei Zhang and others for their help, trainings and friendship that made this journey pleasant and easy. I also would like to thank our collaborators: Dr. Andreas Garcia and Dr. Maria Coronel at Georgia Tech and Drs. Subha R. Das, Phil Campbell, Sai Yerneni and others at Carnegie Mellon University for the opportunity to work with these brilliant minds. I am also thankful to Melissa for helping me with blood withdraw. Special thanks to other helping hands, Dr. Rajdeep Bomjan and my parents, my brother and my dear wife for their continuous support and encouragement to pursue my goals and career aspiration. Finally, I would like to thank the department of Microbiology and Immunology, the Institute for Cellular therapeutics for providing great working environment.

ABSTRACT

TRANSIENT DISPLAY OF CHIMERIC PROTEINS ON BIOLOGICAL SURFACES AS AN EFFECTIVE STRATEGY FOR MODULATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES

Pradeep Shrestha

April 8, 2020

The major premise of this dissertation was to transiently display novel immunological ligands on biological membranes as a localized means of modulating innate and adaptive immune responses with applications to bone marrow and pancreatic islet transplantation. In Chapter two, we engineered donor allogeneic bone marrow cells to transiently display a novel form of FasL, SA-FasL, to efficiently purge out alloreactive donor T cells to prevent acute GVHD. In Chapter three, we engineered pancreatic islets with a novel form of CD47, SA-CD47, to modulate instant blood mediated inflammatory reaction (IBMIR) to prevent immediate islet graft loss following intraportal transplantation.

GVHD is initiated and perpetuated by mature T cells in the bone marrow inoculum following transplantation into conditioned recipients. Upon activation, T cells upregulate Fas receptor and become sensitive to FasL-mediated apoptosis. Thus, we hypothesized that the display of SA-FasL on T cells in bone marrow will result in their apoptosis potentially in autocrine fashion following activation in response to recipient alloantigens and engagement of Fas with SA-FasL on the T cells, thereby resulting in the prevention of

acute GVHD. We demonstrated that SA-FasL engineered T cells underwent apoptosis following response to alloantigens both in vitro and in vivo. Most importantly, in an haploidentical rodent setting where C57BL/6 bone marrow cells containing T cells transplanted into lethally irradiated F1 recipients, engineering cells with SA-FasL resulted in the prevention of lethal acute GVHD in 80% of recipients long term (>100 days). We extended this observation to xenogeneic acute GVHD setting, where mice receiving SA-FasL-engineered human PBMCs were significantly protected.

Significant islet mass loss following intraportal transplantation is a major barrier for clinical islet transplantation. IBMIR is initiated and perpetuated by innate immune cells. CD47-SIRPα axis known as innate immune checkpoint delivers "don't eat me signal" to prevent phagocytosis and activation of myeloid cells. Thus, we hypothesized that engineering islets to transiently display SA-CD47 as an innate immune checkpoint will mitigate IBMIR and enhance engraftment following intraportal transplantation. In a syngeneic marginal mass model of intraportal transplantation, SA-CD47-islets showed better engraftment and function as compared with the control group (87.5 vs 14.3%). Engraftment was associated with low levels of intrahepatic inflammatory cells and mediators of islet destruction, including HMBG-1, tissue factor, and IL-1β. Overall, we show that transient display of immunological ligands on biological membranes is effective in modulating innate and adaptive immune responses with significant translational implication for multiple immune-based disorders.

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CHAPTER 1: INTRODUCTION

Overview on hematopoietic stem cell transplantation (HSCT)

Hematopoietic stem cells or bone marrow transplantation is a well-established treatment care for hematologic and non-hematologic malignancies that are not curable by conventional treatment procedures[1,2]. Over the last 50 years, HSCT procedure has evolved from a highly experimental technique to an effective cellular immunotherapeutic treatment against a number of malignancies, metabolic deficiencies, and autoimmune diseases[3,4]. With advances in patient-donor selection, reduced toxicity conditioning regimen, stem cell sources and supportive care, HSCT procedure has seen a significant progress. Annually an estimated 50,000 procedures are done worldwide and more than 1 million transplantations have been performed with 40% being allogeneic in nature[2,5].

The HSCT may be autologous or allogeneic in nature. Autologous transplantation procedure involves infusion of patient's own stem cells. The procedure is mainly performed as a strategy to rescue from lethal chemotherapy against underlying malignancy. Allogeneic transplantation, where immunologically disparate hematopoietic stem cells and immunologic repertoire are infused into irradiation or chemotherapy conditioned patient to establish donor derived hematopoiesis and immunity[6]. Allogeneic transplant modality is currently extended to maintain mixed donor chimerism to establish solid organ graft tolerance[7-9]. Despite the advances, allogeneic HSCT is associated with a major life-

threatening complication, graft-versus-host disease (GVHD), thus limiting the use of this important procedure. GVHD occurs when the immunocompetent donor cells in the graft recognizes the recipient as foreign. The resulting immune response activates donor T cells to attack and eliminate recipient cells as foreign antigen bearing cells[2].

There are two main clinical presentation of GVHD: acute and chronic GVHD with different etiologies and pathophysiology. Acute GVHD was initially defined as a condition appearing within 100 days post transplantation whereas chronic GVHD being later[1,10]. However, this distinction is not tenable anymore as acute GVHD may present beyond 3 months, whereas chronic GVHD may contract within 100 days post transplantation. The present diagnosis criteria involve the specificity of sign and symptoms, rather than the time of onset[11,12]. Acute GVHD manifests as infiltration of inflammatory T cells with target tissue destruction particularly skin, gastrointestinal tract, and liver. On the other hand, chronic GVHD involves the Th2 immune response, autoimmune disease characteristics including autoantibody formation in skin and mucosal surfaces. Acute GVHD is primarily driven by activated T cells and pro-inflammatory cytokines, whereas chronic GVHD is more complex and involving the interaction of innate immune cells with dysregulated B and T cells[2,13].

Immunobiology of acute graft-versus-host disease

Acute GVHD is a severe inflammatory complication of allogeneic HSCT. Despite the advance in preventive and post-transplant strategies, acute GVHD is still considered a significant cause of morbidity and mortality in allogeneic HSCT recipients. Billingham proposed three distinct requirements for GVHD reaction: i) immune responsive donor cells

in the graft; ii) Antigenic disparity between donor and recipient; iii) immune suppressed recipient system that cannot eliminate transplanted donor cells[12,14]. It is now well established that immune competent cells are donor T cells that primarily drive GVHD and can occur in different clinical settings when tissue (bone marrow, blood products or solid organ) containing T cells are transplanted to recipient that is unable to eliminate those cells[2,12,15].

Three-phase model of acute GVHD

The progression of acute GVHD can be summarized in a three step process with an afferent and efferent phase. Phase 1 involves the effect of conditioning in which irradiation and chemotherapy as conditioning regimen induces injury to host epithelium and endothelium generating proinflammatory cytokines and recruits innate immune cells. Phase 2 involves the process of allorecognition, activation and proliferation of donor T cells in the inflammatory milieu induced in phase 1. These two phases make afferent phase of acute GVHD. Whereas in phase 3 as efferent phase the activated multiple effector cells, cytotoxic T cells (CTLs), natural killer (NK) cells and large granular lymphocytes cause significant damage and further injury in specific and non-specific ways[12,16,17]. The three-phase model of acute GVHD is summarized in figure 1.

Phase 1: Effects of conditioning

HSCT recipient undergoes conditioning regimens before transfusion of donor grafts. The procedure involves irradiation and/or chemotherapy. The conditioning therapy is rather toxic to recipient tissues and induces tissue damage. Underlying disease and conditioning induce tissue injury and respond by releasing proinflammatory cytokines (TNF-α, IL-1 and IL6).

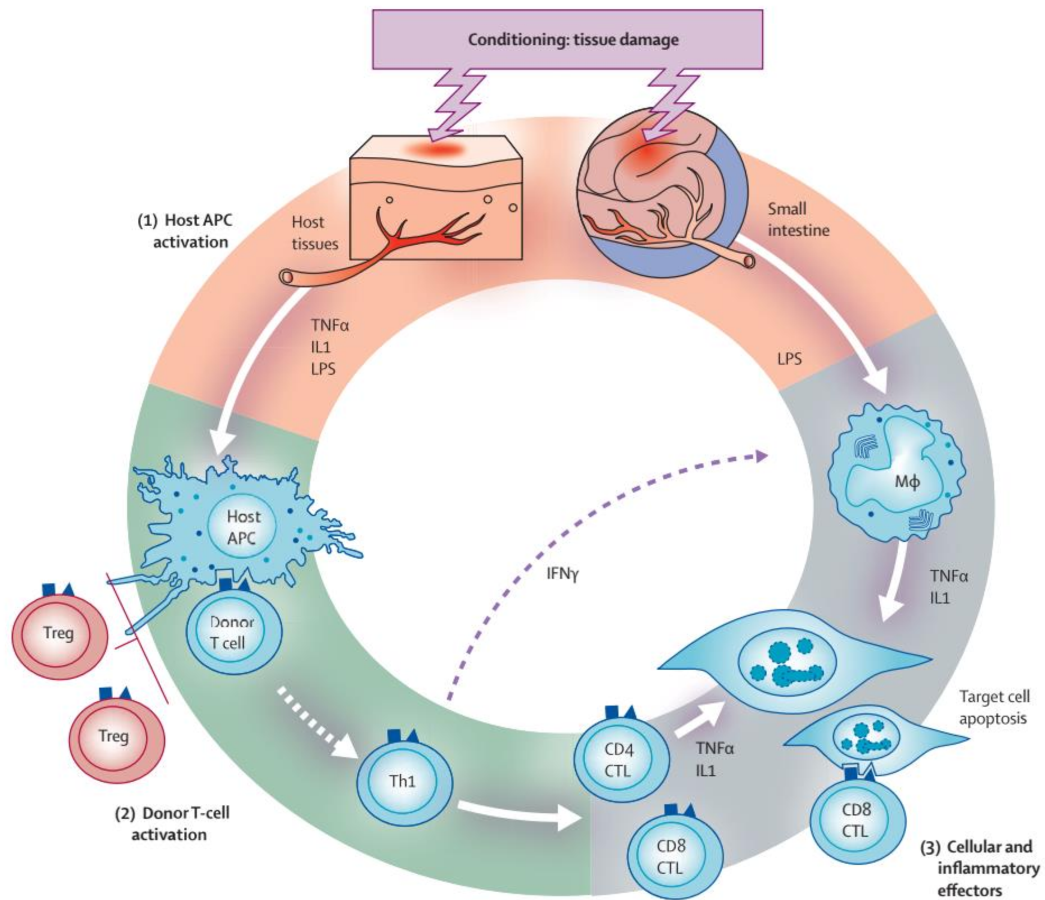

Figure 1: Pathophysiology of acute GVHD. The three phases of acute GVHD. 1. Activation of host APCs because of tissue damage due to conditioning regimen. 2. Activation, differentiation and proliferation of donor T cells by host APCs. 3. The effector phase where complex cascade of effector cells and inflammatory mediators in presence of chemokines and cytokines mediate host tissue damage. (Adapted from Ferrara *et. al*. Lancet 2009).

The proinflammatory cytokines upregulates the expression of chemokines and other adhesion molecules, MHC antigens, costimulatory molecules in antigen presenting cells (APCs)[2,12,18,19]. In addition, gastrointestinal tract is particularly susceptible to injury during the conditioning process. This leads to systemic infusion of inflammatory stimuli such as lipopolysaccharide or other pathogen associated molecular patterns that stimulates gut associated lymphocytes and macrophages to produce TNF-α and IL-1. This directly contributes to "cytokine storm"[2]. Alongside, danger signals such as ATP, uric acid, high mobility group box-1, heparan sulfate released during tissue damage can activate inflammasomes[2,20]. This leads to cleavage of pro-IL-1β to its bioactive form IL-1β and thus enhancing GVHD. This surge of cytokines along with danger signals is important for initiation of primary and secondary immune response. Direct correlation between intensive conditioning regimen and acute GVHD severity is well established and clinical studies suggests that reduced intensity conditioning is associated with significantly reduced morbidity and less early acute GVHD[17,21].

Phase 2: Activation of donor T cells

Interaction between donor T cells and recipient APCs play central role in acute GVHD. This interaction leads to their subsequent activation, proliferation and differentiation and is the second afferent stage in progression of acute GVHD. Donor T cells can be activated directly by host derived or indirectly by donor derived APCs. Presentation of alloantigens directly by host APCs appear to be critical in inducing acute GVHD although indirect presentation by recipient APCs also plays role in activation of donor T cells[22,23]. Dendritic cells (DCs) are the potent APCs during the process.

Inflammatory cytokines (TNF-α, IL-1), danger associated molecular patterns (DAMPs) and pathogen associated molecular pattern (PAMPs) produced during phase 1 (conditioning effects) play important roles in maturation of DCs and induce activation of donor T cells whereas immature DCs induce T cell tolerance[2,12,24].

In addition to engagement of suitable TCR to alloantigen presenting MHC molecule, second costimulatory signal is required for full activation of T cells. Costimulatory signals lower the T cell activation threshold, inhibit apoptosis signaling, maintain cytokine production and support the metabolism of effector T cells. Multiple co-stimulatory pathways including ICOS, CD28, OX40, and 41BB[2,25,26] and negative regulatory pathway including CTLA-4, PD1-PDL1, have been shown to regulate progression of acute GVHD[26,27].

Finally, cytokines play important role in maintenance of T cell activation and survival that drives acute GVHD. Multiple cytokines including IL-1β and Th1 cell cytokines (IFN-γ, IL-2 and TNF-α) mediate T cells differentiation and GVHD pathogenesis[12]. For instance, IFN-γ plays a crucial role in pathophysiology of acute GVHD. T cells isolated in experimental and clinical acute GVHD produce large amounts of IFN-γ[28]. IFN-γ upregulates chemokine expression, MHC molecule and adhesion molecules such that it facilitates antigen presentation and effector recruitment[17]. Also, IFN-γ plays important role in regulating apoptotic death of activated T cells by regulating Fas receptor, thus regulating GVHD[29-31]. In addition, IFN-γ exposure significantly reduces the threshold of LPS required to production of proinflammatory cytokines and Nitric oxide (NO) by macrophages[17,32].

Phase 3: Cellular and Inflammatory effector phase

Multiple cellular and inflammatory mediators mediate host tissue damage during the effector/efferent phase. It is a complex cascade where inflammatory agents (eg. NO. TNF-α, IL-1) and cellular mediators (eg. Cytotoxic T cells, NK cells) work in synergy to amplify host tissue damage thus promoting inflammation and target tissue injury. The primary cellular effectors are CTLs and NK cells that mediate direct tissue damage[33]. They can mediate cytotoxicity by two main pathways. Contact dependent ligation of Fas-FasL results in activation of death-inducing signaling complex (DISC). Activation of DISC results to activation of caspases ultimately leading to apoptotic cell death[34-36]. During GVHD, hepatocytes, epithelial cells on bile ducts upregulate expression of Fas receptor making them susceptible to FasL mediated cytotoxic effect. In contrast, gastrointestinal damage is preferentially mediated by perforin-granzyme-B cytotoxic pathway[37,38].

Activated mononuclear phagocytes are major source of inflammatory mediators including TNF-α, IL-1 that promote direct tissue damage in acute GVHD. TNF-α plays a central role in pathogenesis of acute GVHD. It plays role in activation of DCs and promotes antigen presentation. Also, regulates the recruitment of effector cells (neutrophils, monocytes, and effector T cells) to target organ by induction of inflammatory chemokines (CCL2-CCL5, CXCL2, CCL17)[12,17]. In addition, TNF-α can induce apoptosis and necrosis directly on tissues[33,39]. Nitric oxide (NO) is another inflammatory mediator in acute GVHD. NO promotes pathophysiology of acute GVHD by inducing immunosuppression an inhibiting repair mechanism of target tissue. This results in inhibition of proliferation of epithelial stem cells in gut and skin and induction of direct tissue damage[32,40,41].

Thus, the complex synergistic interaction between cytotoxic effector cells and inflammatory mediators in presence of chemokines and cytokines results in amplification of local tissue injury thus further promoting inflammatory response that ultimately results in observed tissue destruction in the transplant recipient.

Preventive and therapeutic strategies against acute GVHD

Cyclosporine-A, a calcineurin inhibitor and a cytotoxic agent methotrexate (MTX) or mycophenylate mofetil (MTF) are commonly used as standard preventive pharmacologic agents against acute GVHD by multiple clinical centers. MTX/MTF and cyclosporine exert their antiproliferative effect on activated donor T cells by interfering with purine synthesis and calcium dependent TCR signaling respectively. With discovery of tacrolimus, another calcineurin inhibitor with similar mechanism to cyclosporine-A, that has similar or superior efficacy to cyclosporine is now widely used in clinical HSCT[2,12,15]. Tacrolimus/ cyclosporine exert their effect by inhibiting the calcineurin that is required for the activation of nuclear factor of activated T cell (NFAT) family transcription factor that is required for transcription of IL-2 and activation of T cells[42]. Sirolimus, an mTOR inhibitor, is more potent inhibitor of activation and proliferation of conventional T cells than regulatory T cells (Treg cells) owing to dependence of conventional T cells on mTOR-kinase B. High dose of cyclophosphamide is currently used in haploidentical HSCT settings. Two doses of cyclophosphamide are infused immediately post transplantation to eliminate highly proliferating alloreactive T cells but concomitantly sparing of stem cells and Treg cells. Expression of high level of aldehyde dehydrogenase in Treg cells and stem cells is a major in vivo mechanism to cyclophosphamide resistance[43].

Besides the use of pharmacologic agents, manipulation of donor T cells as a prophylactic strategy has been employed in multiple experimental and clinical HSCT settings. One such strategy employs T cell depleted bone marrow graft transplant followed by delayed donor lymphocyte T cell infusion[44-46]. Delayed T cell add back strategy circumvented acute GVHD and restored graft-versus-leukemia (GVL) effect; however, the antileukemic effect was not as efficient as T cell replete transplants[44]. Another strategy involves T cell depletion from the graft. These methods include *ex vivo* negative selection (e.g. monoclonal antibody, lectin agglutination), *ex vivo* positive selection of $CD34^+$ cells, or *in vivo* depletion (anti T cell antibody preparation). However, these strategies substantially limited acute GVHD incidences but did not translate to improved overall survival because of graft failure, Epstein-Bar-Virus associated lymphoproliferative disorder, and disease relapse[2,45,47]. A more subtle strategy to define anti-host GVHD causing T cells and their depletion have been reported. This strategy targets activation induced antigens; whose expression is upregulated on alloreactive T cells upon stimulation in allogeneic mixed lymphocyte reaction culture. Selective depletion of activated cells based on activation markers (such as CD25, CD69, HLA-DR, CD134 and CD137) using fluorescence activated cell sorting (FACS) or CFSE dilution[48] or CD25 conjugated immunotoxin[49] have been reported to improve the outcome of HSCT[47,48,50,51]. These specific alloreactive T cell depletion approach significantly reduces GVHD without losing T cell associated graft-supporting properties and early immune reconstitution[50,52].

Multiple cellular therapy approaches have been tested. Adoptive transfer of ex vivo expanded Treg cells along with T cell replete graft was associated with significantly reduced incidence of acute GVHD with better survival[53]. In addition, adoptive transfer of

donor lymphocytes co-cultured with IL-10 treated host APCs in presence of TGF-β to enrich IL-10 producing type 1 regulatory T (Tr1) cells showed enhanced immune reconstitution with reduced acute GVHD[2].

Despite the prophylaxis, acute GVHD still evolves and the first line of therapy is systemic glucocorticoids like prednisone that have potent anti-lymphocyte and anti-inflammatory activity. Patients refractory to steroid treatment have dismal long term prognosis with overall survival rate of only 5-30%[2]. More importantly, acute GVHD restrict the HSCT to haploidentical population. Thus, there is an acute need for the development of less toxic and more efficacious approaches for bone marrow transplantation that goes beyond haploidentical.

Type 1 diabetes

Diabetes mellitus is a chronic metabolic disorder characterized by persistently increased blood glucose level (BGL). The higher BGL in an individual being defined as fasting glucose level ≥ 125 mg/dl or random non-fasting glucose level ≥ 200 mg/dl or hemoglobin A1c (HbA1c) ≥ 6.5%[54,55]. Persistent higher BGL can cause ketoacidosis, vascular injury, kidney failure, heart disease, stroke and blindness. The world prevalence of diabetes in adults was 6.4% in 2010 and expected to rise to 7.7% by 2030[55]. Every year diabetes is associated with 200,000 deaths and costs $245 billion in US alone[56].

Type 1 diabetes (T1D) is a classical T cell mediated autoimmune disorder associated with loss of pancreatic β-cells. Significant loss of β-cells leads to insulin deficiency resulting in hyperglycemia and ketoacidosis[54]. Although it is termed as "juvenile diabetes" due to more frequent diagnosis in children, majority of patients with T1D are adults and represents 10-15% of total diabetes mellitus cases worldwide. Annually, in average 78,000 youths are diagnosed with T1D worldwide and in US alone > 20 cases per 100,000 people and the incidence rate is expected to rise[56-58].

Etiology of T1D

T1D is a polygenic heritable disease and at least 20 different chromosomal regions are associated to its susceptibility. For instance, identical twin concordance rate of T1D is 30-70%, sibling risk of 3-7% and risk of 1-9% for children with parents who have T1D[59,60]. Multiple genetic factors contribute to both susceptibility as well as resistance to precipitation of T1D. One of the largest contributions comes from several genes located in MHC complex on chromosome 6p21 (i.e *IDDM1* locus). Two HLA class II haplotypes *HLA-DR3/4-DQ2/8*, involved in antigen presentation, are associated with high risk or

disease heritability, whereas *HLA-DR2-DQ6* allele is protective[57,59-61]. Genome-wide association studies have identified multiple non-HLA alleles, *VNTR, PTPN22, CTLA4, IL2RA*, associated with susceptibility to T1D. These genetic variants are involved in immune response that contributes to dysfunctional immune responsiveness, including development and maintenance of tolerance[60]. Despite the genetic factors, the concordance rate of T1D among monozygotic twins is only 50%, implicating the potential role of environmental factors in development and precipitation of T1D. One of the major candidates is viral infection. Extensive data suggests coxsackieviruses, an enterovirus infection precipitates T1D[62]. With evidence of enterovirus in pancreas of recent onset T1D patients and epidemiological studies suggesting significantly more enterovirus infection among diabetic patients than in controls supports the notion that viral infections are the environmental triggers. Many viruses can cause chronic or latent infection. Direct evidence comes from a study where coxsackievirus can infect β-cells and cause insulitis and diabetes in murine models[63]. With the landmark finding by Foulis et al.[64] it is suggested that β-cell tropic viral infection upregulates HLA class I and IFN-α as "viral molecular signatures". In addition, significant sequence similarity between coxsackievirus protein 2C and glutamic acid decarboxylase (GAD) protein, a major T1D autoantigen, postulates the viral mimicry as an etiology of T1D[65]. Coxsackievirus induced upregulation of CXCL10 chemokine on pancreatic islets plays important role in recruitment of CXCR3 positive autoreactive T cells following viral infection[66]. Cumulatively, available data suggest the significant role of environmental triggers, viral infections, microbiome composition and metabolites, milk and wheat proteins, in unleashing autoimmunity, leading to destruction of beta cells that ultimately results in T1D.

Silent immune events targeting the destruction of pancreatic β-cells may take years before clinical symptoms of T1D becomes apparent. Autoantibodies and activated autoreactive T cells infiltrate the pancreas to destroy insulin producing β-cells. The persistent destruction of pancreatic β-cells is a slow process and may become undetectable until the majority (>80%) of pancreatic islets are destroyed or rendered dysfunctional, making an individual hyperglycemic and dependent on exogenous insulin[54,62]. $CD8^+$ T cells are the predominant population in the insulitis region, followed by macrophages, $CD4^+$ T cells, and B cells.

Van Belle TL *et al*.[62] proposed that the disequilibrium between immune suppressive mechanisms (Treg cells) and autoreactive T cells occur over time and shifts the balance to islet autoimmunity, leading to significant loss of islets mass and T1D (Figure 2). $CD8^+$ T cells mediated killing of beta cells, by release of perforin and granzyme or Fas-FasL dependent interaction, is likely the central mechanism of beta cell destruction. $CD4^+$ T cells likely contributes to activation of B cells and $CD8^+$ T cells by secretion of cytokines (IL-21) or by positive feedback mechanism (CD40-CD40L)[62,67].

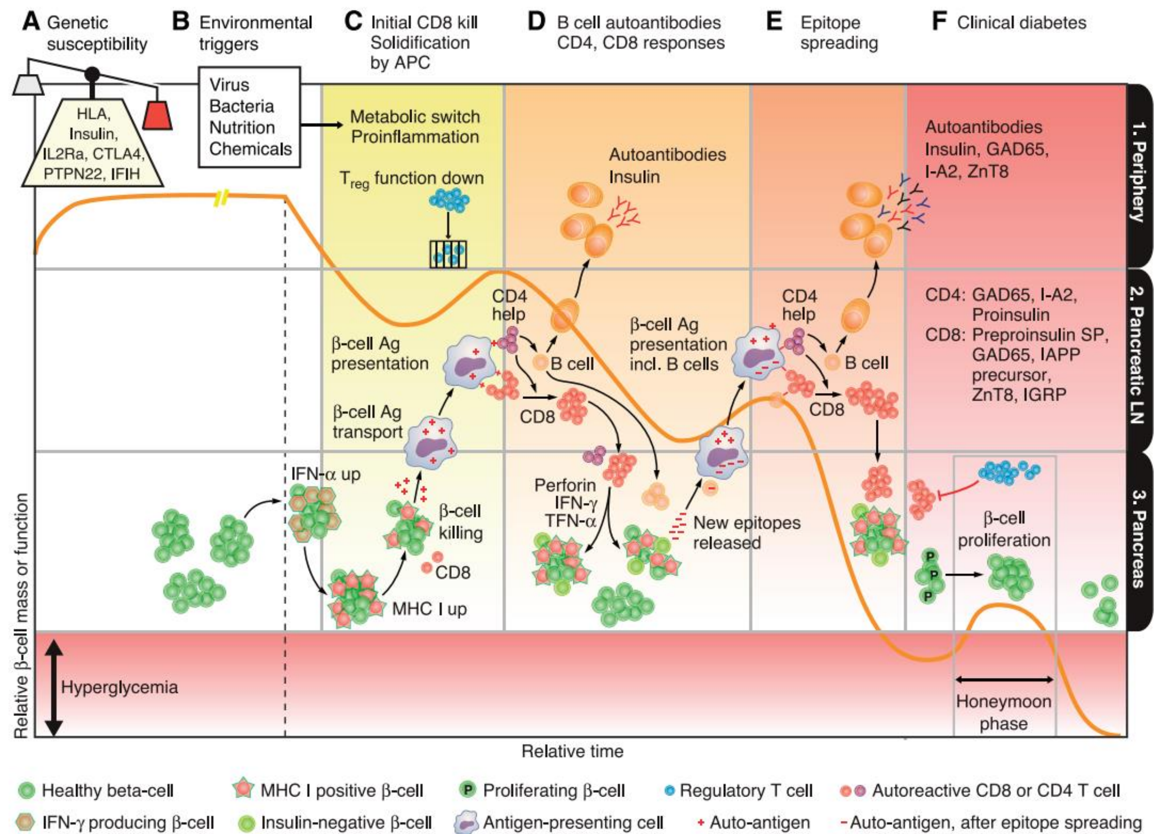

Figure 2: Progression of type 1 diabetes. (Adapted from Van Belle TL *et al.* 2011.)

Medical management of T1D

Once T1D is diagnosed, the main objective is to maintain the BGL near normal level. Insulin remains the mainstay of the therapy although insulin analogs are available to manage BGL. Frequent glucose monitoring and multiple dose insulin regimen is required for optimal glycemic control. Multiple insulin injections or insulin pumps have their own complications and are limited to controlling medical complications, including hyper or hypoglycemia and vascular injury[54]. Hypoglycemia and ketoacidosis are potentially life-threatening complications. Genetic predisposition with serological evidence for multiple autoantibodies is enough to predict the susceptibility of T1D. Multiple preventive strategies

that target autoimmune responses are being tested to prevent progression of T1D. Most strategies broadly implement immunomodulatory agents to maintain tolerance. Nevertheless, it is still at the primacy for prevention and reversal of T1D with no therapeutic agents being approved[59,68,69]. Following the breakthrough protocol for islet transplantation, the procedure has become standard of care treatment for individuals who have developed end-stage renal failure. Pancreatic islet transplantation involves infusion of donor islets into the liver via portal vein.

