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Immune Modulatory Strategies to Prevent Costimulation Blockade Resistant Rejection

By

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Doctor of Philosophy
Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

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Abstract

Immune Modulatory Strategies to Prevent Costimulation Blockade Resistant Rejection

By Jakob G. Habib

Costimulation blockade (CoB) is a promising new transplant immunosuppression strategy offering improved long-term patient and allograft survival without the nephrotoxicity of calcineurin inhibitors. However, increased risks of acute rejection have impeded the widespread adoption of CoB. This dissertation sought to determine how modulating the current CoB regimen in two