Pancreatic islet transplantation

Pancreatic islet transplantation (PITx) involves isolation of islets (containing β cells along with other endocrine and non-endocrine cells) and infusion into the liver of recipient via hepatic portal vein. Since the first islet transplantation was performed two decades ago, PITx is now considered safe and real therapeutic option for patients with chronic pancreatitis (autotransplantation) or in selected patients with affected by T1D (allotransplantation)[70,71].

Chronic pancreatitis is a syndrome with progressive inflammatory condition in pancreas leading to permanent damage leading to impaired endocrine and exocrine function[72]. This disease is the result of multiple environmental and genetic factors and involves replacement of pancreatic secretory parenchyma by fibrous tissue. Chronic pancreatitis is debilitating, painful, and eventually leads to diabetes. Total pancreatectomy followed by auto islet transplantation is performed to eliminate pain of pancreatitis and mitigate resultant pancreatogenic diabetes. So far more than 500 islet autotransplantation have been performed for cases of chronic pancreatitis. The procedure involves complete resection of pancreas followed by isolation of healthy islets from the diseased pancreas and

reinfusion of isolated islets to patient via hepatic portal vein for intrahepatic engraftment without use of immunosuppressive drugs[72,73].

In addition to patients with chronic pancreatitis, islet transplantation is also proposed for selected patients with other clinical indications: i) brittle T1D with unaware episodes of hyperglycemic and hypoglycemia; ii) combined organ transplantation (kidney) in T1D patients with kidney failure; iii) T1D patients under immunosuppressive therapy for other autoimmune diseases[70]. Pancreatic islet transplantation along with whole pancreas transplantation can restore normoglycemia as well as achieve exogenous insulin independence. However, whole pancreas transplantation is associated with significant morbidity and mortality at early transplant period. Instead pancreatic islet transplant is minimally invasive and is carried out by infusion of islets into the liver via portal vein[74]. With current advances, pancreatic islet transplantation is equally efficient to whole pancreas transplantation in normalizing BLG. The excellent therapeutic effect is the accurate blood glucose level responsive insulin secretion. The success of islet transplantation was realized after "Edmonton protocol" was established. The protocol uses glucocorticoid free immunosuppression and the long-term outcome results in 50-70% of patients achieve insulin independence at 5 years. More than 1500 procedures have been performed worldwide as of date. However, limited pancreas supply and use of chronic immunosuppression are the major contributing factors that restricts the applicability of this procedure[75].

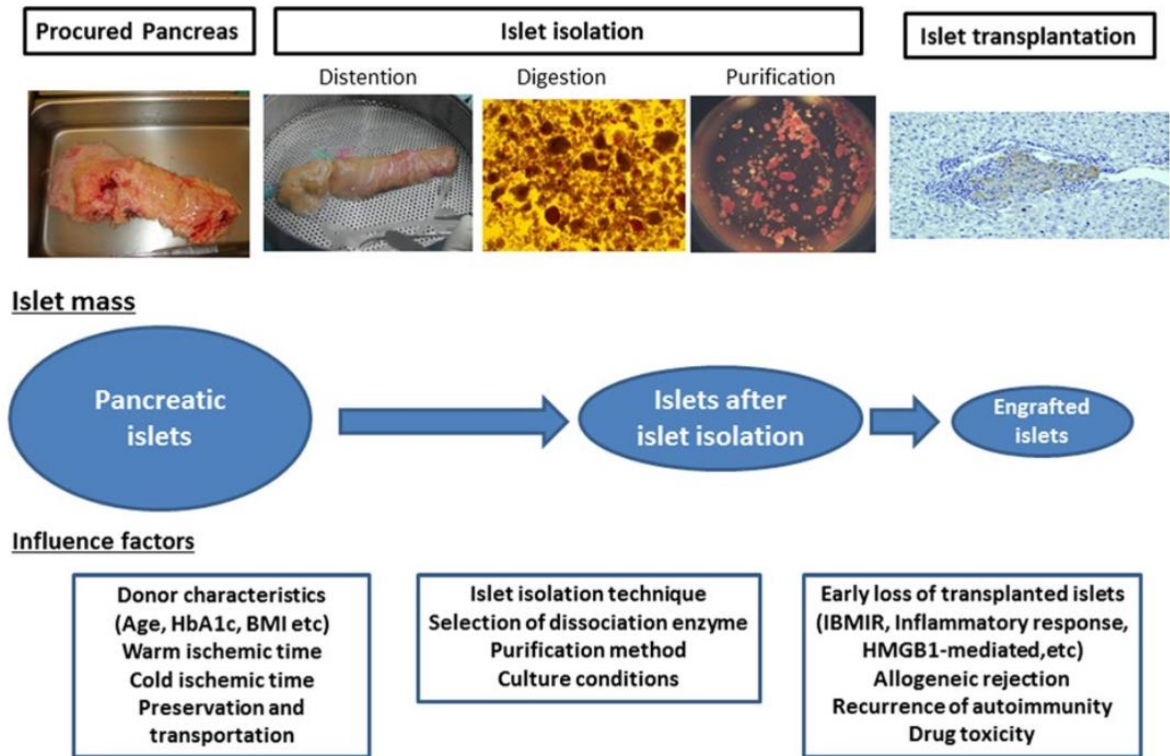

Figure 3: Pancreatic islet transplant procedure and factors contributing to loss of islets. (Adpated from Anazawa *et al*. 2019)

Transplantation into the liver via portal vein is the only clinically approved site for islets. Despite the progress in islet isolation and transplantation procedure, intraportal islet transplantation suffers from significant loss (50-70%) of functional islet mass during the peritransplant period[76,77], thus requiring islets from more than two donors to stabilize BGL[78]. Despite being transplanted from multiple donors, transplanted recipients have β-cell function of only 20% of that in healthy individuals[79]. Multiple factors contribute to this that include localized hepatic ischemia/reperfusion, low oxygenation rate, endogenous liver immune response, and instant blood mediated inflammatory reaction (IBMIR).

Instant blood mediated inflammatory reaction (IBMIR)

IBMIR is a complex non-specific innate immune response generated by contact of islets to blood when islets are infused to liver via hepatic portal vein. IBMIR is characterized by rapid activation of coagulation and complement system, rapid activation of platelets, neutrophils and binding. This generates islet-thrombus and intra-islet infiltration of dense lymphocyte and macrophage. This cascade of inflammatory reaction ultimately leads to disrupted islet integrity, morphology and loss of islet mass[80,81].

Activation of coagulation cascade and complement includes the initial steps in IBMIR. Islet isolation step involves treatment of whole pancreas with collagenase and isolation of "naked islets". This process exposes molecules (collagen, laminin) that are thrombogenic in nature. In addition, isolated islets also lack membrane regulators (heparan sulfate)[81,82]. Cumulatively, the exposure of naked islets to blood facilitates activation of coagulation and complement. Further, stress during the procurement procedure, isolation and culture and brain death of donor induces pro-inflammatory signature with significant upregulation of tissue factor (TF), monocyte chemoattractant protein-1 (MCP-1), IL-1β and TNF-α. Strong coagulation cascade peaks as early as 6-12 hrs post transplantation and associated with upregulated levels of different pro-coagulating factors. Sequentially, IBMIR is also associated with activation of complement. Deposition of IgG, IgM along with C1q, C4, C3 and C9 result in formation of pro-inflammatory anaphylatoxins C3a and C5a[80,81,83,84]. Activated thrombin promotes the secretion of adhesion molecule (p-selectin), thus activating platelet aggregation. This results in generation of islet-thrombus.

A panel of cytokines (IL-6, IL-8) recruit neutrophilic granulocytes and macrophages into the hepatic graft site. Under hypoxic condition, islets express pro-

inflammatory and danger signals like high mobility group box-1 (HMGB-1), IFN-γ, IL-8, MCP-1. The intraislet macrophages, kuppfer cells, and neutrophils secret IL-1β that directly impairs insulin secretion and induce islet apoptosis[85,86]. Moberg *et al.*[87] provides direct evidence of IBMIR mediated islet loss using in vitro loop assay. Accordingly, rapid platelets deposition occurs as early as 5 mins reaching maximum by 30 mins. Whereas $CD11b^+$ neutrophilic granulocytes are predominant cell types infiltrating the islets. The cells occur as early as 15 mins and peaks by 2 hrs[87]. Activated neutrophilic granulocytes generate reactive oxygen derivatives. ROS along with proteases liberated from the granules of activated granulocytes implement direct damage to the islets[84,87,88]. In addition, they secrete MCP-1 to attract macrophages. Macrophages exert killing by phagocytosis, but simultaneously also act as APCs communicating with lymphocytes in the activation phase of specific allo-immune responses[76].

Strategies to prevent IBMIR

Multiple approaches have been tested to abrogate IBMIR, however all strategies focus on the coagulation cascade to prevent generation of islet-thrombus. Clinically, heparin infusion is performed to prevent coagulation. Final islet product is infused in suspension of transplantation media containing heparin (70 units per Kg of recipient body weight). Systemic heparin infusion is performed for 48 hrs (3 U/Kg/hr) to prevent IBMIR[75]. However, Bennet *et al.*[89] suggested, in *ex vivo* settings, that even using a high level of heparin (4 U/ml) was not enough to prevent extensive fibrin deposition and infiltration of $CD11b^+$ cells. In addition to the risk of systemic bleeding, the relatively short half-life of systemic heparin impacts the therapeutic potential. Alternatively, Cabric *et al.*[90] used biotin/avidin strategy to display 40 U of heparin complexes per islet to mitigate IBMIR in

murine and porcine models. They reported prolonged syngeneic islet graft survival when 300 islets were transplanted under the kidney capsule in a mouse model. However, under clinical settings islets are transplanted to a patient's liver via hepatic portal vein. IBMIR is only observed in intraportal settings where islets come in direct contact with recipient's blood circulation. Other anti-coagulation strategies involving low-molecular-weight dextran sulfate[91] and the combination of Tirofiban and activated protein C (APC)[92] have been reported to ameliorate IBMIR in *ex vivo* models. A study by Contreras *et al*.[93] used a systemic bolus of recombinant APC to mitigate the early loss of islets. However, the use of anti-coagulating agents is always linked to high risk of bleeding and hepatic hematoma[94,95]. Strategies targeting anti-inflammatory[88,96,97] and chemokine inhibitors[98] have also been reported to mitigate the effect of IBMIR.

CD47-SIRPα, an innate immune checkpoint

CD47, also known as integrin associated protein (IAP), was identified as missing protein in Rh-hull red blood cells. The same protein was also isolated with αvβ3 integrin in leukocytes and placenta[99]. CD47 is ubiquitously expressed by virtually all cells in the body. It interacts in *cis* with integrins, as well as acts as ligand for two members of signal regulatory protein (SIRP) receptor family[100]. CD47 is also a receptor for secreted protein thrombospondin-1 (TSP-1). CD47 is known to modulate immune responses in neutrophils, macrophages, dendritic cells and T cells[101].

CD47 is a member of immunoglobulin (Ig) superfamily with heavily glycosylated IgV-like extracellular domain at its N-terminus, highly hydrophobic five putative membrane spanning transmembrane domain, and alternatively spliced C-terminal

cytoplasmic domain. The extracellular domain is required for functional and physical interaction with integrins, TSP-1 and SIRP-α[99,102]. However, functional domain for enzymatic or protein interaction in the cytoplasmic tail is still at large. Signal regulatory protein (SIRP) belongs to Ig family of glycoproteins. SIRP family consists of an inhibitory receptor SIRPα (SHPS1), activating receptor SIRPβ1 and nonsignaling receptor SIRPγ[100,103]. SIRPα is widely expressed on myeloid cells, macrophages, dendritic cells and precursor including hematopoietic stem cells. SIRPα is an inhibitory receptor and interact with CD47 via three Ig-like extracellular domain. It contains tyrosine based immunoreceptor tyrosine based inhibitory motif (ITIM) domain in cytoplasmic tail. Upon ligand binding, the cytoplasmic domain gets phosphorylated and mediates recruitment and activation of Src-homology domain 2 containing phosphatase SHP-1 and SHP-2 and ultimately negatively regulates intracellular signaling[100,103].

CD47 modulates multiple cell activities including activation of neutrophils and platelets, transmigration of leukocytes, cell mobility and adhesion, and phagocytosis. The importance of CD47 in immunoregulation was revealed by the observation that pox virus upregulate homolog of CD47 as virulence factor to evade host immune responses[104,105].

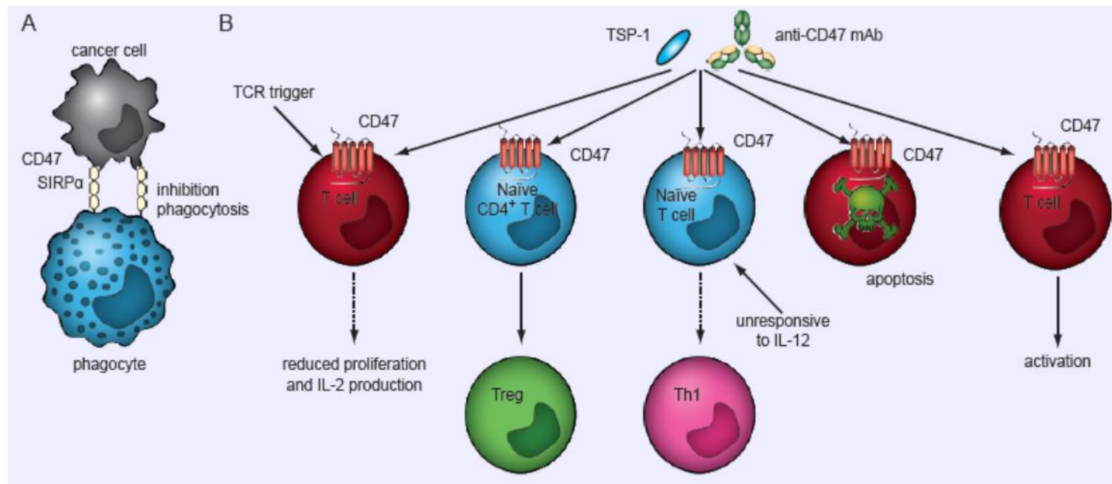

Figure 4: Multifactorial immunoregulatory role of CD47 (Wiersma, Valerie R., et al. *Atlas of Genetics and Cytogenetics in Oncology and Haematology* 2015.)

CD47 delivers "don't eat me" signal

One of the well-studied aspect of CD47 is "marker of self" or "don't eat me" signal delivered to macrophages and dendritic cells via SIRPα[100,106-108]. Macrophages and dendritic cells are important regulators of innate and adaptive immune system. They constantly screen for distinction between self and non-self, such that recognition of pathogens by pathogen associated molecular patterns (PAMPs) will trigger their activation, leading to initiation of innate immune response. Interaction of CD47 as "marker of self" on healthy cells with SIRPα expressed on macrophages and dendritic cells results in inhibition of phagocytosis, hence coined as "don't eat me signal"[105,109]. Evidence of this phenomenon came from observations that CD47-deficient red blood cells (RBCs) are efficiently cleared within hours by splenic macrophages, whereas healthy RBCs have long half-lives in circulation[110,111]. Also, CD47 is transiently upregulated by hematopoietic stem

cells upon mobilization or after strong inflammatory signal[112]. This transient upregulation is necessary for HSC to avoid being phagocytosed and cleared during their mobilization from bone marrow to blood. Studies also suggests that leukemic cells upregulate CD47 expression as an effective evasion mechanism. Increased expression of CD47 was associated with worse overall survival prognosis in patients and contributes to progression and pathogenesis of the disease due to engagement with SIRPα to inhibit and evade phagocytosis[113].

Studies suggests expression of CD47 on DCs is important for homeostasis and migration across lymphatics and during inflammatory condition. CD47 is necessary for maturation of DCs and efficient T cells priming[114]. $CD47^{-/-}$ murine had selective reduction of marginal zone $CD4^{+}$ DCs and blunted immune responses, suggesting the important role of CD47 in DC maturation and homeostasis. Furthermore, CD47 deficient DCs had impaired migration to draining lymph nodes, despite the normal expression level of chemokine receptor[115]. CD47 has been reported to play a role in activation and transmigration of neutrophils and platelets. Finley *et al.*[116] showed that blood conduits modified with recombinant CD47 inhibited activation and adhesion of platelets and neutrophils. Similar studies suggested CD47-SIRPα signaling can inhibit activation and mobilization of neutrophils and macrophage in CD47 adhered surfaces[117]. In addition, the axis is also important for antibody dependent trogocytosis, a mechanic destruction of target cell plasma membrane, of cancer cells by neutrophils[118].

CD47-SIRPα interaction is highly species and strain specific[100] and plays significant role in graft tolerance[119,120]. Xenografts activate recipient phagocytes due to the lack of interaction of donor CD47 with recipient SIRPα. However, xenografts displaying

recipient CD47 significantly diminishes phagocytic response with survival of grafts[119,121]. This is evidenced by significant human hematopoietic engraftment in non-obese diabetic (NOD)-severe combined immunodeficiency (SCID) mice because NOD-SIRPα shows enhanced binding to human CD47[122].

ProtEx^{TM} technology as a facile and effective platform for localized immunomodulation

Immunomodulation by definition encompasses all therapeutic or preventive interventions intended to modulate immune responses[123]. Multiple modalities are employed to manage immune responses based on disease condition. For instance, augmentation of immune response is desirable to combat infection, to prevent infection in states of immune deficiency, as well as to fight cancer. In contrast, in settings of transplantation, allergy, or autoimmunity strategies to dampen the immune response are desirable[123-125]. Multiple drugs or therapeutic agents are often used as single agents or in combination to modulate the immune system. These agents may interfere with immune responses at different levels, antigen presentation, T cell activation, proliferation of regulatory cells, or induction of apoptosis of selective cell types.

In principle, the immunomodulatory biologics are used systemically, i.e., the infusion into the system by various routes of injection. This schema has significant drawback as systemically introduced biologics are rapidly cleared from the system. Alternatively, genes encoding these biologics can be introduce to target cell/tissue as an attractive platform for the treatment of various inherited and acquired immune disorders[126]. However, there are multiple challenges for clinical applicability of gene therapy, including

safety issues, inefficient delivery of gene of interest, and regulation of target gene expression[126,127]. As an alternative approach, our lab has pioneered the ProtEx™ technology that provides a platform to generate functional immunological ligands chimeric with core streptavidin and their transient and rapid display on biotin-modified biological surfaces for localized immunomodulation. Another major advantage of the platform is the ability to simultaneously display multiple immunomodulatory proteins for improved efficacy.

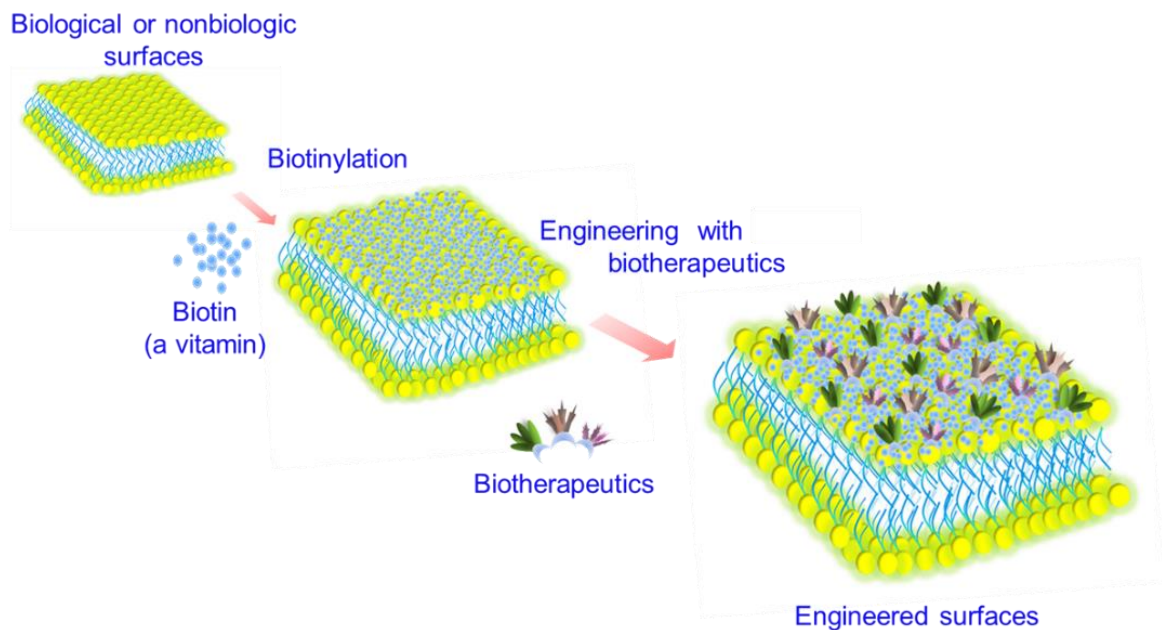

Figure 5: ProtEx™ technology to display multiple immunomodulatory proteins on the biological or nonbiological surfaces.

Multiple functional immunomodulatory proteins, including SA-FasL, SA-PDL1, and SA-4-1BBL, have been generated using this platform and been used to induce localized tolerance to islet grafts[128-130] and cardiac grafts[131], efficient engraftment of stem cells[132,133] and development of cancer vaccines[134,135]. Here, in this set of study we extend our observation as efficient immunomodulatory approach in acute GVHD and IBMIR by targeting SA-FasL and SA-CD47, respectively.

CHAPTER 2

TARGETING FAS-FASL PATHWAY FOR SELECTIVE ELIMINATION OF ALLOREACTIVE DONOR CELLS FOR PREVENTION OF ACUTE GRAFT VERSUS HOST DISEASE

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established curative treatment modality for multiple high-risk hematologic malignancies as well as uncurable non-malignant hematological and genetic disorders[2,4,51]. However, the beneficial efficacy of this treatment is significantly limited by graft-versus-host disease (GVHD). GVHD represents a complex disease; while several factors contribute to its pathogenesis, the major mechanisms underlying the disease are well-elucidated. Interaction of T cell expressing suitable T cell receptor (TCR) with antigen presenting cells (APCs) that express recipient allogeneic MHC molecules is the driving force for the development of acute GVHD. T effector cells activated in response to recipient alloantigens expand and inflict damage to the recipient tissues. T cells are the main effectors of targeted tissue damage[2]. The GVHD causing alloreactive cells constitute a minor subset of total T lymphocytes[48], which presents technical challenge for their complete elimination. Although, rigorous pan-T cell depletion from the donor graft can significantly reduce the incidence and severity of acute GVHD, the procedure is associated with multiple complications including failure in graft engraftment, leukemia relapse, and delayed immune reconstitution[47,48,51]. Thus, the major challenge is to control donor immune reconstitution in the recipient post-

transplantation, conserve beneficial donor T cell immune function against leukemia and infections and reduce the severity of acute GVHD.

One of the distinctive features of the immune system is homeostatic control involves a phase of contraction after clonal expansion of antigen-activated lymphocytes to titrate the level back to the base line[35,36,136,137]. This is achieved by fine tuning between expansion and death triggered by apoptosis, activation induced cell death (AICD). AICD is mediated by Fas/FasL (CD95/CD95L) pathway and is an important physiologic strategy to control the expansion of antigen-activated T cells. AICD requires repeated antigen-specific TCR re-engagement in the presence of IL-2 and does not affect bystander T cells[30,52,136,138]. Resting T cells do not express Fas-receptor and express multiple apoptotic inhibitors (e.g. Flice like inhibitory protein, FLICE, or surviving), whereas activated antigen-specific T cells upregulate Fas receptor and significantly downregulate anti-apoptotic molecules[36,52,139]. This marked difference makes alloreactive T cells prone to Fas/FasL mediated apoptosis, while endowing resistant to resting or bystander T cells. In addition, multiple studies suggest that HSCs[133,140-143] and immunosuppressive regulatory T cells[144] are highly resistant to FasL-mediated AICD. The differential response of activated, naïve, regulatory T cells, and HSC to Fas/FasL-mediated apoptosis provides an attractive approach to use FasL to selectively eliminate alloreactive T cells without a major negative impact on HSCs as well as resting T cells that aid in engraftment and T regulatory cells that modulate alloreactive immune responses.

In our previous studies, we have reported a novel form of FasL protein chimeric with streptavidin (SA)-FasL that exist as tetramers and oligomers with potent apoptotic activity on Fas-expressing cells[130,145]. The protein can be efficiently and rapidly displayed

on the surface of biotinylated cells and biologics[128,130,133,143,145]. Most importantly, SA-FasL engineered cells or biologics could be employed to eliminate alloreactive T cells for systemic or localized immunomodulation[128,130,131]. Since upon activation alloreactive T cells upregulate Fas expression and become susceptible to FasL-mediated apoptosis, we hypothesized that the transient display of SA-FasL on donor cells will induce apoptosis in T cells following activation in response to the recipient alloantigens, thereby blocking acute GVHD. In line with our expectation, there was significant elimination of donor T cells upon allogenic stimulation in *in vitro* as well as *in vivo* settings. To test our strategy in mitigating lethal acute GVHD, we employed the haploidentical parent (C57BL/6) to F1 (C57BL/6xBALB/c) setting. We report that recipients that received SA-FasL-engineered bone marrow cells containing mature T cells were protected from lethal acute GVHD and survived long term (>100 days), whereas all controls displayed clinical signs of acute GVHD and met end point by 40 days. The long-term recipients were immunocompetent as they rejected third party, but not donor, skin grafts. Importantly, this concept also showed efficacy in a humanized mouse model for xenogeneic GVHD where human PBMC engineered with SA-FasL was used for transplantation. Thus, this approach has significant translational potential for the prevention of acute GVHD as a single modality or in combination with other clinically used approaches.

Materials and methods

Animals

C57Bl/6 ($H2^b$), BALB/c ($H2^d$), C3H ($H2^k$) and NOD-*scid*-IL2γR^{null} (NSG) mice were purchased from the Jackson Laboratory and breed in our specific pathogen free facility. C57BL/6.FoxP3^{hCD2} (hereafter referred as B6.hCD2)[146] and B6.SJL-4C.TCR-tg (hereafter referred as 4C.SJL)[147] animals were generously provided by Drs. H. Waldmann of Oxford University and TV Brennan of Duke University respectively. F1 (C57BL/6 x BALB/c, $H2^{b/d}$) were breed in our facility. All animals were maintained in our specific pathogen free vivarium at the University of Louisville. All experiments were performed in accordance to approved protocols by Institutional Animal and Use Committee, University of Louisville.

Modification of cell surface to engineer with SA-FasL protein

Spleens from C57BL/6 or 4C.SJL were harvested and processed into single-cell suspension using frosted slides. Red blood cells were lysed using a home-made buffered ammonium chloride solution. SA-FasL engineering of spleen cells was done following previously reported protocols[145]. In brief, cells were incubated in 5 µM EZ-Link™ Sulfo-NHS-LC biotin (hereafter referred to as biotin) solution (ThermoFisher Scientific, Ref# 21335) in sterile PBS at room temperature for 30 min. After washing with PBS, cells were incubated in PBS containing indicated amount of SA- or SA-FasL protein for 30 min in a cold room by constant rocking. Cells were washed before further experimental use.

MLR cultures and proliferation assay

Standard mixed lymphocyte culture assay was performed[128,130] where spleen cells from 4C.SJL was used as responders against 2000 cGy irradiated BALB/c spleen cells. Briefly, SA/SA-FasL engineered at indicated levels or unengineered 4C.SJL spleen cells were incubated at 37 °C for 45 mins to collect pan-T cells. Non-adherent pan-T cells were collected, washed and co-culture with irradiated BALB/c spleen cells (10^5 cells each/well) in 96 well U-bottom plate in complete MLR medium[128]. For [^{3}H]thymidine incorporation assay, the cultures were pulsed with [^{3}H]thymidine (1μCi/well) for last 16 hrs before harvest. Cultures were then harvested with Tomtec cell harvester and analyzed in a beta plate counter to assess DNA-associated radioactivity as measure of proliferation [counts per minute (cpm)]. For flowcytometric analysis, the cells were harvested, and surface stained as described below.

For in vitro apoptosis assay, spleen cells from 4C.SJL mice were engineered with SA-FasL (100 ng). SA-FasL engineered or unmodified 4C.SJL spleen cells were fluorescently labelled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE; ThermoFisher Scientific Ref# C34554) or 2.5 μM Cell trace violet (CTV; ThermoFisher Scientific Ref# C34557), respectively, following manufacturer's instruction. Fluorescently labelled 4C.SJL pan-T cells were mixed together in equal ratio to generate 4Cmix (1:1) cell suspension. Irradiated BALB/c spleen cells were cultured with 4Cmix cells at different indicated ratio in complete MLR medium. At indicated time points cells were harvested and analyzed for $H2K^{d-}$ cells by flow cytometry.

In vivo monitoring of adoptively transferred cells

Spleen cells from 4C.SJL or B6.hCD2 animals were harvested and engineered with SA-FasL or SA (control) proteins at indicated levels. Engineered cells were fluorescently labelled with 2.5 μM cell trace violet (CTV). Each F1 recipient was intravenously injected with $5x10^6$ CTV labelled SA- or SA-FasL-engineered 4C.SJL cells or $10x10^6$ SA/SA-FasL-engineered B6.hCD2 spleen cells. Animals that received 4C.SJL or B6.hCD2 spleen cells were euthanized 48 or 72 hrs post transplantation and spleen cells were analyzed by flow cytometry for donor cells.

Collection of human PBMC

Human Peripheral blood mono-nuclear cells (PBMCs) were isolated as reported before[148] from healthy donors under signed informed consent approved from Institutional review board of University of Louisville. Peripheral blood was collected in heparin containing vacutainer (BD Bioscience, Ref# 364606). PBMCs were purified and collected from buffy coats by Ficoll-paque (GE, Ref# 17-1440-03) density centrifugation and washed by sterile PBS before further use. Engineering of human PBMCs was done as described before[145].

Induction of acute GVHD in the parent-to-F1 setting

A clinically relevant haploidentical setting was used to assess lethal acute GVHD as described before with some modifications[39]. Female or Male F1 ($H2^{b/d}$) recipients, 10-12 weeks old, were lethally irradiated with single dose of 1000 cGy (Gammacell 40 Exactor,

^{137}Cs source) and 4 hrs later transplanted with 10x10^6 whole bone marrow cells and 20x10^6 whole spleen cells from female or male C57BL/6 (H2^b) mice. For engineering the graft inoculum, only whole spleen cells were engineered with SA-FasL as described above. Animals were followed twice a week for acute GVHD scoring, including five clinical parameters as explained before[149,150]. Each parameter received score of 0 (minimum) to 2 (maximum). A clinical GVHD score index was generated by summation of the five criteria score (maximum value=10). Animals that reached total score of > 6 and lost body weight >25% was considered as at the end point. All animals were supported with soaked food throughout the study.

Skin transplantation

Skin grafts were procured from tail of euthanized BALB/c (H2K^d) donor or C3H (H2K^k) third party mice. Each section of tail skin graft from each donor was transplanted onto prepared skin bed site of an isoflurane anesthetized long term animal. The site was covered with an adhesive bandage, which was removed after 7 days. Animals were followed post transplantation and followed until complete loss of skin grafts[151].

Xenogeneic GVHD protocol

Humanized xenogeneic GVHD was induced as described before[148]. Briefly, 8-10 weeks old NSG females received sublethal dose (200 cGy) of total body irradiation and injected 4 hrs later with 10x10^6 engineered or unmodified human PBMCs via tail vein. Fresh human PBMCs were used in all experiments. Animals were followed for body weight twice a

week and the development of xenogeneic GVHD was assessed by five parameters: loss in body weight, posture, mobility, fur texture and skin texture[152]. Each parameter received a score of 0 (minimum) to 2 (maximum). Animals that reached significant body weight loss (> 25%) and total GVHD score > 6 were considered to reach end point and euthanized.

Antibodies and Flow cytometry

Fluorochrome conjugated monoclonal antibodies (Table 1 and 2) were titrated for optimal concentration before use. For cell surface staining, cells were incubated with the respective antibodies for 30 mins at 4 °C. For intracellular cytokine staining, cells were stimulated with PMA (50 ng/ml) and Ionomycin (1 µg/ml) for 6 hrs in presence of Golgi plug (BD Biosciences, Ref# 51-2301KZ) for last 4 hrs. After incubation, cells were surface stained followed by fixation (eBiosciences, Ref# 00-5123-43) and permeabilization (eBiosciences, Ref# 00-8333-56). For intracellular staining, cells were incubated with respective cytokine antibodies for 1 hr at room temperature. For intranuclear FoxP3 staining, Fix/Perm buffer (eBiosciences, Ref# 00-5523-00) was used per manufacturer's instructions and staining with anti-FoxP3 antibody for 30 min at 4 °C. Flow cytometric analysis was done by using LSR II with FACS Diva software. Data analysis was done by using FlowJo software (Treestar).

Table1. Phenotypic analysis of mouse cells by flow cytometry

Marker	Clone	Vendor	Cat#
CD3	500A2	BD Biosciences	560771
CD4	RM4-5	BD Biosciences	557956
CD8	53-6.7	BD Biosciences	557654
CD44	IM7	eBiosciences	48-0441-82
CD62L	MEL-14	BD Biosciences	563252
PD1	RMP1-30	eBiosciences	48-9981-82
CTLA4	UC10-44B9	eBiosciences	12-1522-83
CD25	PC61.5	eBiosciences	25-0251-82
FoxP3	FJK-16s	eBiosciences	53-5773-82
CD45	30-F11	eBiosciences	45-0451-80
Fc block	93	Biolegend	101320

Table 2. Phenotypic analysis of human cells by flow cytometry

Marker	Clone	Vendor	Cat#
CD45	HI30	BD Biosciences	564047
CD3	UCHT1	BD Biosciences	563109
CD4	L200	BD Biosciences	560836
CD8	SK1	BD Biosciences	561423
CD25	M-A251	BD Biosciences	557741
CD45RO	UCHL1	BD Biosciences	563749
CD197	150503	BD Biosciences	562555
CD95	DX2	BD Biosciences	556640
CD127	HIL-7R-M21	BD Biosciences	558598
CD20	2H7	BD Biosciences	560735
CD4	SK3	BD Biosciences	566320
CD20	2H7	BD Biosciences	563779
CD16	3G8	BD Biosciences	562874
CD3	UCHT1	BD Biosciences	560835
CD8	RPA-T8	BD Biosciences	555369
CD4	RPA-T4	BD Biosciences	555347

Cells isolation and CD25 depletion

Single cells suspension from spleens were depleted of $CD25^+$ cells using Miltenyi Biotec kit following manufacturer's instructions. Cells were sequentially stained with anti-CD25 PE antibody (BD Biosciences, Ref#553866) and super-paramagnetic microbeads conjugated with monoclonal anti-PE antibodies (Miltenyi Biotec, Ref# 130-048-801). After staining, cells were passed through LS columns (Miltenyi Biotec, Ref# 130-042-401) and eluted cells were collected as $CD25^-$ cell fraction. $CD25^-$ cell fraction was then washed with PBS and followed for SA-FasL engineering as described above.

RNA isolation and quantitative RT-PCR

Total RNA was extracted from liver, colon and small intestine tissues using Trizol reagent (Life Technologies, Ref# 15596018) according to manufacturer's instruction. Total RNA was quantified using NanoDrop ND-2000c spectrophotometer (ThermoFisher Scientific) and cDNA was reverse transcribed from 4 µg of the total RNA using SuperScript^{TM} IV VILO^{TM} Master Mix (ThermoFisher Scientific, Ref# 11756050) following manufacturer's instruction. Quantitative RT-PCR was performed for different genes (Table 3) using TaqMan probe assay on Quant Studio 3 RT-PCR system (Applied Biosystems). Each PCR reaction consisted of 5 µl of TaqMan Fast Advanced master mix, 1 µl of TaqMan primer and FAM/VIC labelled MGB probes, 1 µl of cDNA sample (from 1:10 dilution), and 3 µl of nuclease-free water in a final volume of 10 µl. The thermal cycling conditions were as follows: 1 cycle of 95 °C for 20s, followed by 40 cycles of 95 °C for 1s and 60 °C for 20s. Transcript expression was normalized to GAPDH housekeeping gene and represented as fold change ($2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = \Delta Ct_{experimental} - \Delta Ct_{control}$).

Table 3. TaqMan assay for indicated gene expression in tissues

Taqman Primer	Assay ID (Thermofisher)
CCl2/ MCP1	Mm00441242_m1
IL-1β	Mm00434228_m1
FoxP3	Mm00475162_m1
GATA3	Mm00484683_m1
TNF-α	Mm99999068_m1
IFN-γ	Mm01168134_m1
IL-4	Mm00445259_m1
IL-6	Mm00446190_m1
IL-10	Mm01288386_m1
Rorc/ ROR-γT	Mm01261022_m1
TGF-β1	Mm01178820_m1
Tbx21/ T-Bet	Mm00450960_m1
GAPDH	Mm99999915_g1
IL-12b/ IL-12 p40	Mm01288989_m1
IL-23α	Mm00518984_m1

Histopathology

Tissues from animals on day 21 after HSCT were harvested, fixed in 10% neutral buffered formalin (Leica, Ref# 3800602) and embedded in paraffin. Sections (5 μm) were cut with microtome and subsequently stained with hematoxylin and eosin.

Statistics

Statistical analysis was performed using GraphPad prism software v.8 (Graphpad prism, CA). Comparison of the survival curves was done using log-rank (Mantel-Cox) test. Data are shown as individual data points or as mean ± SEM as depicted in the figure legends. Comparison of the means was performed using unpaired, two tailed test or Man-Whitney t-test as indicated. For multiple comparison, one-way ANOVA with tukey posttest was done. Statistical significance was defined as $p < 0.05$.

Results

T effector cells engineered with SA-FasL are efficiently eliminated in response to alloantigens

Alloreactive T cells upregulate Fas receptor as early as 1-2 days post allo-mixed lymphocyte reaction (MLR) assay rendering them sensitive to FasL by day 2[51]. To test if SA-FasL engineered alloreactive cells will have reduced proliferation upon allogeneic stimulation, we performed [^{3}H]thymidine incorporation assay. Spleen cells from 4C mice, TCR-transgenic on C56BL/6.SJL (CD45.1) background, were used as responders to irradiated BALB/c spleen cells. The T cells in 4C has selective reactivity against BALB/c H2-I-A^d alloantigen, which is widely expressed in mouse tissues[147]. Spleen cells from 4C were engineered with SA-FasL (SA-FasL-4C) at different levels or with control protein SA (SA-4C). SA-FasL-4C cells or SA-4C cells or unmodified (4C) cells were co-cultured with irradiated BALB/c cells. There was robust proliferation of 4C or SA-4C cells by 48 and 72 hours post culture observed by significant incorporation of [^{3}H]thymidine. In contrast, we observed significant inhibition of proliferation of SA-FasL-4C cells at all protein concentrations tested (Fig. 6A). This demonstrate that SA-FasL on the surface of alloreactive T cells is extremely effective, even at the lowest protein concentration tested, in blocking their proliferation in an *in vitro* allo-MLR setting.

To provide evidence that SA-FasL targets both CD4$^+$ and CD8$^+$ T cells for inhibition, we assessed the frequency of these cells using Abs to CD4, CD8, and Vβ13 TCR specific for the target alloantigen in flow cytometry. There was significant reduction in frequency of alloreactive CD4$^+$ and CD8$^+$ T cells expressing TCR Vβ13 in the SA-FasL engineered group as compared with controls (Fig. 6B).

SA-FasL on the surface can induce apoptosis in activated alloreactive T effector cells in three different ways; i) autocrine where SA-FasL engages Fas on the same T cell, ii) paracrine where SA-FasL on a T cell engages Fas on another T cell, or iii) combination of autocrine and paracrine. To investigate the relative contribution of these death pathways we performed co-culture MLR studies. SA-FasL engineered 4C and unmanipulated 4C cells were labelled with CFSE and CTV, respectively. Fluorescence labelled 4C cells were then mixed at 1:1 ratio and used as responders at indicated ratios with a fixed number of irradiated BALB/c cells as stimulators. Unmanipulated 4C cells underwent robust proliferation by 72 hrs with distinct daughter cell generations. In marked contrast, there was minimal daughter cell generation in SA-FasL-4C cells at all cell ratios used (Fig. 6C), suggesting that SA-FasL-4C cells were primarily being eliminated by autocrine apoptosis.

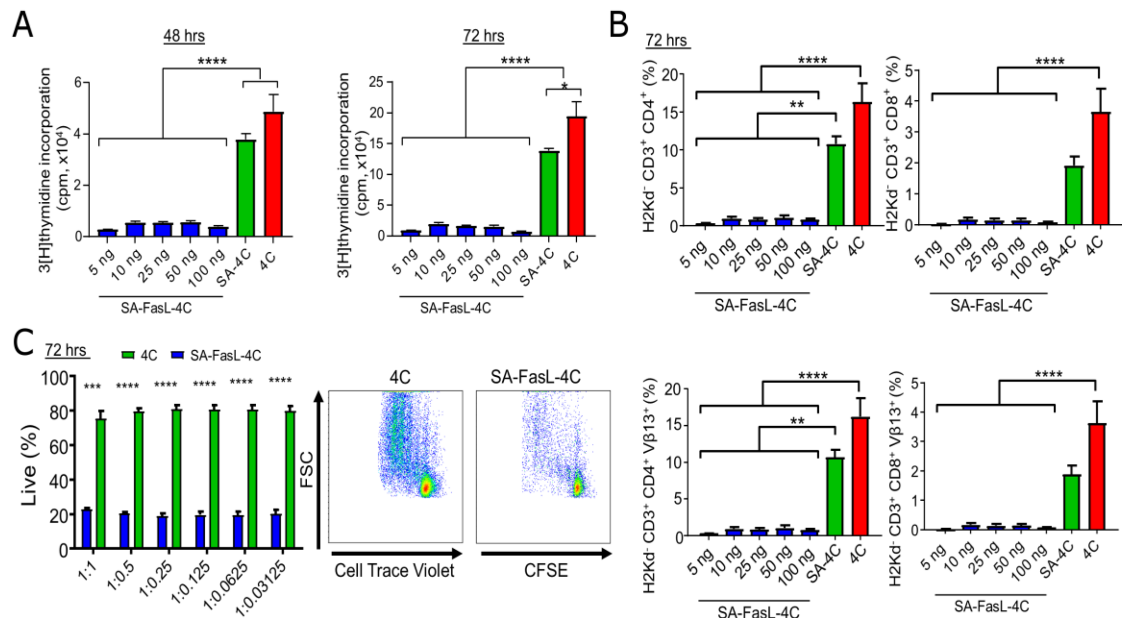

Figure 6. SA-FasL transiently displayed on the surface of T cells is effective in blocking their proliferation in response to alloantigens in vitro. (A) *In vitro* proliferation assay. SA-FasL-engineered or unmodified 4C T cells were stimulated with irradiated BALB/c splenocytes for 48 or 72 hrs. Cultures were pulsed with [^{3}H]thymidine for the last 16 hrs of incubation and harvested using a beta plate counter. Cell associated radioactivity was measured using a scintillation counter. Data were pooled from two independent experiments. (B) Frequencies of live CD4, CD8, and Vβ13 T cell subpopulations in mixed lymphocyte cultures. Experimental conditions are the same as in (A), except instead of pulsing with [^{3}H]thymidine, cultures were harvested at 72 hrs, stained with the Abs to indicated markers and analyzed using flow cytometry. Data were pooled from two independent experiments. (C) SA-FasL induces autocrine death in alloreactive T cells. CTV labelled unmodified 4C cells were mixed one-to-one ratio with CFSE labelled SA-FasL-4C cells and used as responders at the indicated ratios against a fixed number of irradiated BALB/c cells as stimulators. Cells were harvested after 72 hrs of incubation and analyzed for live cells using flow cytometry. Representative flow dot plots of proliferating 4C cells. Date sets pooled from two independent experiments. One-way ANOVA with Tukey multiple comparison was used in (A) and (B). Unpaired two tailed t-test was used in (C). Data are shown as mean ± SEM. cpm counts per minute. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

To recapitulate *in vitro* findings in an *in vivo* model, we used 4C.SJL (CD45.1) transfer to F1 transgenic recipients where 4C cells are expected to respond to the recipient H2-I-A^d antigen. 4C.SJL spleen cells were modified with biotin followed by engineering with SA-FasL (100 ng/10^6 cells) or SA (50 ng/10^6 cells) as the control protein. Each F1 animal was adoptively transferred with 5x10^6 CTV labelled SA-FasL-4C or SA-4C cells. After 48 hrs of transfer, spleen from F1 recipients were harvested and analyzed for $CD45.1^+$ donor cells by flow cytometry. F1 recipients that were transferred with SA-FasL-4C cells had significantly less frequency and absolute numbers of live total donor cells ($7AAD^-$ $CD45.1^+$) and $CD45.1^+CD4^+$ T cells (Fig. 7A and 8A). SA-FasL-4C recipients had 3.8-fold reduction in live donor cells and 5.7-fold reduction in live $CD4^+$ donor cells as compared with SA-4C recipients. In addition, substantially less proliferated donor cells (CTV^{low} $CD45.1^+$) were observed in SA-FasL-4C as compared with SA-4C recipients (Fig. 7A and 8A). This was evident by distinct daughter cell generations in SA-4C, but not in SA-FasL-4C, group (Fig. 8B). Annexin V staining revealed more apoptotic cells in SA-FasL-4C as compared with SA-4C recipients (Fig. 7A), providing in vivo evidence for alloreactive T cells undergoing apoptosis.

To further corroborate our *in vivo* observation, we performed this in vivo tracking study using a non-TCR transgenic model using spleen cells from C57B/6.hCD2 (H-$2K^b$) donors. These mice are transgenic for human CD2 expressed under the FoxP3 promotor of mice, providing a convenient means of tracking Treg cells using an Ab to human CD2[146]. Cells were engineered at indicated doses of SA-FasL or SA proteins, labelled with CTV, and 10x10^6 cells were injected intravenously into F1 ($H2K^{b/d}$) recipients. Animals were euthanized at 72 hrs and splenocytes were subjected to flow analysis. There was

significantly reduced frequency (Fig. 7B) as well as absolute number (Fig. 8C) of live donor total ($H2K^{d-}$ CTV^{+}) cells, $CD4^{+}$ and $CD8^{+}$ T cells as well as proliferating ($H2K^{d-}$ CTV^{low}) donor T cells (Fig. 7B and 8C) at 72 hrs post transfer in F1 recipients of SA-FasL engineered cells as compared with those that received SA engineered cells. Interestingly, there was also a significant drop in the frequency and absolute numbers of T regulatory cells ($hCD2^{+}$) in SA-FasL-spleen recipients (Fig. 7B and 8C). Importantly, these effects were observed for almost all SA-FasL protein doses tested, demonstrating the apoptotic efficacy of SA-FasL plausibly due to its autocrine mode of action.

Taken together, these findings demonstrate that the transient display of SA-FasL protein on T cells is an efficient strategy of purging out pathogenic alloreactive cells with great potential to mitigate acute GVHD.

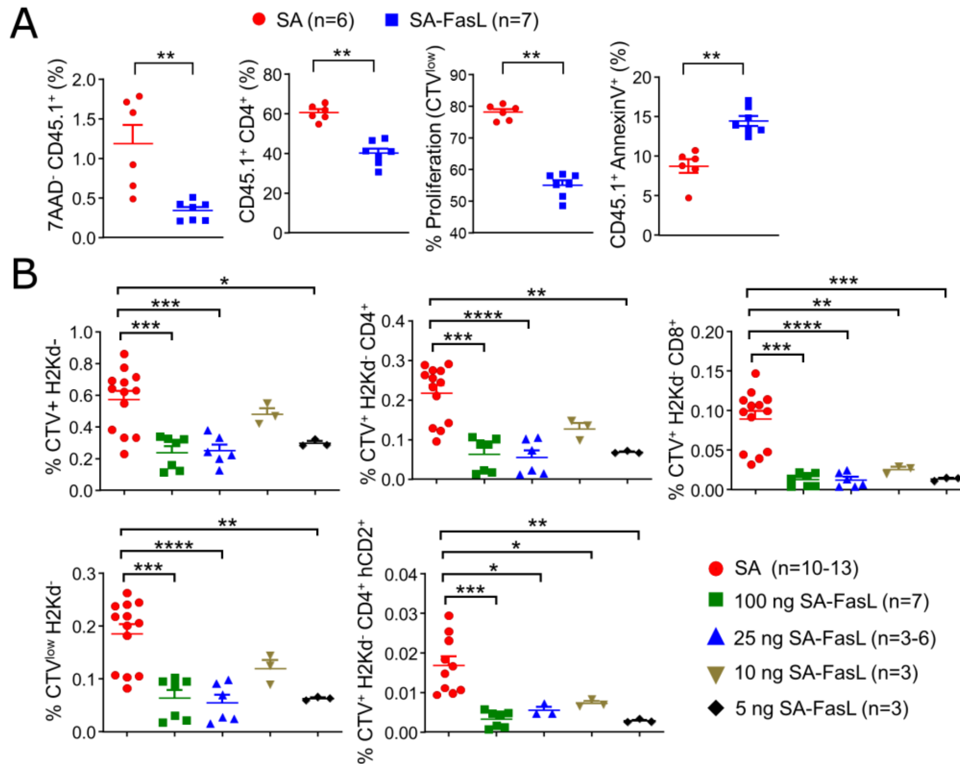

Figure 7. Elimination of alloreactive cells by SA-FasL engineering in an allogeneic adoptive transfer model. (A) Tracking frequency, proliferation, and apoptosis of 4C cells in F1 recipients. 4C.SJL cells were labelled with CTV and engineered with SA or SA-FasL proteins and adoptively transferred into F1 recipients (5x10^6 cells/mouse). After 48 hrs post-transplant, spleen cells of recipients were analyzed for frequency of donor total live (7AAD$^-$ CD45.1$^+$) cells, CD4$^+$ T (CD45.1$^+$CD4$^+$) cells, proliferating (CTV^{low}) cells, and apoptotic (CD45.1$^+$AnnexinV$^+$) cells. Data pooled from two independent experiment, with n=3-4/group. (B) Tracking B6.hCD2 donor cells in F1 recipients. B6.hCD2 splenocytes were labelled with CTV and engineered with SA (50 ng/10^6 cells) or the indicated amounts of SA-FasL protein. Cells were adoptively transferred into F1 recipients (10x10^6 cells/mouse) that were euthanized 72 hrs later to harvest the spleen. Splenocytes were analyzed in flow cytometry by gating on donor cells (H-2K^{d-}) for the frequency of

total cells ($CTV^+H\text{-}2K^{d-}$), $CD4^+$ ($CTV^+H\text{-}2K^{d-}CD4^+$), $CD8^+$($CTV^+H\text{-}2K^{d-}CD8^+$) , and Treg ($CTV^+H\text{-}2K^{d-}CD4^+hCD2^+$) cells as well as proliferating donor cells ($CTV^{low}H\text{-}2K^{d-}$). Data pooled from three independent experiments with n=3-4/group. For comparison of mean, Mann Whitney test was used in (A), One-way ANOVA with Tukey post test was used in (B). Data represented as mean ± SEM. *$p < 0.05$**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

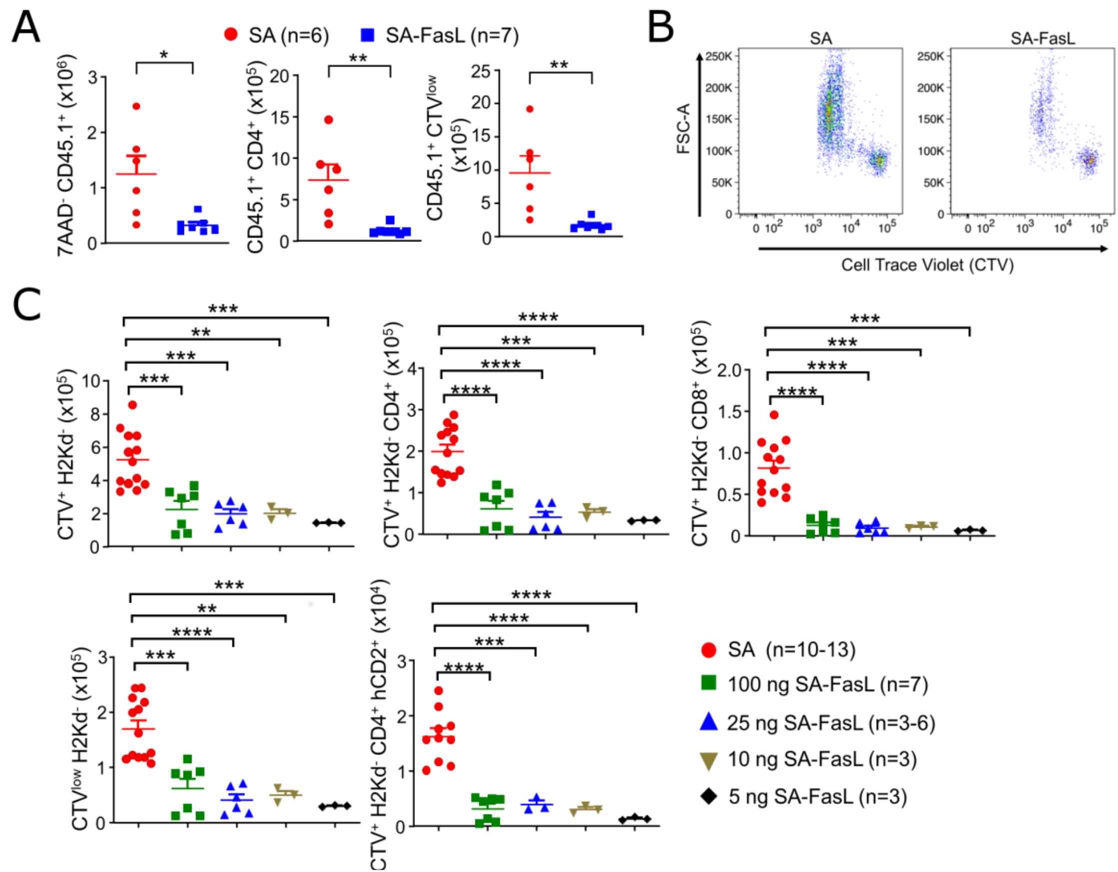

Figure 8. SA-FasL engineered alloreactive cells are eliminated in *in vivo* model (A) Absolute number of 4C cells shown in Fig. 8A. Data pooled from two independent experiments. (B) Representative flow plot showing 4C cell proliferation, equal number of live events were acquired. (C) Absolute number of B6.hCD2 donor cells subpopulations shown in Fig. 8B. Data pooled from three independent experiments with n=3-4/group. For comparison of mean, Mann Whitney test was used in (A), One way ANOVA with Tukey post test was used in (C). Data represented as mean ± SEM. *p <0.05, ***p* < 0.01, ****p* < 0.001, ****p <0.0001

Transient display of SA-FasL on donor T cells Engineering of donor graft to display SA-FasL efficiently prevents lethal acute GVHD

Selective depletion of alloreactive donor cells from graft is an efficient strategy for prevention of lethal acute GVHD[2,153]. Given the significant depletion of alloreactive T cells by engineering of cells to display SA-FasL on their surface, we assessed the efficacy of this strategy to prevent lethal acute GVHD. We used haploidentical parent (C57BL/6, $H2K^b$) to F1 (C57BL/6xBALB/c, $H2K^{b/d}$) model, simulating the broad use of haploidentical bone marrow cells in the clinic. Recipient F1 animals were lethally irradiated at 1000 cGy and 4 hrs later were infused with $20x10^6$ SA-FasL engineered or unmodified spleen cells mixed with $10x10^6$ unmodified whole bone marrow cells. Controls that received only bone marrow cells survived long-term without any signs of GVHD as expected. However, recipients of a mixture of bone marrow cells and unmodified T cells developed acute GVHD symptoms, including bodyweight loss, hunching, and diarrhea, and expired with a median survival time of 26 days (Fig. 9A). The survival of recipients transplanted with SA-engineered donor spleen cells (SA group) and the severity of GVHD were not significantly different from the control group (MST = 25 days; Fig. 9A). The efficacy of SA-FasL was dose dependent as animals receiving T cells engineered with 25 ng SA-FasL per 10^6 cells showed better survival at > 100 days than recipients transplanted with cells engineered with 10 or 5 ng protein (~73%, ~63%, and 20%, respectively, Fig. 9A). Clinical GVHD scores for these groups correlated with the survival rates. Taken together, these results demonstrate the efficacy of this engineering platform with SA-FasL in preventing acute GVHD in a clinically relevant model.

We next analyzed long-term animals for immune composition. Long term animals (>100 days) that received SA-FasL engineered grafts had full donor chimerism (Fig. 10). Immune cells in spleen, peripheral blood and bone marrow were donor derived (H-2K^{b+}, K^{d-}), suggesting efficient engraftment of donor stem cells. Importantly, the frequency and absolute number of immune cells, including Treg cells ($CD4^+CD25^+FoxP3^+$), T effector cells ($CD4^+CD44^{hi}CD62L^-$), NK cells ($NK1.1^+CD3^-$) as well as ratios between Treg and Teff cells in the spleen of long term animals were comparable to the BM control group that did not receive splenocytes (Fig. 9B and 11A). Similar observations were also made with peripheral blood lymphocytes (Fig. 11B). These results demonstrate that long term animals have efficient overall lymphoid reconstitution.

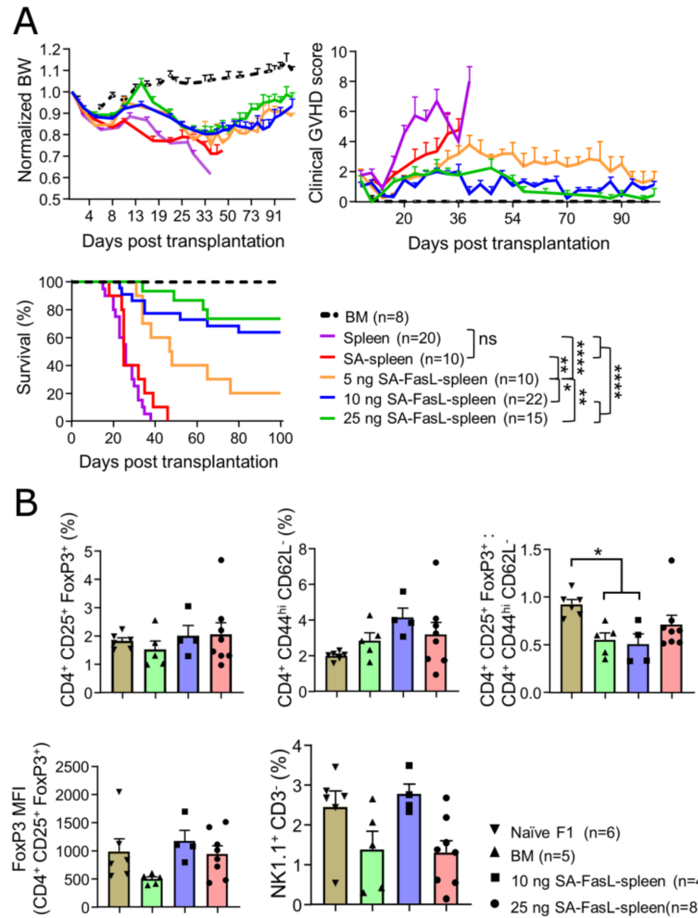

Figure 9. Engineering donor graft with SA-FasL abrogates lethal acute GVHD and show efficient lymphoid reconstitution. (A) Survival of F1 recipients transplanted with a mixture of allogeneic bone marrow and splenocytes. Lethally irradiated F1 animals were transplanted with C57BL/6 bone marrow cells (20x10^6) co-mixed with syngeneic spleen cells (10x10^6) engineered with SA (12.5 ng/10^6 cells) or the indicated amount of SA-FasL. Animals transplanted with bone marrow only (BM) or a mixture of unmodified splenocytes and bone marrow cells (Spleen) served as controls. Animals were followed for body weight, clinical GVHD scores and survival. (B) Frequency of CD4$^+$ Treg cells, Tem and NK cells in long term (>100 days) animals compared with bone marrow only recipients and unmanipulated naïve F1 animals. For comparison of survival curves log-rank (Mantel-

cox) test was used in (A). For comparison of means in (B), one-way ANOVA with Tukey posttest was used. Data represented as mean ± SEM. *p <0.05***p* < 0.01, ****p* < 0.001, ****p <0.0001

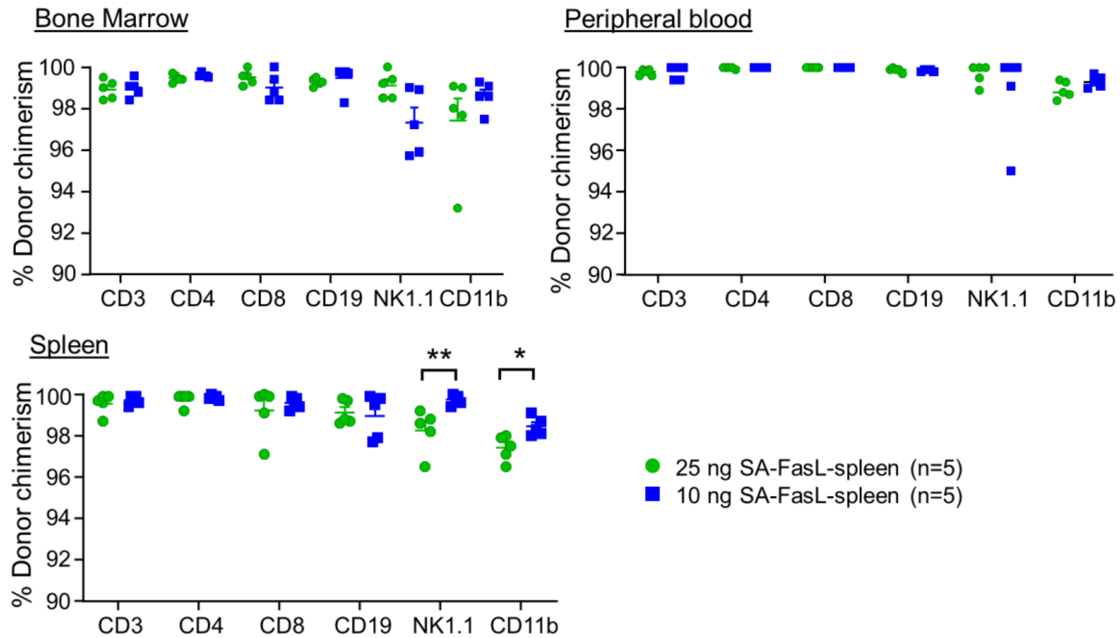

Figure 10. Long term SA-FasL-spleen recipients show full donor chimerism. Long term animals (>100 days) were analyzed for donor chimerism ($H2K^b$ vs $H2K^d$) in bone marrow, peripheral blood and spleen. Frequency of $H2K^{d-}$ (donor cells) in each cell compartment was analyzed by flow cytometry. Data shown as mean ± SEM. Mann Whitney test was used for mean comparison. *p <0.05, **p <0.01

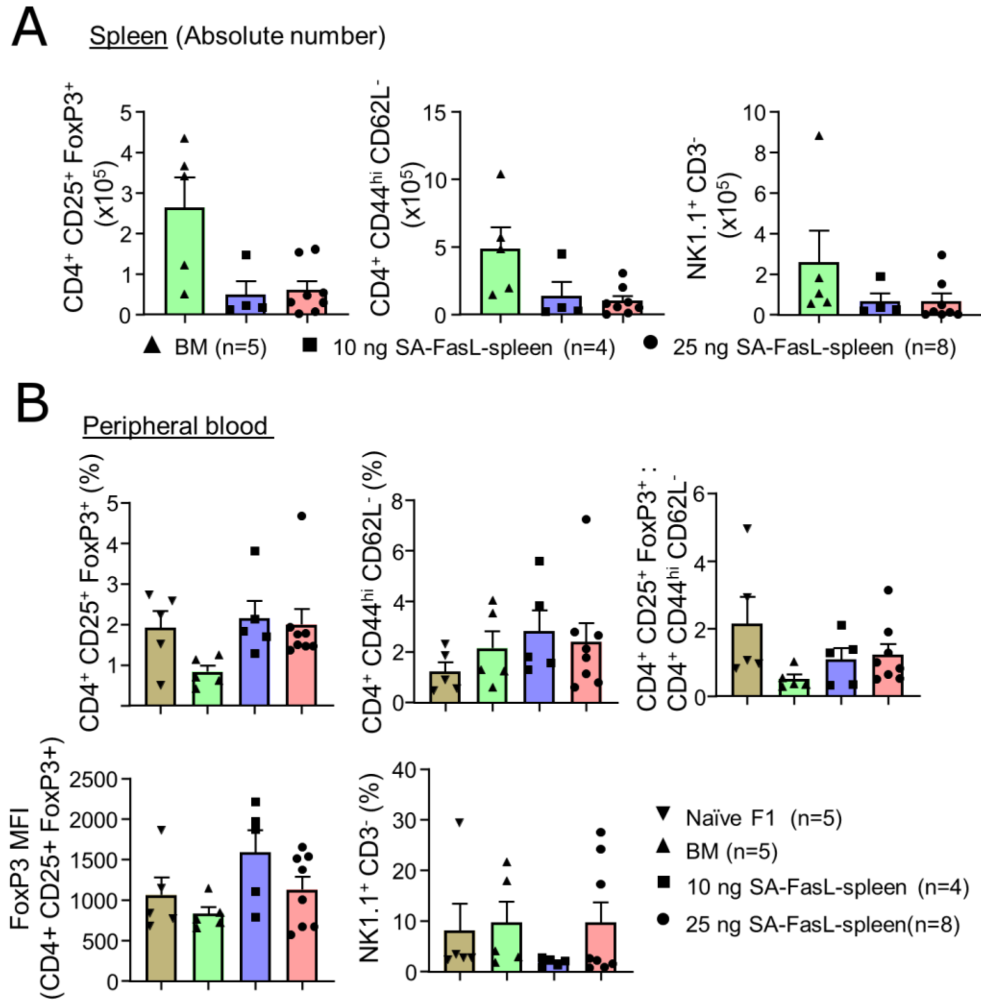

Figure 11. Immunophenotyping analysis of long-term animals. Absolute number in spleen (A) and frequency in peripheral blood (B) of long-term recipients (>100 days) of SA-FasL-spleen cells compared with long-term recipients of bone marrow cells without GVHD causing spleen cells and naïve unmanipulated F1 animals. Data represented as mean ± SEM. One way ANOVA with Tukey posttest was used for statistical analysis.

Long term SA-FasL-engineered donor cell recipients are functionally immune competent.

One of the important aspects in HSCT recipients is the immune competency. The recipients should be competent enough to initiate immune responses against third party antigens, to ward off infections, while maintaining tolerance to allo-antigens. To assess the functional immunity in long term SA-FasL-engineered grafts recipients (>90 days), we tested their ability to reject third party allografts. Two heterotopic skin grafts were applied to trunk of each mouse from TBI + BM group or SA-FasL-group (10 ng). One from BALB/c ($H2K^d$), which is targeting alloantigen in GVHD settings, and one from C3H/HeJ third party ($H2K^K$) donors (Fig. 12). Interestingly, all long-term animals transplanted with BALB/c skin grafts accepted the graft, indicating the presence of systemic tolerance against recipient alloantigens (Fig. 12A and B). In marked contrast, all third party C3H/HeJ skin grafts were rejected in both groups (Fig. 12A) with median survival of 22 days. These results suggest that, at this time after transplantation, T cell immune reconstitution was competent to enable rejection response against third party skin grafts, while maintaining tolerance to recipient allogenic antigens.

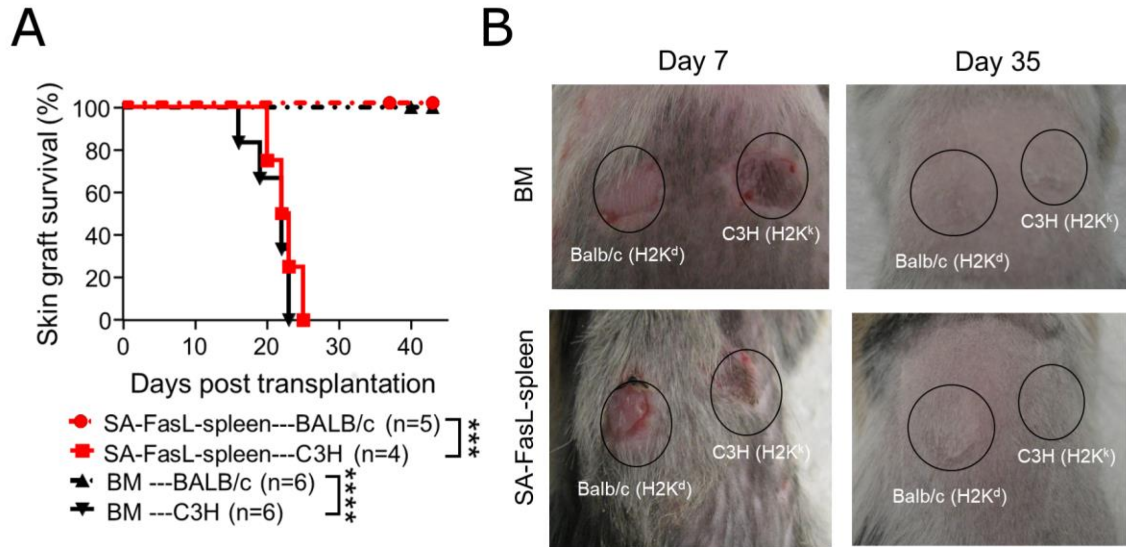

Figure 12. Long term SA-FasL-spleen recipients are immune competent. Long term animals that received bone marrow cells only without GVHD causing spleen cells and bone marrow cells along with SA-FasL-spleen cells were challenged with simultaneous donor (BALB/c, $H2^b$) and third party (C3H, $H2^k$) skin grafts. Animals were followed for complete graft rejection and noted as day of graft survival. (A) Skin allograft survival. (B) Representative pictures of skin grafts on day 7 and 35 post-transplantation. For comparison of survival curve log-rank (Mantel-cox) test was used. ***$p < 0.001$, ****$p < 0.0001$

SA-FasL recipients exhibit reduced activated alloreactive Th1 phenotype.

Although our data provide direct evidence that physical elimination of alloreactive T cells is an important mechanism of the observed prevention of GVHD in our model, apoptosis initiate by SA-FasL may set in motion other immunoregulatory mechanisms that may accentuate the efficacy of SA-FasL in our model. Thus we analyzed liver, mesenteric lymph nodes (mLN) by flow cytometry and target organs (colon, ileum and liver) by qRT-PCR during the efferent phase of acute GVHD, day 21, the time point when control group animals that received unmodified spleen cells had significant loss in body weight. Activated $CD4^+$ T cells ($CD4^+FoxP3^-CD44^+CD62L^-PD1^+$) and $CD8^+$ T cells ($CD8^+CD25^+$ and $CD8^+CD44^+CD62L^-PD1^+$) were significantly higher in frequency in mLN and liver in the control group as compared with the SA-FasL group (Fig. 13A). Similar level of significance was also observed when analyzed as absolute numbers per gram in liver, but not in mLN (Fig. 14B). Interestingly, recipients of unmodified spleen cells had substantially higher frequency and absolute numbers of Treg cells ($CD4^+CD25^+FoxP3^+$) than SA-FasL-spleen recipients in the liver and frequency, but not absolute cell numbers, in mLN (Fig. 13A and 14B). However, the Treg ($CD4^+CD25^+FoxP3^+$) and activated T effector ($CD4^+FoxP3^-CD44^+CD62L^-PD1^+$ or $CD8^+CD44^+CD62L^-PD1^+$) cell ratio was not significant between the control and SA-FasL groups in the tissues analyzed (Fig. 13B). These observations suggest that the primary mechanism that SA-FasL prevents acute GVHD is physical elimination of alloreactive T effector cells, resulting in reduced frequency and absolute numbers in the target tissues.

qRT-PCR analysis of GVHD target tissues (liver, colon and small intestine) from control group that received unmodified spleen cells had significantly higher expression

profile of inflammatory cytokines (IFN-γ, TNF-α, and IL-6), all are known mediators of acute GVHD[154], as compared with SA-FasL-spleen recipients (Fig. 13C). In addition, GVHD control group also had higher level of T-bet transcription factor, a master regulator of Th1 differentiation[155], than SA-FasL-spleen recipients in all GVHD target organs (Fig. 13C). Interestingly, upregulated Th1 response in GVHD control group was associated with augmented expression level of IL-4, mediator of Th2 differentiation, in liver ($p = 0.0084$) and colon ($p = 0.0205$) of SA-FasL-spleen recipients as compared with GVHD control group without an apparent difference in small intestine (Fig. 13C). Similarly, the levels of GATA3, a master regulator of Th2 differentiation[156], transcript was significantly higher ($p < 0.0001$) in large intestine, but not the liver or small intestine, of SA-FasL-spleen recipients than GVHD controls (Fig. 15). GVHD control group also had higher level of other inflammatory mediators, including IL-1β ($p = 0.0111$), but the level of IL23p40 did not reach statistical significance ($p=0.1006$) in the liver (Fig. 15). We observed a similar pattern in the large intestine, but in the small intestine SA-FasL-spleen recipients had significantly higher expression level of IL23p40 ($p = 0.0001$; Fig. 15). SA-FasL-spleen recipients had significantly less transcripts of chemokine CCL2 in liver ($p = 0.0099$) and in small intestine ($p = 0.0172$; Fig. 13). Interestingly, SA-FasL-spleen recipients had significantly higher expression level of RORγt in the liver ($p= 0.0003$) and small intestine ($p= 0.0004$), but significantly lower expression in large intestine ($p < 0.0001$; Fig. 15). In marked contrast, when analyzed for FoxP3, transcription factor for Treg cells, GVHD control animals had higher level than SA-FasL-spleen recipients (Fig. 15).

The qRT-PCR data was further corroborated by intracellular cytokine assay using spleen cells stimulated with phorbol myristate acetate (PMA) and ionomycin. SA-FasL-

spleen cells recipient had significantly lower frequency, but not absolute numbers, of $CD4^+$ cells secreting IFN-γ (p=0.0175) and TNF-α (p=0.0125) (Fig. 16). We did not observe any difference between the groups for IFN-γ or TNF-α or granzyme B producing $CD8^+$ T cells (Fig. 16). Histologically, there was evidence of villous blunting (colon and ileum), intrahepatic infiltration and bile duct epithelial infiltration in the liver of GVHD control groups when compared with SA-FasL-spleen and BM recipients (Fig. 17). Taken together, these data suggest that SA-FasL-spleen recipients are protected from lethal acute GVHD by elimination of alloreactive T cells resulting in significantly reduced activated alloreactive T cells in the target organs by day 21. This was further supported by significantly higher transcripts of proinflammatory cytokines, Th1 skewed T cell phenotype, and other inflammatory mediators in GVHD control animals that received unmodified spleen cells but not SA-FasL-spleen recipients.

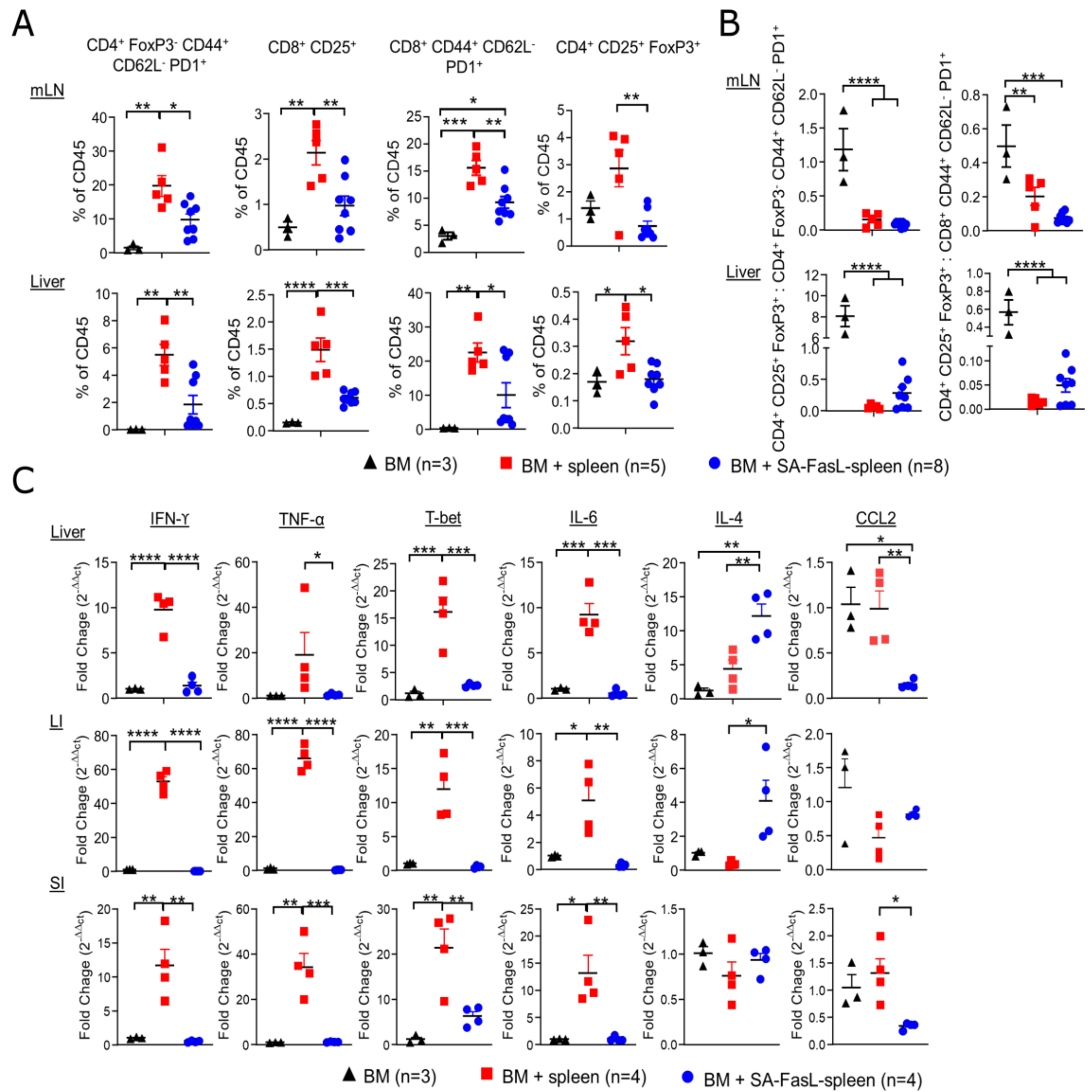

Figure 13. SA-FasL-spleen recipients have less activated cells and inflammatory mediators of acute GVHD at day 21 post transplantation. (A) Frequency of activated cells and regulatory T cells in mesenteric lymph nodes (mLN) and the liver. Intrahepatic immune cells and mesenteric lymph nodes were analyzed for activated CD4 ($CD4^+FoxP3^-CD44^+CD62L^-PD1^+$) and $CD8^+$ T cells ($CD8^+CD25^+$; $CD8^+CD44^+CD62L^-PD1^+$) and Treg cells ($CD4^+CD25^+FoxP3^+$). (B) Ratio of Treg cells to activated $CD4^+$ and $CD8^+$ T cells. (C) qRT-PCR analysis on target tissues [liver, large intestine (LI) and small intestine (SI)]

at day 21 post transplantation. Total RNA from liver, colon and ileum was isolated and subjected to TaqMan based qRT-PCR assay for indicated cytokines, transcription factors, and chemokines. Fold change expression ($2^{-\Delta\Delta Ct}$) was calculated with respect to GAPDH as house-keeping gene and bone marrow only recipients. Data representative of two independent experiments and shown as mean ± SEM. For comparisons, One way ANOVA with Tukey posttest was used in (A), (B) and (C). *p <0.05**$p < 0.01$, ***$p < 0.001$, ****p <0.0001

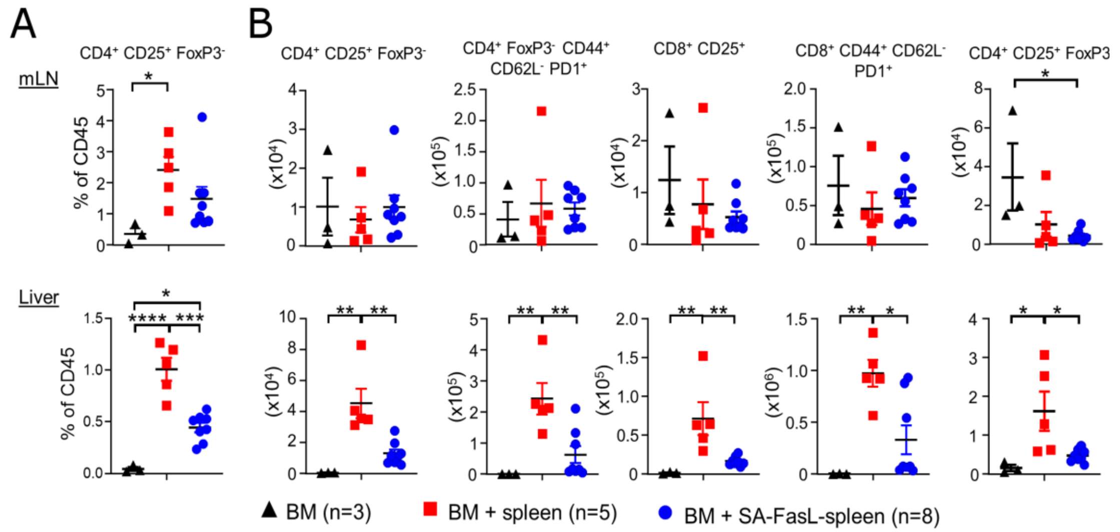

Figure 14. Immunophenotyping of recipients at day 21 post transplantation.

Mesenteric lymph nodes (mLN) and liver infiltrating cells were analyzed at day 21 post-transplantation. (A) Frequency of $CD4^{+}$ activated T cells ($CD4^{+}CD25^{+}FoxP3^{-}$). (B) Absolute number of $CD4^{+}$ and $CD8^{+}$ activated T cells and Treg cells in mLN and liver. Data pooled from two independent experiments and shown as mean ± SEM. For comparison, One way ANOVA with Tukey posttest was used in (A) and (B). *p <0.05***p* < 0.01, ****p* < 0.001, ****p <0.0001

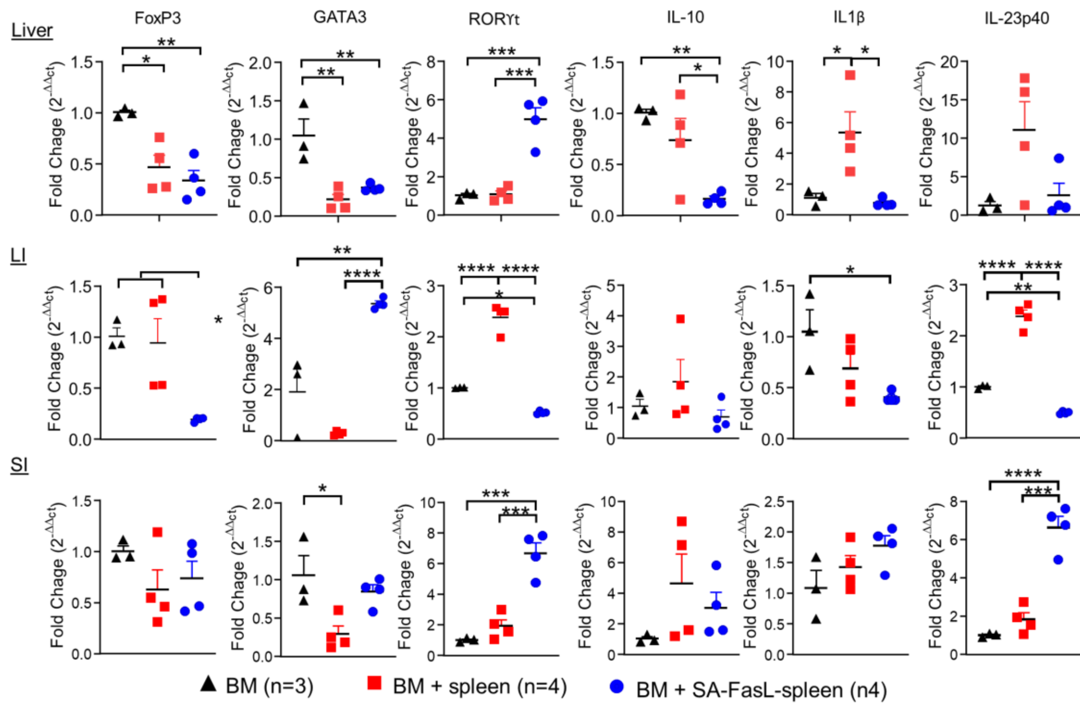

Figure 15. qRT-PCR analysis of GVHD target organs for immune markers. Total RNA from liver, colon, and ileum was isolated and subjected to TaqMan based qRT-PCR for the expression profile of multiple immune genes as indicated. Fold expression ($2^{-\Delta\Delta Ct}$) was calculated with respect to GAPDH, as a house keeping gene, and bone marrow only recipients. Data represented as mean ± SEM. One way ANOVA with Tukey posttest was used for statistical comparison. *p <0.05***p* < 0.01, ****p* < 0.001, ****p <0.0001

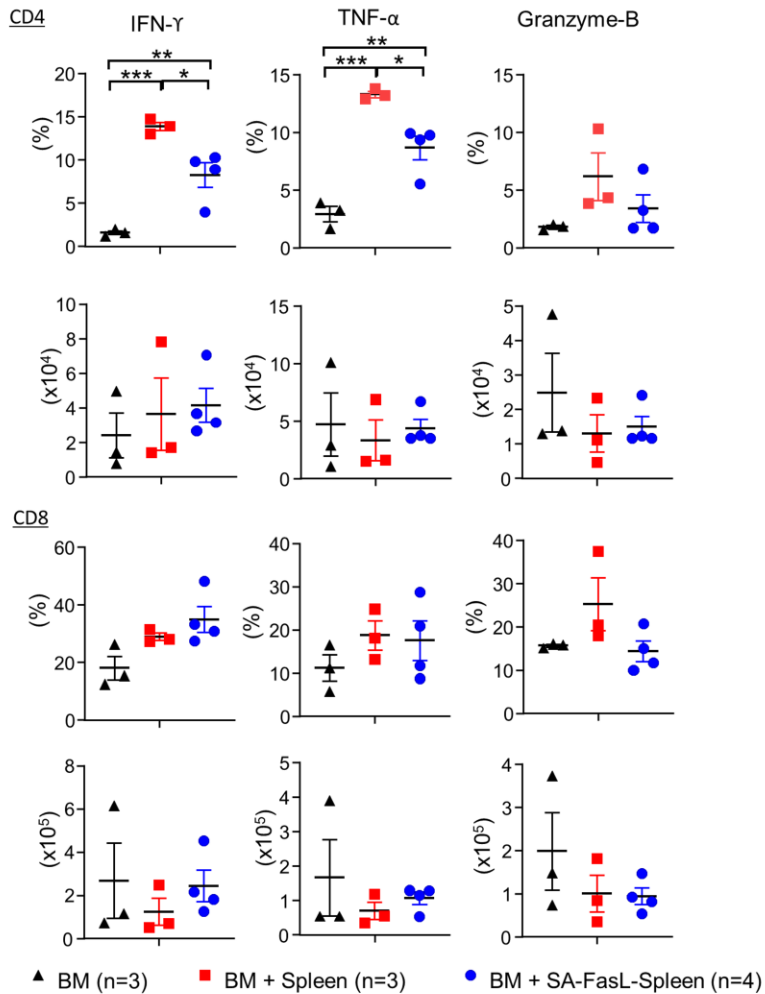

Figure 16. Intracellular cytokine analysis on spleen cells. Frequency and absolute number of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, TNF-α, and granzyme B. After 21 days post transplantation, spleen cells were harvested from the indicated groups, stimulated with PMA and ionomycin, and analyzed using flow cytometry. Data are from cells pooled from multiple animals and represent two independent experiments. Data are represented as mean ± SEM. One way ANOVA with Tukey posttest was used for statistical comparison. *p <0.05***p* < 0.01, ****p* < 0.001, ****p <0.0001

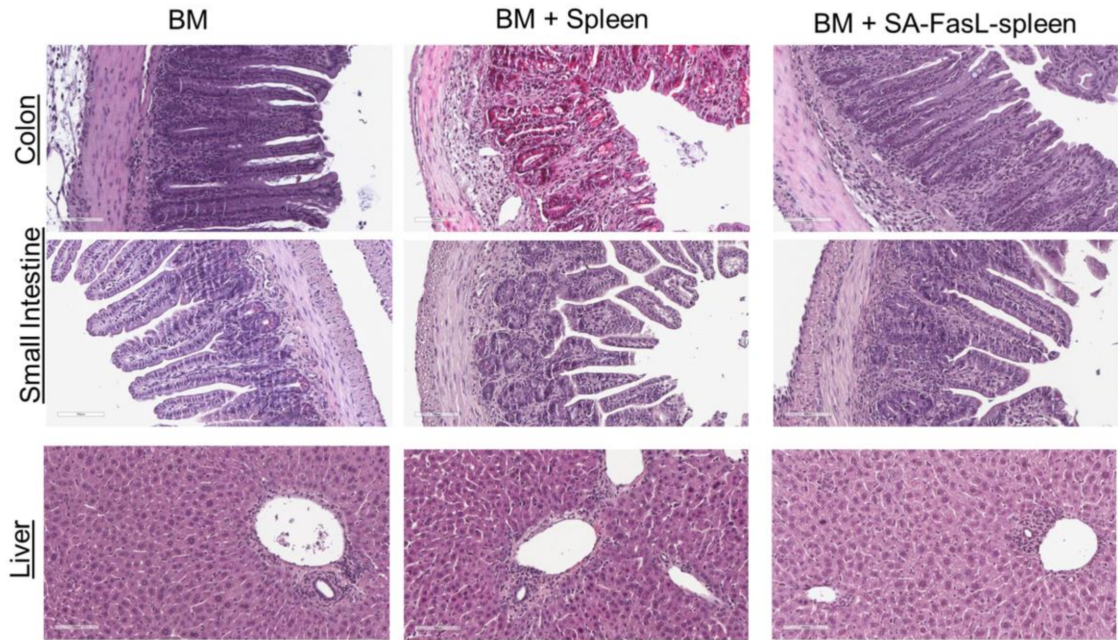

Figure 17. Representative H&E staining of GVHD target organs from each cohort. GVHD tissues were harvested at day 21 post transplantation, formalin fixed, and paraffin embedded. H&E staining was performed on tissue section for the indicated groups.

Donor $CD25^+$ Treg cells are indispensable for the prevention of acute GVHD.

$CD4^+$ $CD25^+$ regulatory T cells can suppress expansion of alloreactive T cells and inhibit lethal acute GVHD[157,158]. However, massive infusion of Treg cells at 1:1 ratio with T effector cells ($CD25^-$) is required for protection[53,158]. To assess the contribution of Treg cells for the protection against acute GVHD in our model, we depleted $CD25^+$ T regulatory cell population from donor cell inoculum (Fig. 18) and then used the Treg-deplete splenocytes for adoptive transfer into lethally irradiated F1 recipients. Recipients transplanted with whole unmodified splenocytes developed fatal signs of acute GVHD with median survival time of 34 days. Whereas, animals receiving whole SA-FasL engineered splenocytes were protected from lethal GVHD with 80% of animals survived long term (> 60 days). Surprisingly, all F1 recipients of Treg-deplete splenocytes engineered with SA-FasL developed signs of acute GVHD with severe diarrhea, hunched posture and decreased motion, and expired with a median survival time of 28 days as compared with 34 days for GVHD controls (Fig. 19; $p=0.043$). Taken together, these results demonstrate the critical role of donor Treg cells in the prevention of acute GVHD in our model.

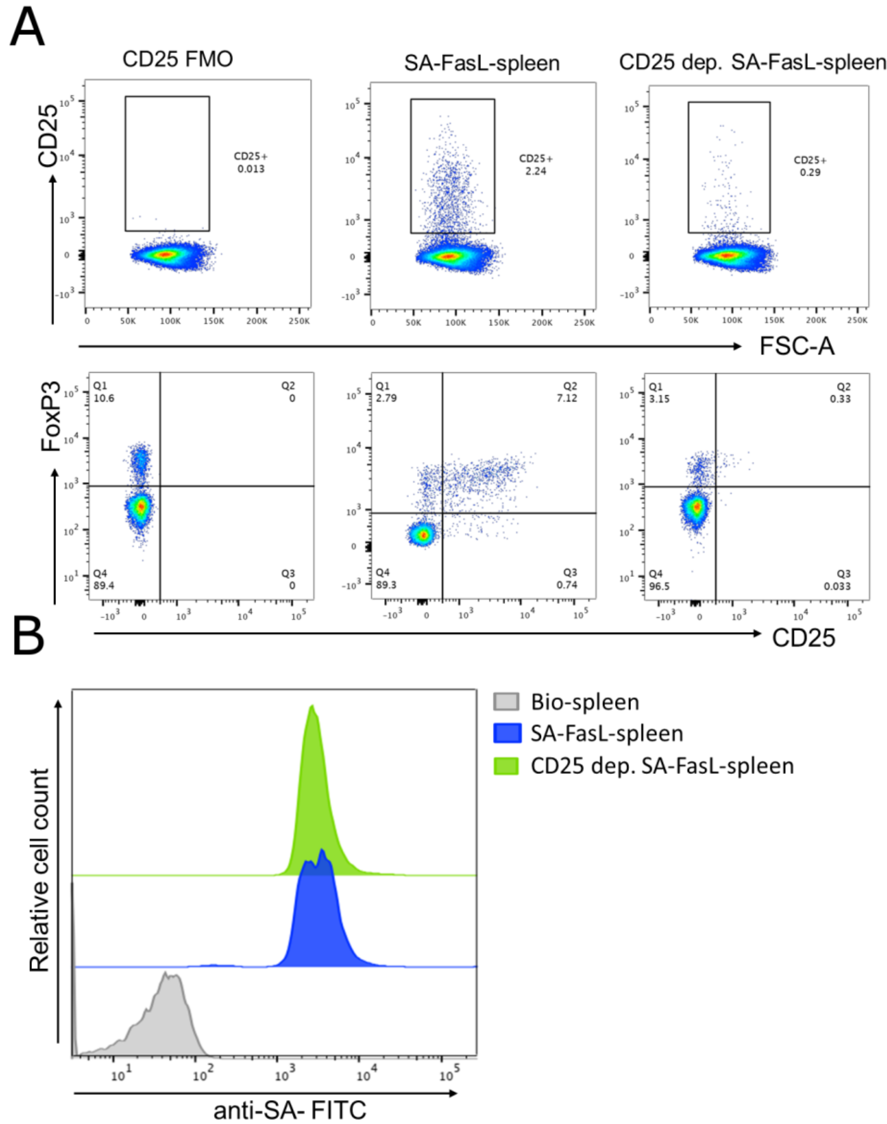

Figure 18. Depletion of CD25⁺ cells from donor inoculum. (A) Representative flow dot plot for CD25⁺ and FoxP3⁺ CD25⁺ cells in SA-FasL-spleen and CD25 depleted SA-FasL-spleen inoculum, indicating significant depletion of CD25⁺ cells. (B) SA-FasL engineering level on CD25+ cells depleted inoculum. Histogram plot suggesting similar level of SA-FasL engineering on CD25⁺ replete inoculum and CD25⁺ deplete inoculum.

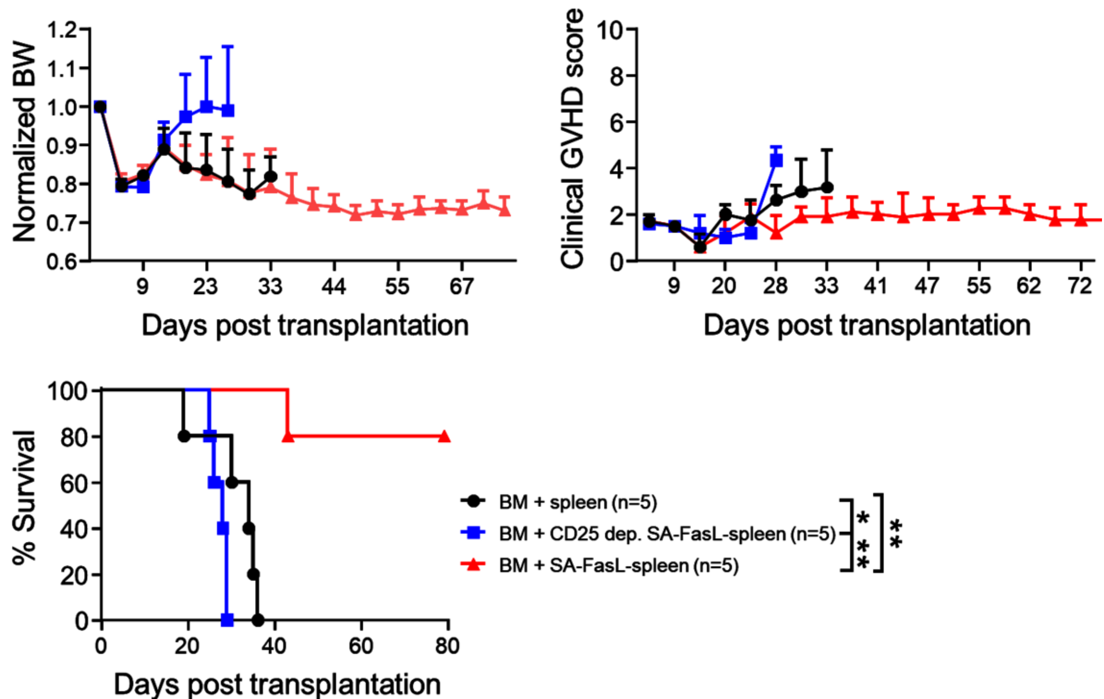

Figure 19. CD25⁺ cells are indispensable for observed protective effect of SA-FasL for acute GVHD. Lethally irradiated F1 mice received bone marrow cells admixed with unmodified or SA-FasL engineered spleen cells or SA-FasL engineered CD25-deplete spleen cells. Animals were monitored for signs of acute GVHD and survival. For survival curve comparison, log-rank (Mantel-Cox) test was used. *p <0.05***p* < 0.01

SA-FasL engineering of human PBMCs abrogates acute GVHD in a humanized mouse model.

To extend the observed effect of SA-FasL engineering on human immune response, we first performed *in vivo* tracking of human cells using NOD-*scid*-IL2γR^{null} (NSG) model. Neutrophil depleted human PBMC were engineered with SA-FasL or SA (control protein). NSG recipients were irradiated at 200 cGy followed by *i.v* injection of $5x10^6$ SA- or SA-FasL engineered human PBMCs. When analyzed 5 days post infusion, spleen of SA-recipients had significantly greater number (Fig. 21A) as well as frequency (Fig. 20) of total human cells and human T cells as compared with SA-FasL group. Importantly, we observed a similar pattern in the liver (Fig. 21A and 20), a major target in human PBMC induced xenogeneic GVHD. Following this observation, we established a model of acute xenogeneic GVHD by transferring human PBMC, adapted from King *et al.*[148]. When 200 cGy preconditioned NSG animals were infused with 10 $x10^6$ human PBMC, animals developed clinical signs of GVHD by day 14, including significant weight loss, hunched posture, ruffled fur and reduced motion with median survival time of 15 days (Fig. 21B). Delivery of SA-engineered human PBMCs had similar trend of developing fatal GVHD with median survival time of 15 days (Fig. 21B). However, engineering the human PBMCs with SA-FasL before transfusion significantly extended the survival ($p<0.0001$) with 27.67% of animals surviving for >60 days (Fig. 21B). There was a significant delay in the onset of GVHD with significant body weight loss by day 25 as compared with day 13 for the SA group. Taken together, these data suggest that engineering alloreactive T cells with SA-FasL is an efficient strategy to eliminate pathogenic cells to mitigate fatal acute GVHD in a xenogeneic humanized NSG model.

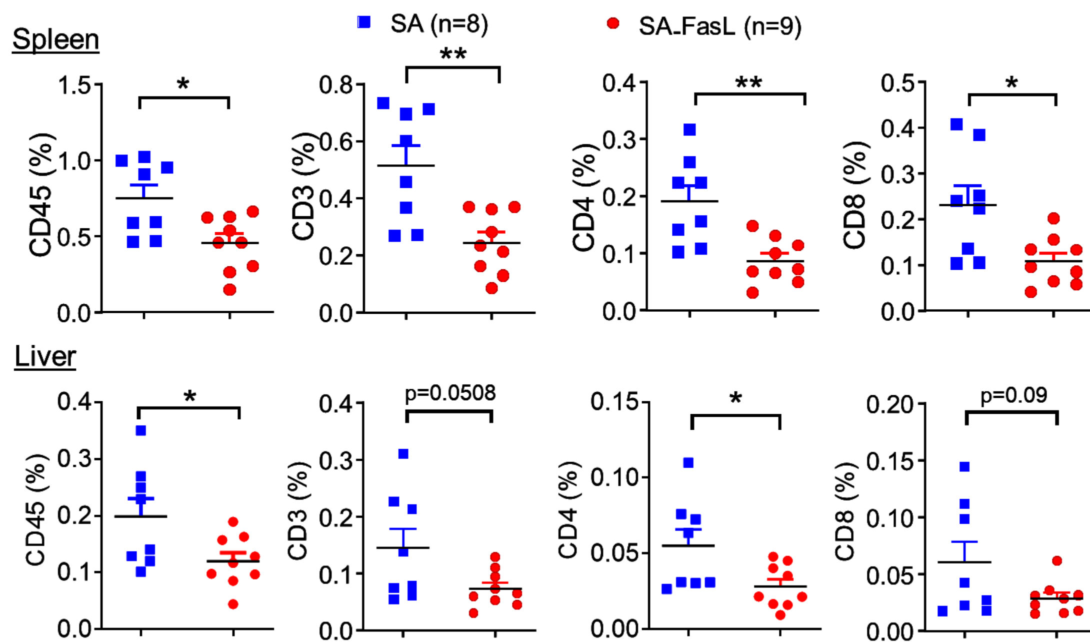

Figure 20. Frequency of human immune cells recovered from preconditioned NSG animals. Preconditioned (200 cGy) NSG animals were transplanted with SA or SA-FasL engineered human PBMC. After 5 days post transplantation, spleen and liver of NSG recipients were analyzed for human immune cells by flow cytometry. For comparison of means, unpaired two tailed T- test was used. Data shown as mean ± SEM. *p <0.05***p* < 0.01

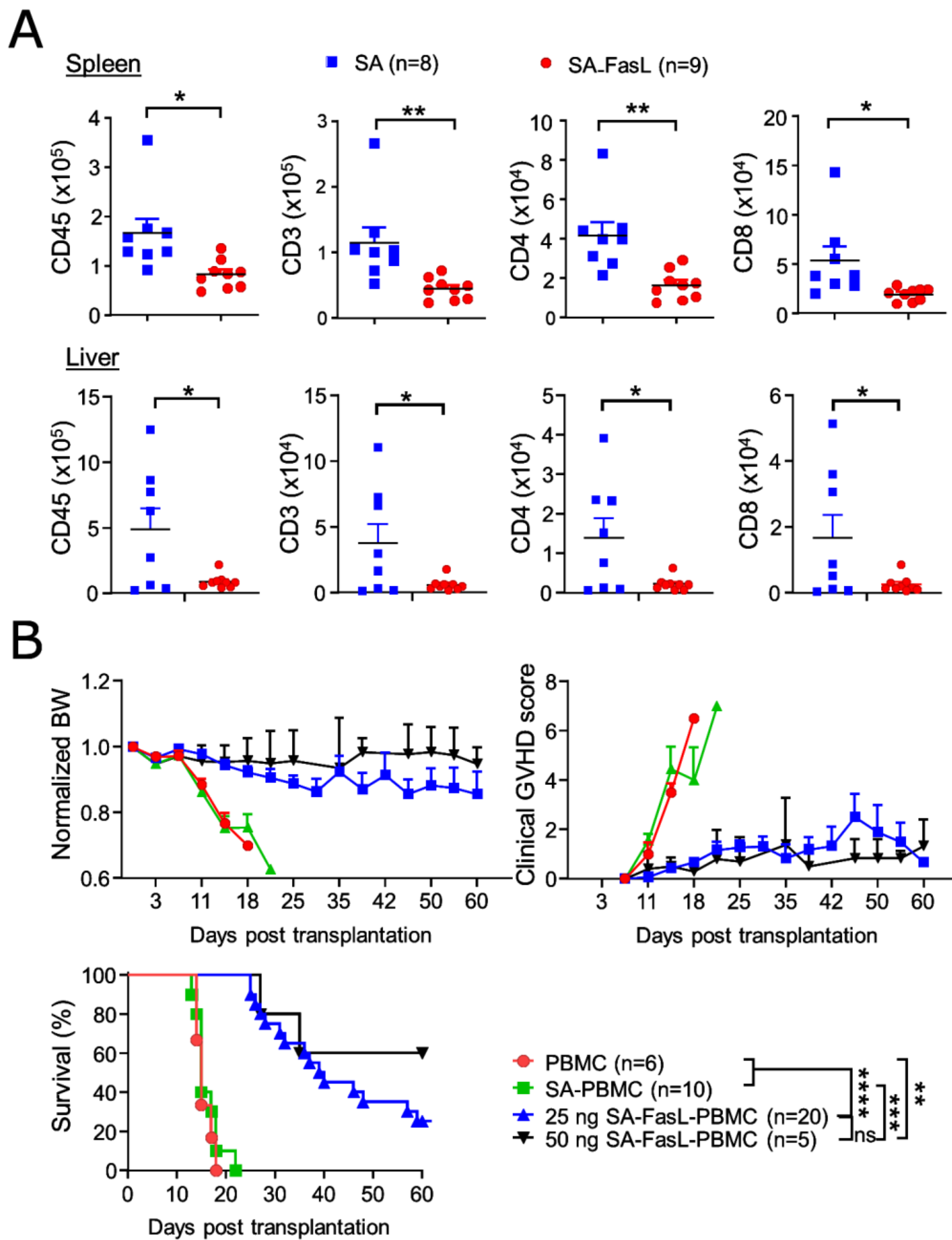

Figure 21. SA-FasL engineering eliminates activated human PBMCs and abrogates xenogeneic GVHD. (A) Absolute number of human cells recovered from preconditioned NSG animals. NSG animals were preconditioned at 200 cGy and SA or SA-FasL

engineered neutrophil depleted PBMCs were i.v injected. After 5 days post injection, spleen and liver were analyzed by flow cytometry. Data pooled from three independent experiments. (B) Xenogeneic GVHD is prevented from SA-FasL engineered PBMCs. Preconditioned NSG animals (200 cGy) were transplanted with unmodifed or SA/SA-FasL engineered human PBMC. Animals were followed for development of xenogeneic GVHD. For comparison of means, unpaired two tailed t-test was used in (A). For survival curve comparison log-rank (Mantel Cox) test was used. Data shown as mean ± SEM. *p <0.05**, $p < 0.01$, ***$p < 0.001$, ****p <0.0001

Discussion

Despite various preventive strategies being implemented in the clinic for modulation of pathogenic function of alloreactive donor T cells, significant incidence of acute GVHD remains a challenge and limits the broad application of HSCT as a therapeutic modality for various disorders. Clinically, the major challenge is to reduce the severity of GVHD without simultaneously compromising the beneficial effects of T cell mediated immune responses. We herein describe a novel strategy to physically deplete alloreactive T cells *in vivo* as an effective means of preventing acute GVHD in a mouse model simulating HSCT in haploidentical clinical setting. We employed Fas/FasL mediated AICD as a strategy to specifically purge out alloreactive T effector cells to prevent acute GVH. AICD via Fas/FasL pathway is an important homeostasis mechanism to limit clonal expansion of activated T cells at the down phase of immune responses. AICD has been demonstrated in human and murine T cells following activation and plays a vital role in the maintenance of peripheral T cell tolerance[35,36,128,159]. Mature T cells acquire specific AICD susceptibility after prolonged activation and are dependent on repetitive TCR engagement with the antigen and cell cycle progression, thus spearing resting T cells[34,136,160]. Using the ProtEx™ platform technology pioneered in our lab[145], we engineered donor T cells to transiently display on their surface a novel from of the Fas death ligand, FasL, molecule chimeric with a modified form of streptavidin, SA-FasL. Our collective results show that this strategy efficiently depletes activated alloreactive T cells, resulting in the prevention of lethal acute GVHD in an allogeneic and a humanized mouse model.

T cells were effectively engineered with SA-FasL and underwent apoptosis following activation by alloantigens both in vitro and in vivo. Coculture of SA-FasL-engineered alloreactive T cells with unmodified cells in a mixed lymphocyte in vitro study demonstrated that apoptosis occurs primarily through an autocrine fashion by engagement of SA-FasL with Fas receptor on the same T cells. Importantly, SA-FasL was effective at femtogram levels in the elimination of alloreactive T effector cells in vivo. These observations are consistent with reports by others demonstrating that membranous, but not soluble, FasL is effective in the induction of apoptosis by crosslinking Fas receptor[161]. Our published and unpublished studies in solid organ and islet transplantation models[129-131] using SA-FasL also corroborate the present findings. Interestingly, SA-FasL also eliminates donor Treg cells in our model. A study by Fritzsching *et al*.[144] demonstrated that freshly isolated naive Treg cells ($CD4^+CD25^+FoxP3^+$) have higher susceptibility to Fas-mediated apoptosis post TCR restimulation confers resistance to Fas-mediated apoptosis in contrast to T effector cells. This suggest that SA-FasL on Treg cells provides apoptotic signals early on before this cell population being activated, whereas SA-FasL deliver apoptotic signals in T effector cells after TCR activation.

We then established a clinically relevant parent (C57BL/6, $H\text{-}2^b$) to F1 (C57BL/6xBALB/c, $H\text{-}2^{b/d}$) haploidentical HSCT model. In as much as transplantation of whole bone marrow cells into a lethally irradiated mouse does not develop GVHD, unlike rats and humans, we admixed bone marrow cells with autologous splenocytes and demonstrated the transplantation of this cell mixture from C57BL/6 into lethally irradiated F1 recipients resulted in acute GVHD. Using this model, we demonstrated that donor cells containing splenocytes engineered with SA-FasL was effective in preventing acute GVHD,

whereas all mice receiving SA control protein engineered cells showed classical signs of GCHD, including hunched posture, decrease in mobility, ruffled fur and skin rashes along with diarrhea, and succumbed to acute GVHD and expired. As a prelude to clinical translation of this approach, we also demonstrated that human T cells engineered with SA-FasL underwent apoptosis in response to mouse xenoantigens in vivo that resulted in significant protection against acute GVHD.

Alloreactive T cells mediated destruction of primary and secondary lymphoid organs is a good indicator of acute GVHD and is responsible for the lack of immune reconstitution following HSCT in conditioned recipients[162]. Inefficient immune reconstitution contributes to frequent opportunistic infections and relapse. When long term surviving animals (>100 days) were analyzed for immune composition, they were fully donor chimeric with efficient engraftment and better frequency of immune cell components as compared with long term surviving animals that received only bone marrow cells, but not GHVD causing spleen cells. Efficient immune competency was further confirmed by demonstrating that long term survivors rejected third party, but not those animals that received donor skin allografts. These findings also indicate that SA-FasL selectively purges out alloreactive T cells, leading to systemic tolerance to donor alloantigens, but preserves non-alloreactive T cells so that they generate an effective immune response to donor-unrelated antigens.

Clinical GVHD involves significant target tissue tropism that includes recognition of either major or minor histocompatibility antigens followed by proliferation in lymphoid organs and target tissue infiltration resulting in significant tissue damage. Multiple studies suggest that the GVHD progression is highly time sensitive. Allogeneic T cell activation

occurs in several lymphoid organs at early time point (afferent phase day 6) followed by dramatic expansion of effector T cells[163,164]. Activation of alloreactive T cells occurs in draining lymph nodes rather than peripheral lymph nodes. Clinical significance is supported by the inability of memory T cells to induce lethal acute GVHD[165], despite their ability to alloreactivity as naïve T cells. Furthermore, $CD62L^+$ Treg, but not $CD62L^-$, cells are capable of suppressing acute GVHD *in vivo*, despite both cells being able to suppress alloreactive T cell proliferation *in vitro*[166,167]. Study by Nguyen *et al.*[167] suggested that alloreactive T cells had similar pattern of early activation, proliferation and localization with respect to Treg cells. However, persistent BLI (bioluminescence imaging) signals indicated that proliferation of alloreactive T cells continued to increase in lymphoid tissues and target organs in parallel with development of signs of GVHD. In addition, they show that Treg cells as cellular therapy to prevent acute GVHD is time and dose dependent, such that earlier infusion of Treg cells led to greater reduction of effector T cells proliferation and better survival. The observed protective effect is dose dependent requiring higher doses of Treg cells to reverse established inflammation. In our study, SA-FasL engineering induced apoptosis of alloreactive T cells post infusion, thus significantly reducing their number and thereby limiting their proliferation. When analyzed at day 21 post transplantation (effector GVHD phase), SA-FasL-spleen cells recipient had significantly less activated $CD4^+$ and $CD8^+$ T cells, but these animals also had substantially less Treg cells. The Treg/T effector ratio is a critical determinant of suppression; however, SA-FasL-spleen and unmanipulated spleen recipients had similar level of Treg/activated T effector ratios. However, the depletion of donor Treg cells before transplantation negated the

beneficial effect of SA-FasL, providing direct evidence that Treg cells play a critical role in our model.

The source of discrepancy between the transplantation of Treg-depleted cells and phenotyping at day 21 post-transplantation in our model is unknown and may be due to several factors. First, FoxP3 as a canonical marker of Treg cells does not define their regulatory capacity. Treg cells show plasticity dictated by the nature of the microenvironment they function in. In particular, Treg cells can assume pathogenic functions in response to excessive inflammation. IFN-γ has been shown to limit Treg cell function[168] and acute GVHD is characterized by a highly inflammatory state. Expression of FoxP3, a master regulator of Treg cells, alone is not adequate for functional stability. The suppressive function of these cell population is well correlated with DNA methylation status of *FoxP3 CNS2* region[169]. Acute GVHD has been considered a Th1-type disease dominated by cytotoxic T cell mediated pathology with increased production of Th1 cytokines. When analyzed by qRT-PCR, SA-FasL-spleen recipients had significantly less Th1 phenotype (T-bet, IFN-γ, TNF-α) and other proinflammatory mediators (IL-6) than control groups. Instead, SA-FasL-spleen recipients had skewed differentiation with upregulated IL-4, GATA-3 and RORγt. This is in line with previous reports demonstrating that the depletion of alloreactive Th1 T cells by AICD polarizes the residual $CD4^+$ T cells to a Th2 phenotype, which may protect against acute GVHD[52,170]. Treg cells has been implicated in therapeutic role to prevent acute GVHD. However, significant number of Treg cells needs to be infused and multiple experimental protocol for ex vivo expansion of donor Treg cells are under trial[53,171]. Interestingly, $CD25^+$ Treg cells were still indispensable in the preventive strategy despite the significant elimination of alloreactive

cells *in vivo*. This observation is similar to the high dose post-transplant cyclophosphamide strategy that requires Treg cells for its preventive effect[172].

In conclusion, we demonstrate human and mouse T cells can efficiently be engineered with SA-FasL protein in a practical and clinically applicable manner and that this strategy is effective in preventing acute GVHD in two clinically relevant settings, myeloablative haploidentical and humanize mouse models. Engineering donor graft with SA-FasL provides efficient, attractive and facile means of modulating immune response to prevent lethal acute GVHD with significant translational potential.

CHAPTER 3

PANCREATIC ISLETS SURFACE ENGINEERED WITH CD47 INNATE IMMUNE CHECKPOINT SHOW ENHANCED ENGRAFTMENT FOLLOWING INTRAPORTAL ISLET TRANSPLANTATION

Introduction

Islet transplantation is an important β-cell replacement therapy for refractory chronic pancreatitis and type 1 diabetes (T1D). A major limitation to clinical beta cell replacement therapy is a significant loss of islets immediate post-transplantation triggered by instant blood mediated inflammatory reaction (IBMIR)[173,174]. IBMIR is initiated when islets come into direct contact with the recipient blood following intraportal infusion and is responsible for the loss of 50-70% of the initial islet mass[175,176]. IBMIR is characterized by the activation of coagulation cascade, complement activation, and infiltration of myeloid cells that express various inflammatory mediators that result in the destruction of islets[80,84]. Tissue factor (TF) and various other proinflammatory cytokines and chemokines, such as IL-1β, IP-10, IL-6, CXCL8, CXCL10, CCL2, expressed by islets or resident antigen presenting cells trigger IBMIR[79,84,88,177,178]. As such, insulin independence rate for autologous islet transplantation at 5 years can be as low as 10% and multiple infusions of islets from different donors may be required to achieve insulin independence in recipients of allogeneic islet grafts[179,180]. Therefore, effective control of IBMIR will overcome an important limitation of clinical islet transplantation.

CD47 is a ubiquitously expressed transmembrane glycoprotein of the immunoglobulin superfamily that serves as a ligand for the signal regulatory protein alpha (SIRPα), an immune inhibitory receptor, expressed on the surface of myeloid cells[119]. The interaction of CD47 with SIRPα serves an important innate immune checkpoint that delivers a "don't-eat-me" signal and acts as a critical regulator of "marker of self"[119]. The importance of CD47/SIRPα axis in self-recognition and inhibition of phagocytosis by macrophages was demonstrated by rapid clearance of red blood cells genetically modified to lack CD47 expression following infusion into CD47 competent syngeneic mice[110].

CD47-SIRPα pathway has been implicated in the regulation of both innate and adaptive immune responses. The system negatively regulates macrophage activation and phagocytosis[119], adhesion and activation of platelets and neutrophils[116], attenuation of antibody-dependent cell-mediated cytotoxicity/phagocytosis (ADCC/ADCP)[181,182], and adaptive immune responses[183-185]. These functional features of CD47 were shown to be extensively exploited for immune evasion by various tumors that highlighted the potential of CD47-SIRPα axis as an important target for cancer immunotherapy[186]. Indeed, various tumor cells were shown to have elevated levels of CD47 expression and its blockade using antibodies resulted in effective therapy in preclinical cancer models that led to current efforts to test this innate immune checkpoint blockade for cancer immunotherapy in the clinic[186-189].

CD47/SIRPα pathway has been shown to play a critical role in innate allo- and xeno-recognition, independent of adaptive immunity[190,191]. Polymorphism in the SIRPα that alters the binding strength of CD47 is associated with rapid clearance of various cellular grafts, including hepatocytes[120,185], insulinoma cells[192], and hematopoietic stem

cells[108,193,194]. Ectopic expression of recipient CD47 in porcine cells blocks their phagocytosis by human macrophages[195]. Similarly, hematopoietic cells from swine genetically modified to express human CD47 showed enhanced engraftment in a humanized mouse model of transplantation[196].

Given the demonstrated role of CD47-SIRPα axis as innate immune checkpoint, we hypothesized that CD47 can be used to enhance syngeneic islet engraftment by mitigating IBMIR. We herein report the generation of a novel form of SA-CD47 molecule and demonstrate for the first time that that the transient display of CD47 protein on the surface of islets enhance engraftment and long-term function in a minimal mass intraportal islet transplantation mouse model by mitigating IBMIR. The rapid and transient display of SA-CD47 protein on the surface of tissues and cells to mitigate IBMIR provides a facile and clinically applicable platform with significant translational potential for transplantation of various tissue and cellular grafts.

Materials and Methods

Animals

C57BL/6 (CD45.2, $H2K^b$) mice were purchased from Jackson Laboratory. Animals were bred and maintained in our specific pathogen-free animal barrier facility at the University of Louisville, KY. All experiments were performed in accordance with the policies of the NIH for Guide for the Care and Use of Laboratory Animals and protocols approved by the University of Louisville Animal Care and Use Committee.

Construction, production, and physical characterization of SA-CD47 protein

A synthetic gene encoding the extracellular domain of CD47 (19-161 bp; GI: AB012693.1) of C57BL/6 murine strain was subcloned C-terminus to a modified form of streptavidin in the drosophila pMT/BiP/V5-His $CuSO_4$-inducible expression vector (Fig. 1)[145]. Drosophila S2 cells were transfected with the construct using the Celfectin II transfection system (Invitrogen) to establish stable transfectants. SA-CD47 expression was induced using 1 mM $CuSO_4$, culture medium was collected 3 days later, and the protein was purified using a metal-ion charged sepharose column taking the advantage of the 6xHis tag engineered into the C-terminus (GE, Amersham). Purified protein was characterized using SDS-PAGE and Western blot analysis per published protocols[130,145].

Cell surface engineering with SA-CD47 protein

Spleens from C57BL/6 or Lewis rat were harvested and processed into single-cell suspension using frosted slides. Red blood cells were lysed using a home-made buffered ammonium chloride solution. SA-CD47 engineering of spleen cells was done following previously reported protocol[131]. Cell surface was modified with biotin by incibation in 5 μM EZ-Link™ Sulfo-NHS-LC biotin (hereafter referred to as biotin) solution (Pierce) in sterile PBS at room temperature for 30 mins. Cells were then washed twice with sterile PBS followed by incubation with 200 ng SA-CD47 or an equimolar amount of SA protein per 10^6 cells at a final volume of 500 μl of sterile PBS for 30 mins in a cold room by constant rocking. Cells were then washed and analyzed for the presence of biotin and SA-CD47 on the cell surface by staining with streptavidin-allophycocyanin (APC) and APC-labelled anti-mouse CD47, respectively, using flow cytometry.

In vitro phagocytosis assay

In vitro phagocytosis assay was done as described before[112,183]. Briefly, SA- and SA-CD47 engineered rat spleen cells were labeled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific) or 2.5 μM Cell Trace Violet (CTV, Thermo Fisher Scientific), respectively, according to manufacturer's protocol and resuspended in complete RPMI (RPMI 1640 supplemented with 10% FBS, Penicillin/ Streptomycin and 2 mM L-Glutamine). For single culture assay, SA-rat spleen cells or SA-CD47-rat spleen cells (2.5 X 10^6/ well) were cultured with mouse RAW 264.7 macrophage-like cell line (0.5 X 10^6/well). For mixed culture settings, both fluorescence labeled target cells were mixed in a 1:1 ratio followed by co-culture (2.5 X 10^6 cells/well) with RAW 264.7 cells (0.5 X 10^6/well). Post incubation non-engulfed cells were washed, and adherent RAW cells were harvested and stained with anti-CD11b antibody prior to flow cytometric analysis. Phagocytosis assessed by measuring % of double positive macrophages ($CD11b^+$ $CFSE^+$ or $CD11b^+$ CTV^+).

Pancreatic islet isolation, engineering, and functional analysis

Islet isolation was performed using Liberase^{TL} enzyme (Roche) according to a standard protocol[128,130]. Islets were engineered with the SA-CD47 or SA as a control protein by incubating first in 5 μM biotin then with the indicated amount of protein for 30 mins[130,145]. Alamar blue (AB, Thermo Fisher scientific) assay, that incorporates redox reagent that changes color when reduced in response to metabolic activity, was used to assess the effect of SA-CD47 engineering on viability and metabolic activity of islets[197]. Briefly, 100

unmodified naïve or SA-CD47-engineered islets were cultured in complete RPMI media supplemented with 10% AB reagent in a 96-well plate. After 6 hours of incubation, the reduced form of reagent soluble in the solution was measured as absorbance at a wavelength of 562 nm on SpectraMax microplate reader (Molecular Devices).

Glucose-stimulated insulin secretion (GSIS) assay

A static incubation protocol for GSIS assay was performed to assess whether engineering with SA-CD47 impacts islet function. Naïve islets or SA-CD47-engineered islets (100 islets per transwell) were incubated at 3 mM glucose solution, prepared in Krebs ringer bicarbonate buffer, for 1 hour for equilibration in a transwell (Millicell, Merck). The transwell was then drained and transferred to a new low glucose solution well, incubated for 1 hour, and the solution was collected as low-glucose insulin secretion. The transwell was then transferred to a high glucose solution (11 mM) well, incubated for 1 hour, and the solution was collected as high-glucose insulin secretion. The insulin content was analyzed by murine insulin ELISA kit (Mercodia). The stimulation index (SI) was calculated as a ratio of insulin secreted in high to low glucose stimulation.

Pancreatic islet transplantation and glucose tolerance test

The impact of SA-CD47 on the modulation of IBMIR and islet engraftment was assessed in a minimal mass intraportal model. Four days before transplantation, male C57BL/6 mice (10-14 wks-old) were rendered diabetic by single intravenous injection of streptozotocin (200 mg/kg, Sigma-Aldrich). Blood glucose level were obtained using a

portable glucose meter (Roche, AccuCheck). Animals with two consecutive non-fasting blood glucose readings of > 250 mg/dl were considered diabetic and used for intraportal transplantation. Each diabetic recipient was transplanted with 125 SA- or SA-CD47-engineerd islets via portal vein. Animals were monitored for blood glucose levels twice a week. Long-term graft recipients with normoglycemia were subjected to a standard intraperitoneal glucose tolerance test (IPGTT). Briefly, animals were injected intraperitoneally with glucose bolus (2 mg/kg) after 6 hrs of fasting and monitored for blood glucose levels at various time points (0, 10, 20, 30, 45, 60, 90, 120, 150 mins). Naïve C57BL/6 mice were used as controls for IPGTT.

Isolation of intrahepatic immune cells

Intrahepatic immune cells were isolated using mechanical disruption as previously described[198]. Briefly, after euthanasia, cardiac perfusion was performed with 10 ml of sterile, cold saline. Mechanical disruption was done using frosted slides (in HBSS) followed by repeated passing through a18-G needle. The suspension was centrifuged at 500 rpm for 1 min and supernatant was transferred to a new tube and centrifuged at 1400 rpm for 10 min. The pellet was resuspended in complete RPMI, filtered, and processed for flow cytometry analysis.

Flow cytometry and antibodies

The optimal concentration for all fluorochrome-conjugated antibodies was determined by titration. Antibodies clones specific against mouse CD45.2 (eBiosciences, 12-0454-82),

CD11b (BD Biosciences, 550993), Ly6C (Biolegend, 128036), Ly6G (Biolegend, 127606), F4/80 (Biolegend, 123122), Gr1 (eBiosciences, 48-5931) were used. For surface staining, cells were first incubated with Fc blocking antibody (anti CD16/32, Biolegend) for 10 min at 4° C followed by incubation with surface antibodies for 30 min at 4° C. The cells were analyzed by BD LSR II and Flow jo (Tree Star, CA).

***In vitro* tube model of islet-blood interactions**

In vitro tube model to mimic IBMIR was performed as described [173]. Fresh autologous blood was harvested and collected in heparin-coated 1.5 ml Eppendorf tubes. SA- or SA-CD47-engineered islets (100 islets) were mixed and incubated with 500 µl of blood (without any additional heparin). Tubes were incubated in a rotator at 37 °C incubator for 3 hrs. Post incubation, serum was collected, and blood-islet clots were preserved in 10% Neutral Buffered Formalin (NBF, source) followed by paraffin embedding for histological analysis.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the liver tissues or islet-thrombus using TRIzol reagents (Invitrogen Corporation, USA) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop ND-2000c spectrophotometer (Thermo Fisher Scientific, Inc.). In addition, cDNA was reverse transcribed from total RNA (2 µg) using SuperScript IV VILO cDNA Master Mix (Thermo Fisher Scientific, USA) with the following thermal conditions: 25 °C 10 min; 50 °C 10 min; and 85 °C 5 min. qRT-PCR was performed using validated TaqMan Gene Expression Assays (Table 1) according to the manufacturer's instruction on Quant Studio 3 RT-PCR system (Thermo Fisher Scientific. USA).

Table 4. TaqMan gene expression assay for islet graft analysis

Gene symbol	Assay ID
F3 (TF)	Mm00438855_m1
IL1b	Mm00434228_m1
MCP-1	Mm00441242_m1
NF-KB (p65)	Mm00501346_m1
GAPDH	Mm99999915_21
HMGB1	Mm00849805_gH

Histological analysis

Paraffin-embedded clots were cut into 4 µm thickness, H&E stained, and then graded for parameters as described[199]. Grade 0: intact islet morphology with uniform nuclear distribution without any fragments/fractures. Grade 1, intact islet morphology with uniform nuclear distribution with minimal fragments/fractures; Grade 2, disrupted morphology with non-uniform nuclear distribution with significant fragments/fractures along with necrotic patches; and Grade 3, significant disruption of morphology with loss in nuclei and significant necrotic patches.

For immunohistochemical analysis, islets bearing liver sections or islets clots retrieved from *in vitro* tube assay were paraffin-embedded, then sectioned at 4 µm thickness on a microtome (Leica). The sections were dewaxed and processed for histological evaluation as reported[87,90,199]. Sections were stained with anti CD11b (Novus biologicals, NB600-137)

or anti-insulin (Dako, A0564) or Hoechst (Molecular probes, H-3570). Fluorescent images were obtained using a Leica confocal microscopy.

Statistical analysis

All data sets were analyzed using Graph pad prism v.7 (GraphPad Inc. CA) and expressed as mean ± SEM. Mean comparison between the groups was performed by using Student's t-test. Survival curves were compared using the Log-rank test (Mantel-Cox) statistical method. $p < 0.05$ was considered significant.

Results

Generation and characterization of chimeric SA-CD47 protein

Transient and positional display of recombinant protein ligands to immune receptors on the cell surface provides a practical and potentially safe alternative to ectopic gene expression for immunomodulation. We have developed the concept of generating recombinant immunological ligands with a modified form of core streptavidin and their transient and positional display on the surface of biotinylated cells and tissues for immunomodulation with demonstrated efficacy in autoimmunity, transplantation, and cancer settings[129,200-204]. Using this scheme, we herein designed a synthetic gene encoding the extracellular domain of mouse CD47 (aa 19-161), required for binding and signal transduction through SIRPα, and a modified form of core streptavidin (SA). The chimeric gene was cloned into the Drosophila copper sulfate-inducible pMT/BiP/V5-HisA expression vector with an N-terminal secretion signal sequence (BiP) and a hexahistidine tag (6xHis; Figure 22A). *Drosophila* S2 cells stably transfected with the construct used for protein expression and purification using a metal affinity chromatography as we reported previously[129]. SA-CD47 was run as a ~37 kDa protein in denaturing SDS-PAGE when the samples were heated at 100° C and as > 250 kDa oligomers without heat treatment (Figure 22B), consistent with the reported structural feature of native streptavidin[145]. Western blot analysis using anti-SA antibody confirmed the identity of the protein (Figure 22C).

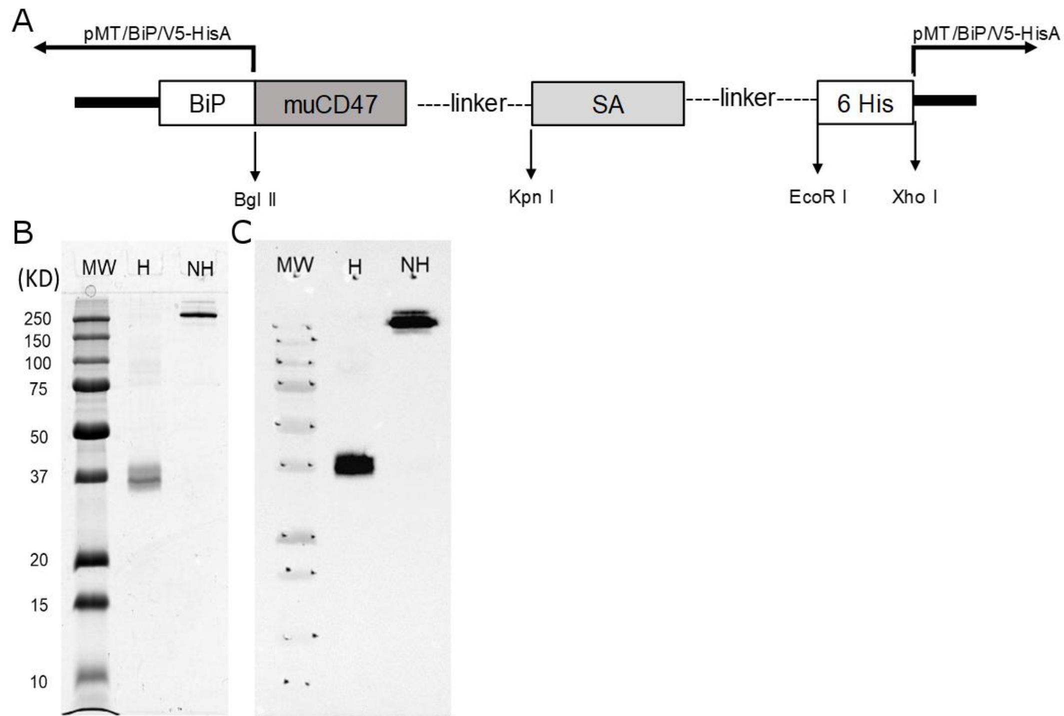

Figure 22. Cloning, expression, and structural characterization of SA-CD47 protein. (A) Schematic representation of SA-CD47 construct. A synthetic chimeric gene encoding the extracellular domain of mouse CD47 N-terminus to core streptavidin (SA) with flexible linkers and a 6xHis tag to facilitate protein purification was subcloned into the $CuSO_4$-inducible pMT-Bip-V5-HisA S2 insect cell expression vector. (B) SDS-PAGE profile of the purified SA-CD47 protein. The protein was purified from culture supernatant of S2 stable transfectants using immobilized metal affinity chromatography and analyzed using SDS-PAGE after heating the samples at 100 ^{o}C (H) or without heat (NH). (C) Western blot profile of SA-CD47 using antibodies against streptavidin. The SA-CD47 chimeric protein runs as a single monomeric band (~ 37 kDa) with heat and as oligomers (> 250 kDa) without heat.

Effective engineering of islets with SA-CD47 protein without negatively impacting their viability or function

We next employed engineering of mouse spleen cells as a flexible and quantitative platform to assess the function of streptavidin domain of chimeric SA-CD47 protein for binding biotinylated surfaces[145]. Mouse spleen cells were first modified to attach a reactive biotin to the cell surface through NHS ester crosslinking followed by engineering with various concentrations of the SA-CD47 protein. There was a dose-dependent attachment of SA-CD47 to the surface of biotinylated cells that reached a plateau at 320 ng protein/10^6 cells (~ 99 % of live cells with a mean MFI of 6260) as assessed by flow cytometry (Figure 23 and 24A).

We next tested if pancreatic islets can be engineered with SA-CD47 without a detrimental effect on islet viability and function. C57BL/6 mouse islets were surface modified with biotin (5 µM) followed by engineering with SA-CD47 protein (400 ng/125 islets). Confocal microscopic analysis of islets using an anti-CD47 Ab demonstrated dense display of the protein on the surface (Figure 24B). Islets were analyzed for viability and metabolic activity using Alamar Blue (AB) assay to ensure the lack of a detrimental effect due to SA-CD47 engineering. Viable and metabolically active cells reduce resazurin, a component of AB, to resorufin by mitochondrial enzymes[197]. The degree of redox reaction is an indicator of the metabolic activity of viable cells[197]. After incubation for 6 hrs in AB, there was no significant difference in the level of reduced resorufin between SA-CD47-engineered islets and unmodified control islets (Figure 24C), demonstrating lack of a detrimental effect causing by engineering. This observation was further corroborated with FDA/PI viability assay performed 24 hr post-engineering. Confocal microscopy analysis

revealed that the majority of SA-CD47-engineered islets (> 90%) were intact and viable as indicated by bright FDA staining (Figure 24D). Engineered islets were also morphologically intact without any hypoxic centers (Figure 25). GSIS assay also showed comparable levels of functional fitness between SA-CD47-engineered islets and unmodified control islets in insulin secretion (Figure 24E). Importantly, no difference in stimulation indices of SA-CD47-islets and unmodified islets were observed (Figure 24E). Taken together, these results demonstrate that pancreatic islets can be efficiently engineered to display the SA-CD47 protein on their surface without a negative impact on their viability, metabolic activity, and insulin secretion function.

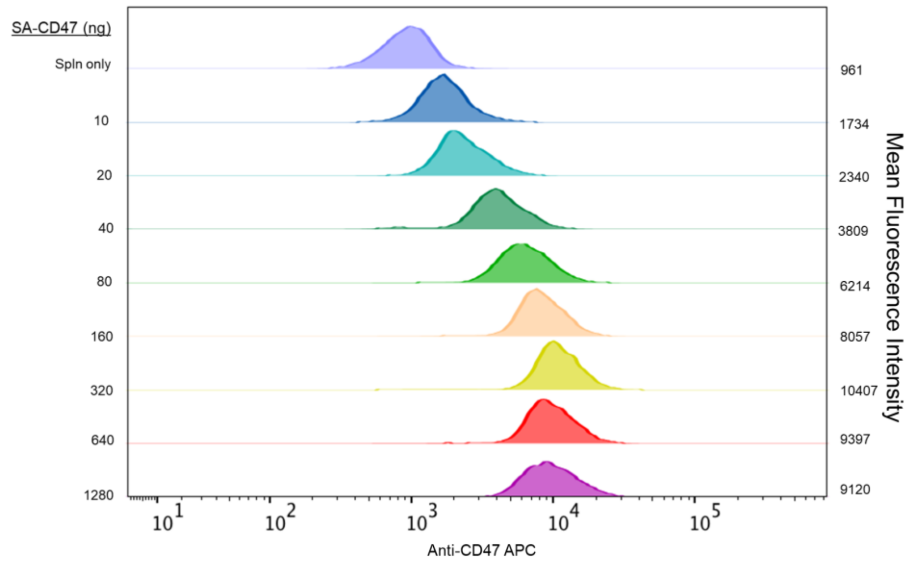

Figure 23. Representative histogram overlay plot of spleen cells engineered with SA-CD47 protein. Spleen cells were biotinylated (5 µM) and then engineered with SA-CD47 protein at the indicated concentrations (ng protein/10^6 cells). Histograms in color show the fluorescence intensity (MFI) at respective engineering levels.

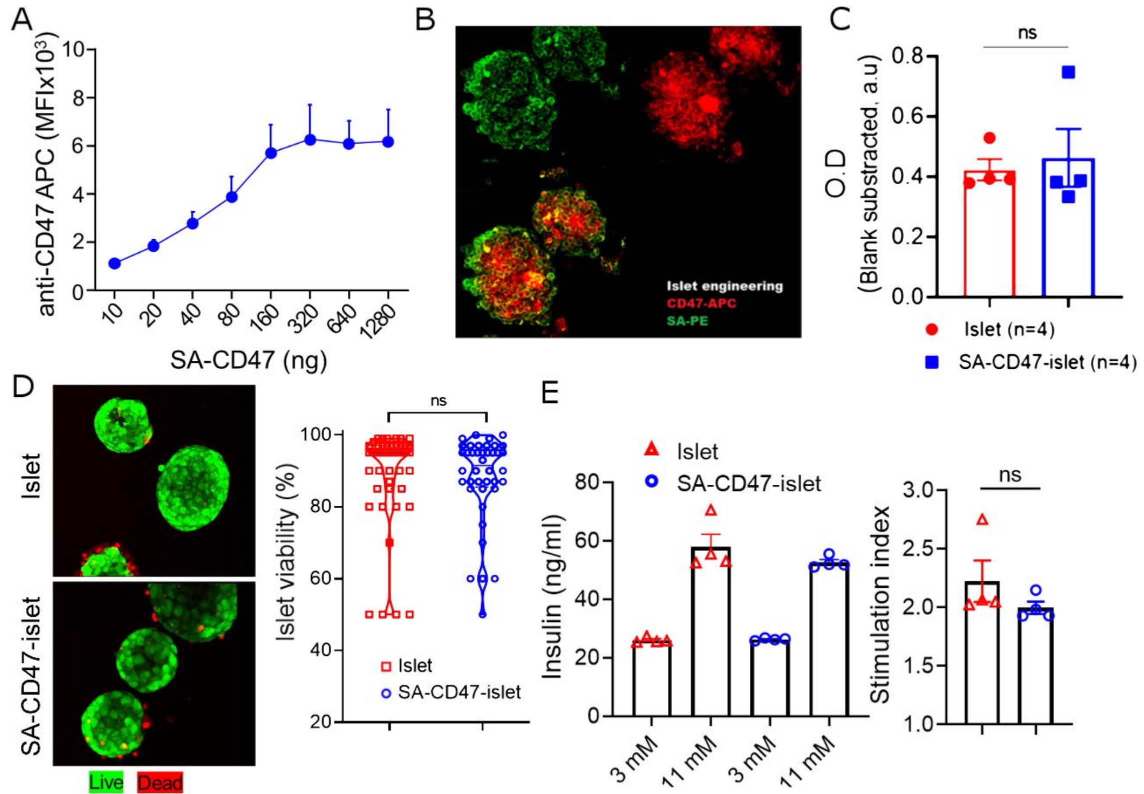

Figure 24. Effective engineering of islets with SA-CD47 protein without a negative impact on their viability or function. (A) Assessment of cell surface engineering with SA-CD47 protein. Spleen cells were biotinylated in 5 μM EZ-Link Sulfo-NHS-LC-Biotin followed by engineering with the indicated amounts of the SA-CD47 protein (ng/10^6 cells). The levels of chimeric protein on the cell surface was assessed using an anti-CD47 antibody in flow cytometry. The MFI values as a function of protein concentrations from three independent experiments are graphed (B) Representative image of islet engineered with SA-CD47 protein. Mouse islets were biotinylated (5 μM) followed by engineering with SA-CD47 protein (400 ng/125 islets). The levels of biotin and CD47 molecules were assessed using SA conjugated PE (SA-PE; green) and an antibody against CD47 (anti-CD47 APC; red), respectively, in confocal microscopy. (C) Engineering with SA-CD47

protein does not impact islet viability and metabolic activity. SA-CD47-engineered islets (SA-CD47-islet) or unmodified naïve islets (Islet) were incubated with alamar blue reagent for 6 hours at 37°C. The level of alamar blue reduction was measured as absorbance at 562 nm. Data are from two independent experiments. (D) FDA/PI viability test showing lack of a detrimental effect of engineering on islets. Representative live/dead confocal microscopy images of unmodified and SA-CD47-engineered islets 24 hours post engineering (green = live; red = dead). A total of 47 unmodified (Islet) and 40 engineered (SA-CD47-islet) islets were analyzed for viability. (E) Glucose-induced insulin secretion assay showing functional fitness of SA-CD47-engineered islets. Data expressed as mean ± SEM. For comparison of means, an unpaired t-test was used for C, D and E. ns, not significant.

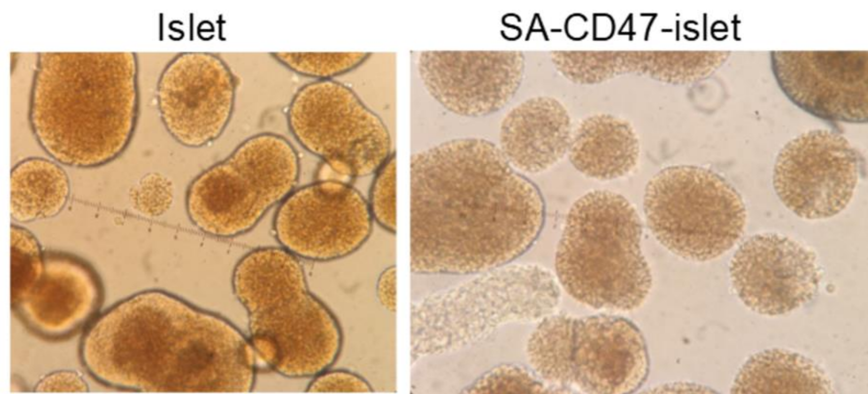

Figure 25. Bright field images showing integrity of SA-CD47-engineered islets. Unmodified (control, left) and SA-CD47-engineered islets (right) were cultured for 24 hours post engineering and then imaged.

SA-CD47-engineered xenogeneic cells are refractory to phagocytosis by macrophages.

CD47 expressed on autologous cells deliver a “don’t eat me” signal through SIRPα, a negative regulator of phagocytosis, expressed on macrophages and dendritic cells[119]. Interaction of CD47 with SIRPα is species-specific and dictates the phagosome formation[205]. Thus, we assessed the anti-phagocytic function of SA-CD47 in a standard xenogeneic *in vitro* phagocytosis assay[112,183]. Rat splenocytes were engineered with SA-CD47 or SA as a control protein (Figure 26A) and then labeled with the fluorescence dyes CTV or CFSE, respectively. Coculturing these cells with the mouse RAW 264.7 macrophage cell line demonstrated significant ($p = 0.0088$) phagocytosis of SA-engineered rat cells over SA-CD47-engineered cells (Figures 26B and 27). To further corroborate this observation, we performed competitive phagocytosis assay in mixed culture settings where RAW 264.7 cells were co-cultured in the presence of both target cells labelled with different fluorescence dyes. Mouse macrophage preferentially phagocytosed SA-rat spleen cells (Figures 26C and 27). Collectively, these results demonstrate that the SA-CD47 protein is functional and when displayed on target cells prevents phagocytosis by macrophages.

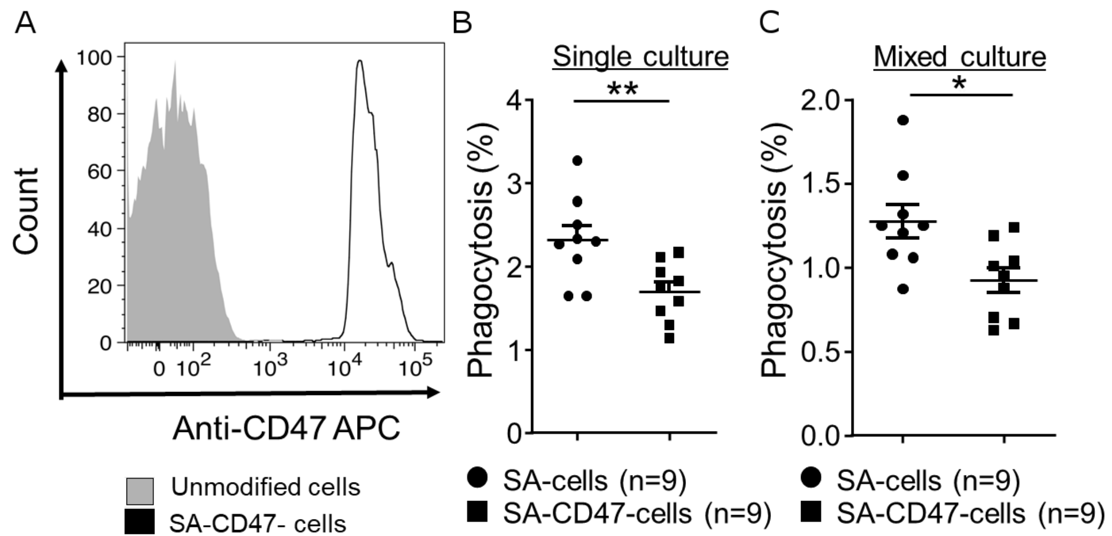

Figure 26. SA-CD47 protein on xenogeneic cells prevents phagocytosis by macrophages. (A) A representative histogram of rat splenocytes engineered with the SA-CD47 protein. Rat splenocytes engineered with the SA-CD47 protein (200 ng/10^6 cells) were analyzed using an antibody against mouse CD47 in flow cytometry (dark line open histogram) with unmodified cells (gray shaded histogram) serving as control. (B) SA-CD47 inhibits phagocytosis of rat splenocytes by macrophages. Mouse RAW 264.7 macrophage cells were cultured with CFSE-labelled SA-engineered rat splenocytes or CTV-labelled SA-CD47-enginered rat splenocytes at 1:5 ratio. After 18 hours of incubation, cells were analyzed by flow cytometry by gating on $CD11b^+CFSE^+$ or $CD11b^+CTV^+$ as percent phagocytosis. (C) Assessment of phagocytosis in mixed cultures. Rat cells engineered with SA and SA-CD47, and labelled with CFSE and CTV, respectively, were mixed at 1:1 ratio and then co-cultured with RAW 264.7 cells under the same conditions for (B). Phagocytosis of rat cells by macrophages was assessed using flow

cytometry. Data shown as mean ± SEM of three independent experiments. *p <0.05, **p <0.01; two-tailed unpaired t-test.

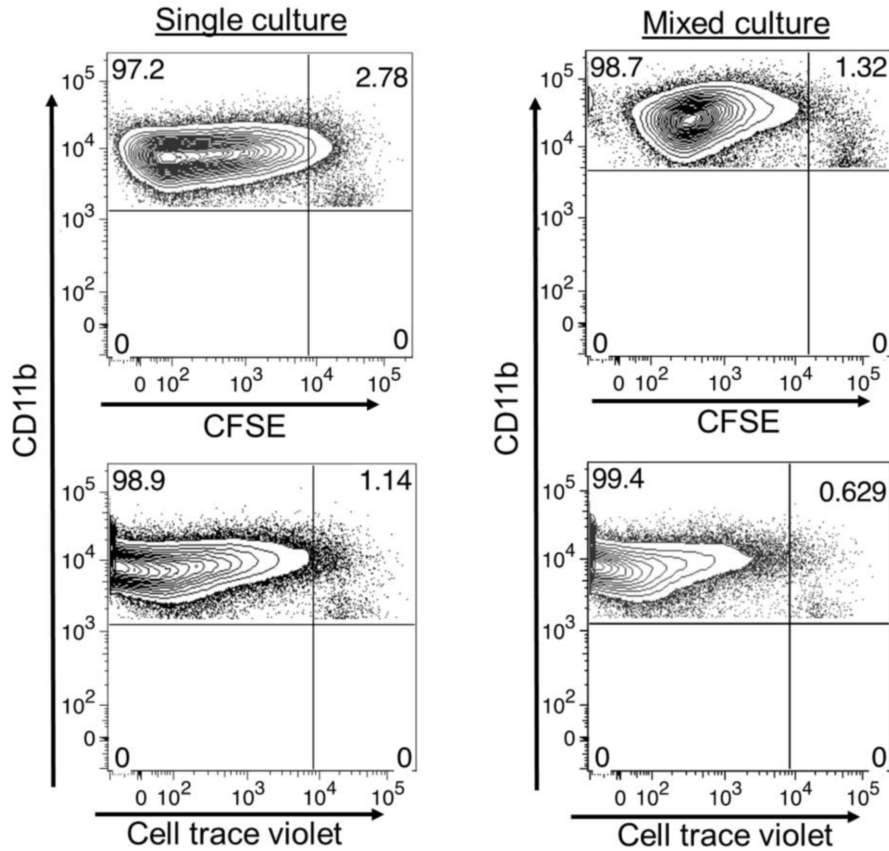

Figure 27. Representative flow plots of in vitro phagocytosis assay. SA-CD47-engineerd rat splenocytes (CTV labelled) are more refractory to phagocytosis by mouse macrophages as compared with SA-engineered cells in single or competitive mixed culture settings.

SA-CD47 protects islets and prevents intra-islet infiltration of CD11b$^+$ cells in an *in vitro* loop assay.

First, to understand the potential role of CD47 in protection of islets from IBMIR mediated destruction, we performed a modified *in vitro* loop assay for islet-blood interaction [173]. Engineering with SA-CD47 did not prevent fibrin deposition or thrombosis after incubation with blood. However, histological analysis of the islet-thrombus revealed relatively intact SA-CD47-engineered islets, whereas SA-engineered islets had significant ($p = 0.0128$) destruction (Figure 28A). Intact islets with regular morphology with uniform distribution of nuclei (Grade 0) were more common in SA-CD47-engineered islets (40%) as compared with the SA-engineered control group (25%) (Figure 28A). SA-engineered group had more Grade 2 islets with disrupted morphology with necrotic centers and Grade 3 islets with highly disrupted morphology as compared with SA-CD47-engineered group (Grade 2: 25% vs. 18%; Grade 3: 24% vs. 11%). Immunofluorescence analysis of islet-thrombus sections demonstrated significantly ($p = 0.0244$) higher infiltration of CD11b$^+$ cells in SA-engineered islets as compared with SA-CD47 group (Figure 28B and 29). Collectively, these results show that SA-CD47 protects islets from IBMIR mediated disruption, potentially by inhibiting the intraislet infiltration of inflammatory cells.

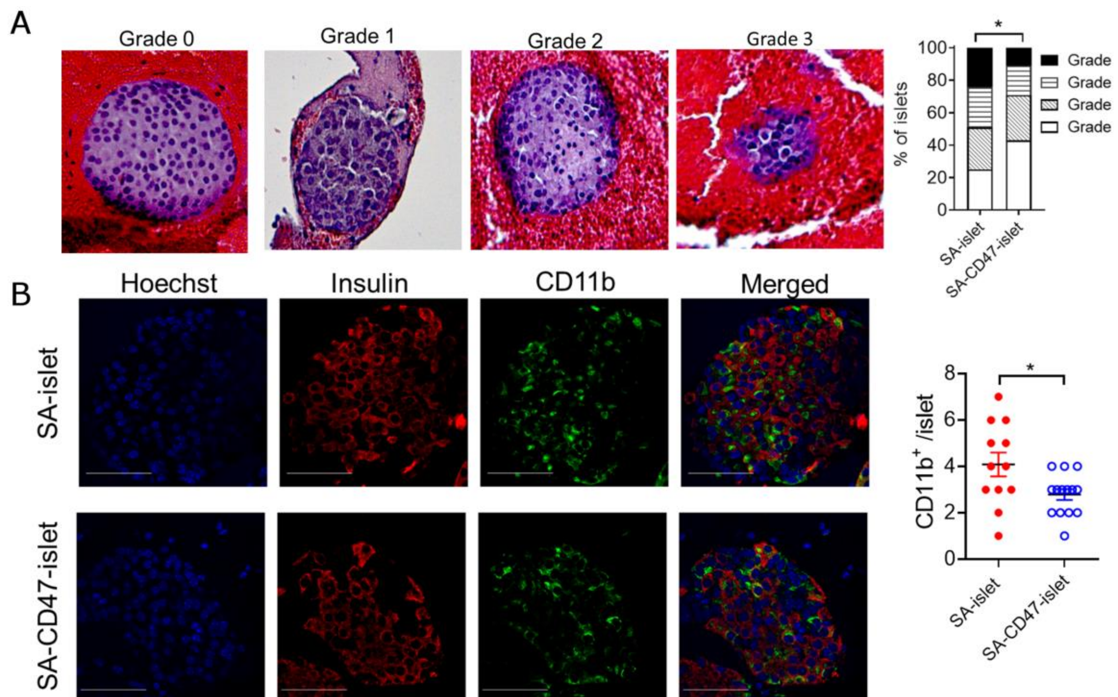

Figure 28. SA-CD47 engineering protects islets from destruction by IBMIR in an *in vitro* loop assay. (A) Histological grading of islets 3 hours post *in vitro* loop assay indicates SA-CD47-engineered islets are protected from blood mediated destruction as compared with SA-engineered control islets. Frequency distribution between the groups was analyzed by chi-square test. (B) Immunofluorescence analysis for intra-islet CD11b$^+$ inflammatory cells. SA-CD47-engineered islets had significantly less infiltration of CD11b$^+$ granulocytes/macrophages as compared to SA-engineered islets. Data pooled from three independent experiments and represented as mean ± SEM. Data means compared by unpaired two-tailed t-test. * $p < 0.05$.

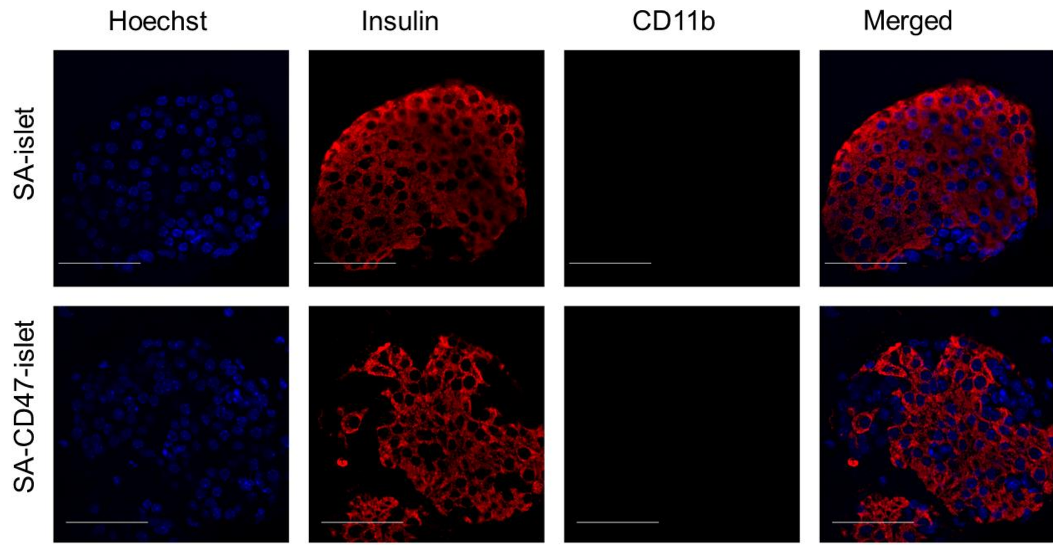

Figure 29. Representative confocal images for CD11b negative staining. Tissue sections were stained with Hoechst, insulin but without CD11b for specificity of anti CD11b antibody (scale bar = 50 μm).

SA-CD47-engineered islets show enhanced engraftment and function following intraportal transplantation

Instant blood-mediated inflammatory reaction (IBMIR) occurs following the exposure of islet grafts to recipient blood and is responsible for 50-70% of islet loss immediate post intraportal transplantation[79,173]. Phagocytes play an important role in IBMIR[80,84,87,173]. To assess the impact of SA-CD47 as an inhibitor of phagocytosis on islet engraftment and long-term function, C57BL/6 islets were engineered with SA-CD47 or SA as a control protein and used for intraportal transplantation in a syngeneic marginal mass (125 islets) model (Figure 30A). Seven out of 8 mice (87.5%) transplanted with SA-CD47-engineered islet graft showed engraftment and long-term function as assessed by blood glucose levels as compared with 1/7 (14.28%) of the SA-engineered islet graft recipients ($p = 0.0088$; Figure 30B, C and 31). The average days required for achieving normoglycemia was 17.86 ± 4.9 (mean ± SEM) in SA-CD47-islet recipients.

Intraperitoneal glucose tolerance (IPGTT) test showed significantly rapid glucose clearance in long-term (>80 days) recipients of SA-CD47-engineered as compared with SA-engineered islet grafts (Figure 32A). Indeed, long-term SA-CD47-engineered islet graft recipients had a glucose clearance response comparable to naïve animals with a similar area under the curve (Figure 32B). Histological analyses demonstrated insulin-secreting functional islet mass in long-term recipients (Figure 32 C and D). Collectively, these results demonstrate that the transient display of SA-CD47 protein on islet surface significantly enhance engraftment and long-term function following intraportal transplantation.

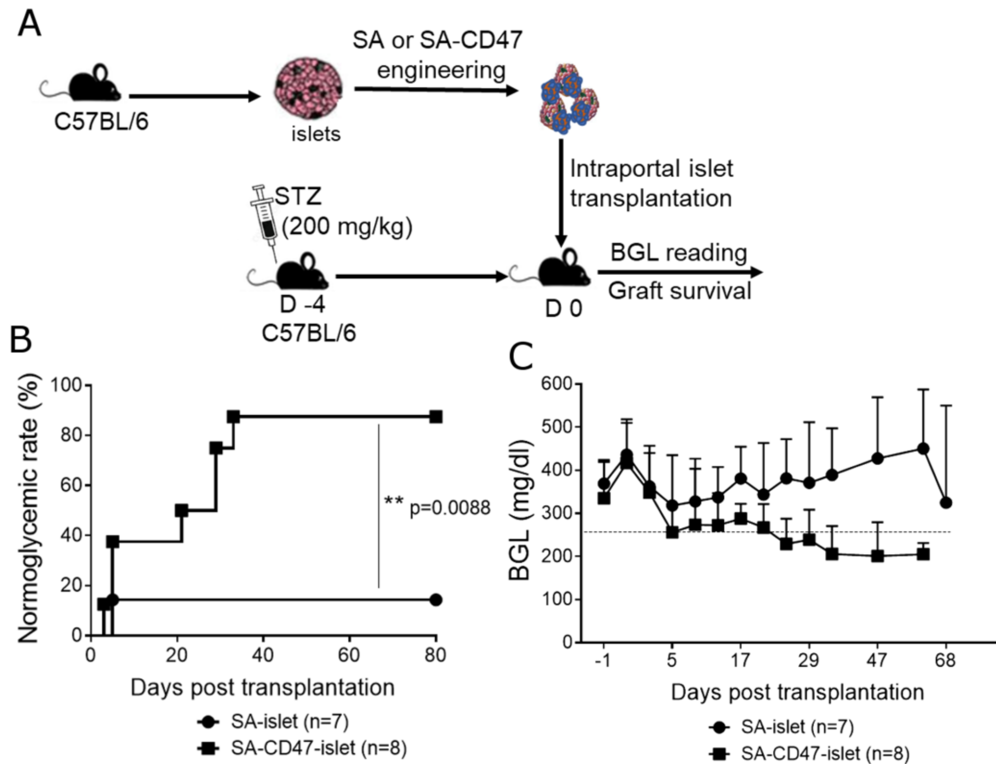

Figure 30. Surface display of SA-CD47 protein significantly improves pancreatic islet engraftment and function in a syngeneic marginal mass intraportal transplantation model. (A) Study design for intraportal islet transplantation. Animals were monitored for blood glucose levels (BGL) post-transplantation and two consecutive daily readings of glucose ≥ 250 mg dl^{-1} was considered as engraftment failure. (B) Kaplan-Meier analysis between the groups shows significantly higher rate of post-transplant normoglycemia in chemically diabetic recipients of SA-CD47-enginered as compared with SA-engineered control grafts. Statistical difference assessed using log-rank (Mantel-Cox) test with **p <0.01. (C) Non-fasting blood glucose level of transplant recipients in (B).

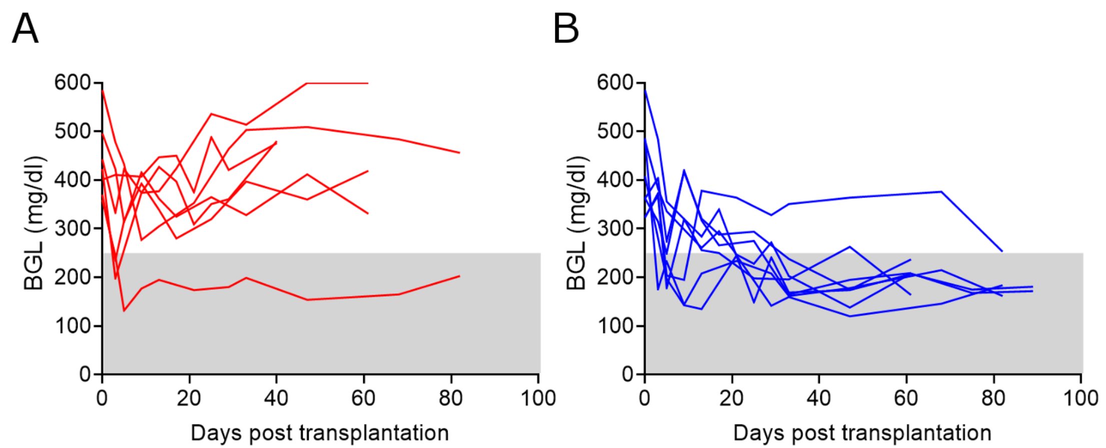

Figure. 31. SA-CD47 engineering improves the intraportal islet transplantation outcome. STZ induced (200 mg/kg) diabetic animals were transplanted with islets engineered with SA (A, n=7) or SA-CD47 protein (B, n=8). Animals were monitored for blood glucose levels (mg/dl) post transplantation. Grey shaded area indicates the euglycemic region (BGL < 250 mg/dl).

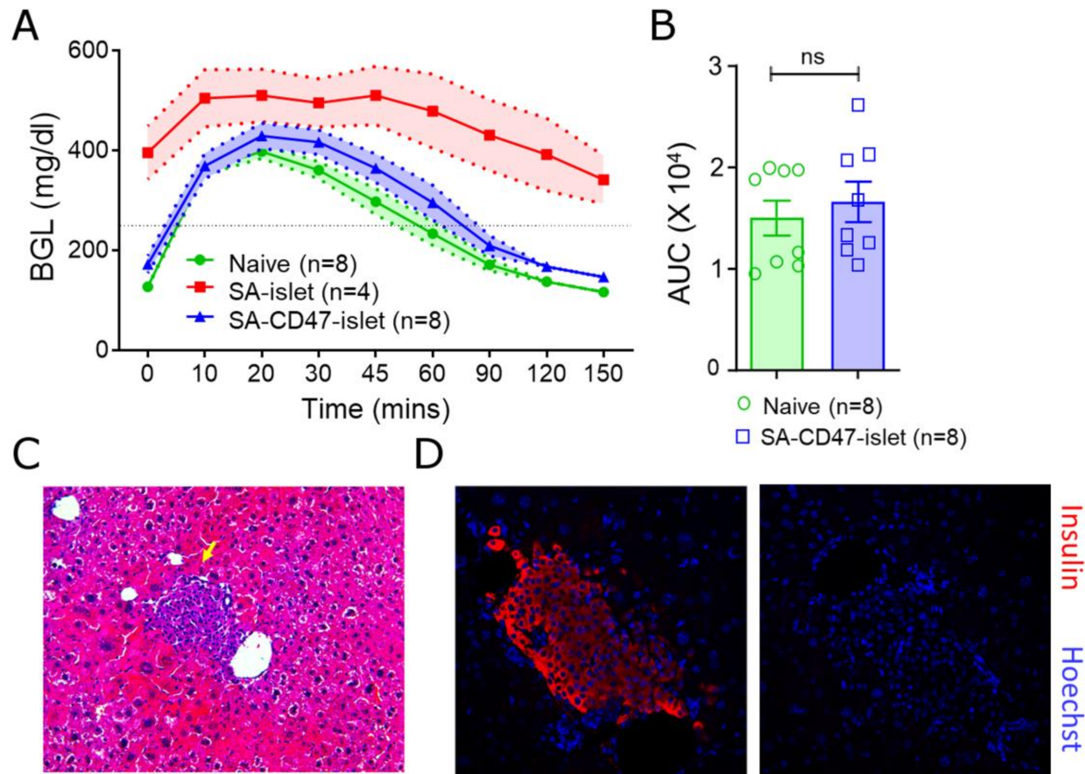

Figure 32. SA-CD47-engineered islet grafts show long-term survival and function following intraportal transplantation. (A) Intraperitoneal glucose tolerance test shows functional islet mass in long-term (>80 days) recipients of SA-CD47-engineered islet grafts. SA-CD47-engineered islet graft recipients (blue) were able to regulate blood glucose at levels comparable to age-matched naïve animals (green). In marked contrast, SA-engineered islet graft recipients (red) failed to regulate blood glucose levels following intraperitoneal glucose challenge. (B) Area under the curve analysis for (A). (C, D) Histological analyses showing insulin positive islets in the liver of long-term graft recipients. Representative H&E (C) and immunofluorescence staining (D) of liver tissues of long-term recipients of SA-CD47-engineered islet grafts. Nuclei are stained with Hoechst (blue) and β-cells with anti-insulin antibody (red). Data mean comparison was done by two-tailed unpaired t-test for B. Shadow indicate SEM; ns, not significant.

SA-CD47 display alters the intrahepatic infiltration of inflammatory cells and inflammatory mediators.

One of the characteristic features of IBMIR mediated early loss of islets is the recruitment of inflammatory myeloid cells into the site of engraftment[87,97]. To test whether SA-CD47 on the surface of islets protect them from IBMIR mediated destruction, livers of graft recipients were analyzed using flow cytometry to assess intrahepatic inflammatory infiltrates 3 hrs post-transplantation. Livers of recipients transplanted with SA-CD47-engineered islet grafts had lower numbers of inflammatory infiltrates, including $CD11b^+$ myeloid cells, inflammatory monocytes ($CD11b^+$ $Ly6C^{hi}$/ $CD11b^+$ $Ly6C^{int}$), neutrophils ($CD11b^{hi}$ $Gr1^{hi}$), and macrophages ($F4/80^+$) as compared with SA-engineered islet controls (Figure 33A and 34). However, when analyzed at 24 hrs post transplantation, there were similar levels of intra hepatic inflammatory infiltrates in both SA-CD47- and SA-engineered islet graft recipients. This observation is in line with previous studies reporting appearance of inflammatory infiltrates in islet grafts as early as 15 min with massive infiltration by 2 hrs post transplantation[84,87,89].

We further analyzed the liver tissue samples by quantitative RT-PCR to assess the expression of inflammatory mediators involved in peri-transplant islet mass loss. There was no significant difference in transcript levels for various inflammatory mediators between SA-CD47- and SA-engineered islet graft recipients when assessed at 3 hr post transplantation. In marked contrast, when analyzed 24 hrs post transplantation, liver samples from SA-engineered islet recipients had significantly higher expression of transcripts for high mobility group box-1 (HMGB-1), tissue factor (TF), IL-1β, which are responsible for early islet loss as compared with the SA-CD47-engineered islet group

(Figure 33B). Although there was reduction in transcripts levels for MCP-1 and NK-κB in the SA-CD47-engineerd islet group as compared with the SA-engineered group, differences did not reach statistical significance. Taken together, these results show that the presence of SA-CD47 protein on islet grafts mitigates IBMIR by reducing the infiltration of inflammatory cells and their expression of inflammatory factors, resulting in enhanced engraftment and sustained function.

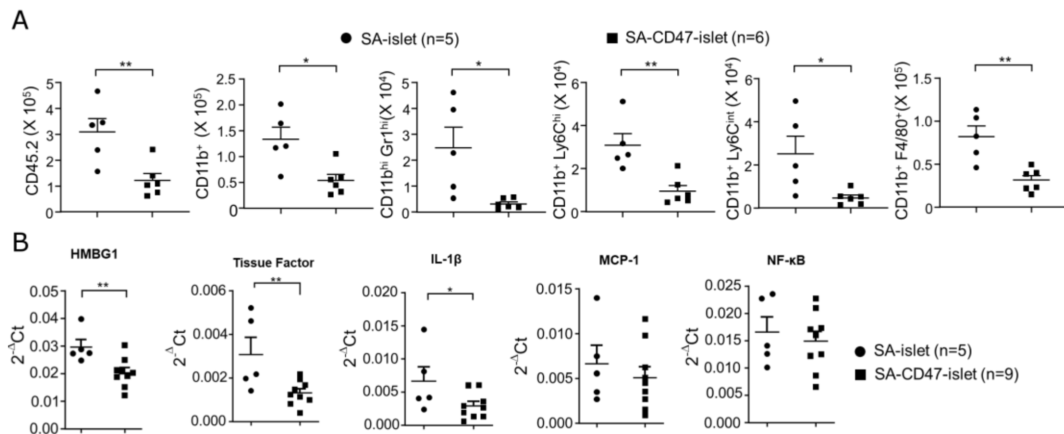

Figure 33. SA-CD47-islet recipients have significantly reduced infiltration of inflammatory cells and mediators in liver following intraportal transplantation. (A) Intrahepatic immune cells analysis 3 hr post-transplantation show substantially less infiltration of immune cells in SA-CD47-engineered islet graft recipients as compared with control SA-engineered islet recipients. Data points represent absolute cell number per gram of liver pooled from two independent experiment with n = 2-3 per group. (B) RTqPCR analysis of liver tissue samples 24 hr post-transplantation shows heightened expression of inflammatory mediators in SA-engineered islets as compared with SA-CD47-engineered islets. Data points represent relative expression in comparison to GAPDH pooled from two independent experiments, each with n=2-4 per group. For comparison of means unpaired one-tailed t-test was used in (A) and (B). *p < 0.05, **p < 0.01. Data shown as mean ± SEM.

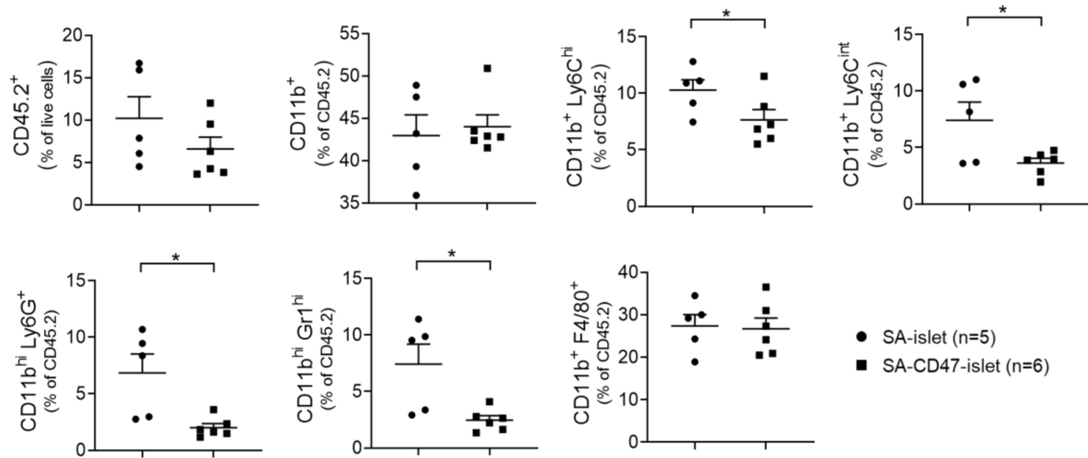

Figure 34. SA-CD47-engineered islet graft recipients have substantially less intra-hepatic infiltration of inflammatory cells. Data point indicates frequency of respective cells (as frequency of total CD45.2$^+$ cells) 3 hrs post-transplantation. Data pooled from two independent experiments, with n=2-3 in each group, represented as mean ± SEM. *p <0.05, **p < 0.01. For comparison of means unpaired one-tailed t-test was performed.

Discussion

IBMIR mediated primarily by myeloid cells is responsible for significant islet mass loss immediate post infusion into the liver[174-176]. Despite being transplanted from multiple donors, the functional islet mass in insulin-independent patients is equivalent of 30% of a non-diabetic healthy individual[90]. CD47/SIRPα axis is a critical innate immune checkpoint that inhibits activation and phagocytic function of myeloid cells as a critical mechanism of self/non-self-discrimination[186]. Tumor cells express CD47 on their surface as an effective means of immune evasion and the blockade of this molecule using antibodies was shown to galvanize innate immune responses against tumor with remarkable efficacy for the clearance of cancer in various preclinical models[186-189]. In this study, we assessed whether tumor immune evasion mechanisms can be simulated using CD47 as a means of mitigating IBMIR. We herein report for the first time that the transient display of a novel form of this molecule, SA-CD47, on the surface of islets results in enhanced engraftment and long-term function in an intraportal minimal mass syngeneic mouse model. Long-term engraftment and function were associated with peri-transplant reduced intragraft inflammatory innate immune cells and transcripts for proinflammatory mediators.

As a practical and alternative approach to ectopic expression of CD47 in islets, we generated a novel construct, SA-CD47, that contained the extracellular functional domain of mouse CD47 chimeric with core-streptavidin using a previously published scheme for FasL as a regulator of adaptive immunity[129]. Consistent with the structural features of streptavidin[206], SA-CD47 protein exists as oligomers and was displayed on the surface of biotinylated cells and islets in a rapid and efficient manner taking advantage of the high affinity interaction (K_d ~10^{-14} M) between biotin and streptavidin[206]. Importantly, the

engineering of islets with SA-CD47 did not negatively impact their viability, metabolic activity, or insulin secretion. These findings are consistent with our published studies transiently displaying SA-FasL on the surface of islets for the regulation of alloreactive immune responses[204,207]. In an *in vitro* xenogeneic system, SA-CD47-engineered rat cells overcame phagocytosis by mouse macrophages. This observation is consistent with studies demonstrating that porcine cells genetically modified to express human CD47 circumvent phagocytosis by human macrophages[195]. Engraftment of human hematopoietic stem cells is sensitive to SIRPα alleles expressed in mice as NOD expressing the high affinity allele for human CD47 show better engraftment as compared with mice expressing alleles with low affinity[194]. Furthermore, mouse hematopoietic stem cells were shown to transiently upregulate CD47 on their surface in response to mobilizing cytokines and inflammatory agents and the level of expression was directly correlated to their ability to evade phagocytosis[208].

In vitro loop assay that simulates *in vivo* IBMIR, that involves direct contact of peripheral blood with islets, showed well-preserved morphology with uniform distribution of nuclei and minimal fracture of SA-CD47-engineered islets as compared with SA-engineered controls. There were significantly ($p=0.0244$) fewer $CD11b^+$ cells in the islet-thrombus as compared with SA-engineered islets. This result suggested that SA-CD47 can potentially abrogate IBMIR effects. Following this observation, in intraportal islet transplantation, islets engineered with SA-CD47 protein showed significantly enhanced engraftment and long-term function (87.5%) as compared with SA control protein-engineered islets (14.2%) following intraportal transplantation. These observation provides strong evidence for the ability of SA-CD47 to mitigate IBMIR and is consistent

with a study reporting improved engraftment of human hepatocytes transduced with the mouse CD47 in immunodeficient mice following intraportal transplantation[209]. Also, transplantation of hepatocytes from CD47 knockout mice into syngeneic wild type recipients resulted in the activation of macrophages and poor graft survival[120]. Previous studies have shown that IBMIR is initiated immediately after islet infusion[80,84,87]. Rapid platelets deposition is observed within 30 mins on the islet surface along with infiltration of neutrophils/macrophages ($CD11b^+$) that appear as early as 15 min post-transplantation and peak at 2 hr[87]. When analyzed at 3 hrs post-transplantation, SA-CD47-engineered islet graft recipients had significantly less intragraft inflammatory cells, particularly inflammatory monocytes ($CD11b^+$ $Ly6C^{hi}/Ly6C^{int}$), neutrophils ($CD11b^+$ $Gr1^{hi}$), and macrophages ($CD11b^+$ $F4/80^+$) as compared with SA-engineered controls. SA-CD47-engineered islet grafts also had significantly reduced intragraft expression of proinflammatory HMGB1, TF, and IL-1β, all have been implicated in early islet loss[79,83,210,211]. These observations indicate that the presence of SA-CD47 protein on the surface of islets protects them from IBMIR by preventing transmigration of inflammatory cells and reducing inflammatory mediators in the graft site.

The current study offers a novel, effective, and practical strategy to ectopic expression of CD47 for the enhancement of autologous islet engraftment and long-term function following infusion into the liver. However, the application of this facile approach is not limited to islets or autologous transplantation as it can be applied to any cellular auto or allotransplantation. Although in the present study, we focused on the efficacy of SA-CD47 in modulating innate immune responses, CD47 has also been implicated in regulating adaptive immune responses. CD47 interaction with SIRPα on DCs activates

STAT3, a critical regulator of IL-6, IL-10 and IDO expression, resulting in polarization of T cells toward a regulatory phenotype[212,213]. Interaction of CD47 with SIRPα expressed on DCs inhibits their activation and impair Th1 response[214]. CD47 interaction with SIRPα on DC was shown to be critical to cardiac allograft tolerance achieved by CD154 blockade and donor-specific transfusion[215]. Lack of CD47 on donor cells resulted in rapid, alloantigen-independent activation of DCs, uncontrollable with CD154 blockade. Furthermore, intrasplenic infusion of CD47-deficinet allogeneic hepatocytes resulted in accelerated rejection of donor-matched skin grafts, whereas CD47-competant hepatocytes enhanced skin allograft survival that was associated with reduced alloreactive T cell responses, enhanced production of regulatory cytokines, IL-4 and IL-10, as well as significant expansion of myeloid-derived suppressor cells (MDSC)[216]. Indeed, CD47 was shown to play a critical role in the expansion and regulatory function of MDSC in a costimulatory blockade-induced tolerance to kidney allografts. The blockade of CD47/SIRPα pathway in this model resulted in rejection of long-term tolerant kidney grafts that was associated with overexpression of MCP-1 and inflammatory macrophage signature[217]. However, it remains to be investigated if the transient display of SA-CD47 on allogeneic islets or cellular grafts is sufficient as a single agent or in combination with a modulator of adaptive immunity, such as SA-FasL[204,207,218,219], is effective in inducing tolerance.

CHAPTER 4

SUMMARY, IMPLICATION AND FUTURE DIRECTION

In this dissertation, we show transient display of functional chimeric proteins as a novel immunomodulatory approach in two distinct experimental models. In chapter two, we use previously reported novel form of FasL, SA-FasL, to engineer donor graft in hematopoietic stem cell transplantation. In haploidentical and xenogeneic GVHD models, we provide evidence that engineering donor graft with SA-FasL can efficiently attenuate the GVHD causing ability of the graft, potentially by eliminating alloreactive cells. This strategy involves relatively very short procedure and is clinically translatable. Unlike previously reported strategies that involves ex vivo culture of donor grafts with recipient antigens for days, this method involves very minimal modulation of donor grafts. This strategy is highly impactable for procedures involving HSCT for leukemic patients or to establish donor chimerism for solid organ allo-graft transplantation. However, resistance of some T effector cells from AICD cannot be ignored. Also, SA-FasL-spleen recipients contract GVHD at some degree and some animals recovered as such the total protection is not 100%. This may be beneficial with respect to graft versus leukemia or graft versus tumor effect, where GVHD at some degree is required to maintain anti-leukemia effect. In addition, this may be important for other T cell functions like efficient engraftment and immunity against infections.

The proposed strategy can be modified in multiple ways to increase the impact. First, we show that engineering whole grafts leads to significant depletion of Treg cells without a negative impact on the efficacy of the protocol in our model. However, this may not be the case in the clinic. To avoid this potential pitfall, our approach could be used in combination with other clinically applied strategies, which is attractive and easily achievable given the efficient and practical nature of engineering. For example, we have shown that rapamycin as an immunoregulatory drug with beneficial impact on Treg cells, works in synergy with SA-FasL in islet and heart transplantation models[130,220]. Rapamycin is used widely in the clinical transplantation with great safety profile and can be used in combination with SA-FasL engineered cells to mitigate GVHD. Alternatively, SA-FasL can be used in combination with IL-33, another immunoregulator with beneficial effect on Treg cells, or chemotherapy-based conditioning, such as cyclophosphamide that also positively impacts Treg cells and extensively used for HSCT in the clinic.

On the second part of the dissertation, we report novel implication of CD47-SIRPα axis in attenuation of IBMIR. We show that islets engineered with SA-CD47 can modulate innate immune responses, resulting in the prevention of early islet loss, maintaining functional islet mass, and promoting efficient engraftment. This strategy has significant implication in islet transplant settings either in allogeneic or autologous transplantation. This is the first study of its kind where we target innate immune cells, myeloid cells, to modulate immune response to prevent early loss of islets due to IBMIR. To increase the impact of the observation, allogeneic study is very important to understand if similar results will be observed. In addition, this strategy needs to be tested with other clinically used agents, such as heparin to assess its beneficial impact. Extensive mechanistic studies are

warranted in autologous as well as allogeneic settings to better understand how SA-CD47 functions that will allow further refinement of this approach and its combinatorial use with agents that may work in synergy for a better outcome. Most importantly, this approach needs to be tested for settings involved cell transplantation, such as HSC or hepatocytes, as a single agent and also in combination with other modulators. In this context, the ProtEx^{TM} technology is a rather effective platform to display both SA-FasL and SA-CD47 and the implication of these both molecules on HSCT and allogeneic islet transplantation will be important to explore.

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CURRICULUM VITAE

Pradeep Shrestha

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EDUCATION

Doctor of Philosophy in Microbiology and Immunology **April, 2020**
University of Louisville; Louisville, KY

Master of Science in Microbiology and Immunology **Aug, 2017**
University of Louisville; Louisville, KY

Master of Science in Biotechnology **Jan, 2014**
Tribhuvan University; Nepal

Bachelor of Science in Microbiology **Dec, 2009**
Tribhuvan University; Nepal

RESEARCH EXPERIENCE

Graduate researcher at University of Louisville **2016-present**
Department of Microbiology and Immunology
Mentors: Dr Haval Shirwan and Dr. Esma S. Yolcu

Dissertation project
Title: *Targeting FasL-engineered donor cells as strategy to prevent acute Graft-versus-host disease (GVHD).*
We employed a novel strategy to eliminate alloreactive cells to prevent acute GVHD. Using haploidentical and xenogeneic settings we show that, engineering the donor cells with novel form of FasL, can significantly eliminate pathogenic alloreactive T cells, thus successfully preventing lethal GVHD.

Side projects

Title: *Localized immunotolerance in islet transplantation setting by employing FasL engineered microgels.*

The goal of the project was to establish localized tolerance for islet grafts in allogeneic transplantation settings under transient cover of immunosuppression. We showed that targeting alloreactive cells by FasL-engineered microgels and short-term treatment with rapamycin is efficient enough to establish localized tolerance in murine model. The study resulted in one co-first author paper published in Nature Materials (2018). Pre-clinical study in non-human primate model is ongoing with collaboration to Harvard Medical school and Georgia Tech university.

Title: *Novel form of CD137 as cancer immunoprevention agent in multiple murine cancer models.*

The goal of the project was to test if novel form of CD137 (SA-4-1BBL) can prevent tumor development in murine model. We showed that animals treated with SA-4-1BBL, when challenged with cancer cells, are protected from cancer development. The study was published in Cancer Research journal (2019).

Title: *Modification of extracellular vesicles as a strategy for nanotherapeutics.*

The ongoing project is in collaboration with Carnegie Mellon group where extracellular vesicles can be effectively engineered with desired proteins and can be established as nanotherapeutics. As proof of concept, FasL-displaying extracellular vesicles can significantly eliminated alloreactive cells which can be employed in autoimmune disease models. This study has, so far, been published in ACS Nano (2019).

Graduate research at Tribhuvan University **2012-2013**

Central department of Biotechnology

Mentor: Dr. Deepak Raj Pant

- *In vitro axillary proliferation and propagation of Swertia Chirayita, a medicinal plant.* This research work was supported by Thesis support grant, UGC, Nepal.

- *Phytochemical evaluation of different medicinal plants*. This research work was granted as faculty research grant to Dr. Deepak Raj Pant as primary investigator by UGC, Nepal. I served as an associate researcher for the project.

AWARDS

- Travel award, AAI (2018).
- Graduate Fellowship (2015-2017), Integrated Programs in Biomedical Sciences, University of Louisville, Louisville, USA.
- Korean Government Scholarship Program (2014)
- Dissertation Grant, University Grant Commissions, Bhaktapur, Nepal (2012-2013).

MEMBERSHIPS

- American Association for the Advancement of Sciences (AAAS)
- American Association of Immunologists

PROFESSIONAL APPOINTMENTS/ RESPONSIBILITIES

- Trained and mentored several people including high school students, rotating graduate students and postdoctoral fellow in the lab.
- Lecturer at Kantipur Valley College, Kathmandu, Nepal (2014-2015)

PROFESSIONAL IDENTIFIER

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PUBLICATIONS

- **<u>Pradeep Shrestha</u>**, Lalit Batra, Tariq Malik, Min Tan, Orlando Grimany-Nuno, Esma. S. Yolcu, Haval Shirwan. Immune checkpoint CD47 molecule engineered islets mitigate instant blood-mediated inflammatory reaction and show improved engraftment following intraportal transplantation. (Resubmitted with minor revision, *American Journal of Transplantation*)

- Lalit Batra*, **<u>Pradeep Shrestha</u>***, Hong Zhao, Kyle B. Woodward, Min Tan, Orlando Grimany-Nuno, Alper Togay, Maria M. Coronel, Andrés J. García, Haval Shirwan, Esma S. Yolcu. Localized immunomodulation with PD-L1 results in sustained survival and function of allogeneic islets without chronic immunosuppression. (In Press *Journal of Immunology*)

- Woodward KB*, Zhao H*, **<u>Shrestha P</u>**, Batra L, Tan M, Grimany-Nuno O, Bandura-Morgan L, Askenasy N, Shirwan H, Yolcu ES. Pancreatic islets engineered with a FasL protein induce systemic tolerance at the induction phase that evolves into long-term graft-localized immune privilege. *American Journal of Transplantation*. 2019 Dec 18.

- Yerneni SS*, Lathwal S*, **<u>Shrestha P</u>**., Shirwan H, Matyjaszewski K, Weiss L, Yolcu ES, Campbell PG, Das SR. Rapid On-Demand Extracellular Vesicle Augmentation with Versatile Oligonucleotide Tethers. *ACS nano*. 2019 Aug 22;13(9):10555-65.

- Barsoumian HB*, Batra L*, **Shrestha P.**, Bowen WS, Zhao H, Egilmez NK, Gomez-Gutierrez JG, Yolcu ES, Shirwan H. A Novel Form of 4-1BBL Prevents Cancer Development via Nonspecific Activation of CD4+ T and Natural Killer Cells. *Cancer research*. 2019 Feb 15;79(4):783-94.

- Headen DM*, Woodward KB*, Coronel MM*, **Shrestha P.***, Weaver JD, Zhao H, Tan M, Hunckler MD, Bowen WS, Johnson CT, Shea L. Local immunomodulation with Fas ligand-engineered biomaterials achieves allogeneic islet graft acceptance. *Nature materials*. 2018 Aug;17(8):732.

- Williams, C. L., Yolcu,E. S., Woodward, K. B., Zhao, H., Bowen, W. S., **Shrestha, P.**, Batra, L., Askenasy, N. and Shirwan, H. FasL mediated apoptosis as an effective means of inducing tolerance to allogenic pancreatic islets. *Turkish J Biochem*. 41:S4 2016.

  * First authorship shared

PUBLICATIONS UNDER PROGRESS

- **Pradeep Shrestha**, Ali Turan, Lalit Batra, Christine Akimana, Esma S. Yolcu, Haval Shirwan. Expression of FasL on donor grafts as a novel approach to prevention of acute graft-versus-host disease. (In preparation for submission to Blood)

- Wang, F.*, **Shrestha, P.***, Woodward, K.B.*, Zhao, H., Williams, C., Bowen, W.S., Shea, L., Garcia, A.J., Hering , B., Yolcu, E.S, and Shirwan, H. Agonists of Fas and IL2 receptors work in synergy to induce robust tolerance to allografts. (manuscript in preparation)

* First authorship shared

ABSTRACTS/PRESENTATIONS

- Lalit Batra, Hampartsoum B. Barsoumian, **Pradeep Shrestha**, Jenci L. Hawthorne, William S. Bowen, Hong Zhao, Nejat K. Egilmez, Jorge G. Gomez-Gutierrez, Haval Shirwan, and Esma S. Yolcu. A novel agonist of CD137 immune checkpoint stimulator serves as a cancer immunoprevention agent with efficacy against various tumor types. American Association of Immunologists - *Immunology 2019*, San Diego, California, USA.

- Haval Shirwan, Hampartsoum B. Barsoumian, Lalit Batra, **Pradeep Shrestha**, William S. Bowen, Hong Zhao, Nejat K. Egilmez,Jorge G. Gomez-Gutierrez, Esma S. Yolcu. A novel form of 4-1BB agonist shows robust immune protection against various tumor types through $CD4^+$ memory-like T and NK cell axis. American Association for Cancer Research (*AACR-2019*) annual meeting, Atlanta, Georgia, USA.

- Headen, D., Woodward, K., Weaver, J., Coronel, M.M., **Shrestha, P**., Bowen, W., Johnson, C., Shea, L., Yolcu, E., Garcia, A. and Shirwan, H., 2017, December. Allogeneic Graft Tolerance without Immunosuppression via Engineered Immunomodulatory Signal Presentation. In TISSUE ENGINEERING PART A (Vol. 23, pp. S41-S42). 140 HUGUENOT STREET, 3RD FL, NEW ROCHELLE, NY 10801 USA: MARY ANN LIEBERT, INC.

- Devon Headen, Jessica Weaver, Esma Yolcu, Kyle Woodward, Hong Zhao, **Pradeep Shrestha**, Haval Shirwan, Andrés J. García. Microgels presenting SA-FasL achieve allogeneic islet graft acceptance without chronic immunosuppression. 2017 Society for Biomaterials Annual meetings. Minneapolis, Minnesota, April 5-8, 2017

- Batra L, **Shrestha P**., Yolcu ES, Zhao H, Bowen WS, Woodward KB, Coronel MM, Tan M, Garcia AJ, Shirwan H. The transient display of a chimeric PD-L1 protein on pancreatic islets promotes indefinite survival in allogeneic recipients. 27th International Congress of the Transplantation Society, Madrid, Spain, June 30th to July 5th, 2018.

- **Shrestha P**, Bowen WS, Batra L, Tan M, Yolcu ES, Shirwan H. CD47 Overcomes Early Loss of Pancreatic Islet Grafts Transplanted Intraportally. 27th International Congress of the Transplantation Society, Madrid, Spain, June 30th to July 5th, 2018.

- Woodward KB, Headen DM, **Shrestha P**, Tan M, Bowen WS, Coronel MM, Hunckler MD, Weaver JD, Yolcu ES, Garcia AJ, Shirwan H. Immunomodulation with FA-FasL-engineered mucrogels achieves long-term survival of allogeneic islet grafts. 27th International Congress of the Transplantation Society, Madrid, Spain, June 30th to July 5th, 2018.

- Batra, L., **P. Shrestha**, E.S. Yolcu, H. Zhao, W.S. Bowen, K.B. Woodward, M. M. Coronel, M. Tan, A.J. García, and H. Shirwan. PD-L1-engineered pancreatic islet grafts overcome rejection in allogeneic recipients. *J. Immunol.* 194:176.6 (Abstr.)

- **Shrestha, P**., W.S. Bowen, L. Batra, M. Tan, E.S. Yolcu, and H. Shirwan. Display of CD47 protein on pancreatic islet grafts improves engraftment following intraportal transplantation. *J. Immunol.* 194:55.38 (Abstr.)

- Haval Shirwan, Kyle B. Woodward, Devon M. Headen, Hong Zhao, **Pradeep Shrestha**, Min Tan, William S. Bowen, María M. Coronel, Michael D. Hunckler, Jessica D. Weaver, Esma Yolcu, Andrés J. García. SA-FasL-engineered PEG microgels as a novel means of modulating immune response to allogeneic islet grafts. *J. Immunol.* 194:55.36 (Abstr.)

RESEARCH SKILLS and TECHNIQUES

- **In vivo animal procedures (murine model).**
    - Islet transplantation
    - Bone marrow transplantation and graft versus host disease models
    - Microsurgical procedures
    - Injections (subcutaneous, intravenous, intraperitoneal)
    - Tissue harvest and analysis
- **Flow cytometry** (Proficiency in 14 parameters panel optimization and analysis)
- **Humanized murine model**
    - Human lymphocyte isolation and immunophenotyping
    - Islet transplantation
    - Acute GVHD model
- **Molecular Techniques**
    - PCR
    - RT-qPCR
    - ELISA
- **Histological analysis**
    - Tissue processing and sectioning
    - Immunohistochemistry
    - Hematoxylin and Eosin staining
- **Processing and analysis of tissues and organs**
    - Tissue harvest and processing (Spleen, lymph nodes, liver, intestine)
    - In vitro Cytokine, Mixed lymphocyte proliferation assays
- **Softwares**
    - Microsoft office
    - Graph pad prism
    - Flowjo
    - GIMP & Adobe photoshop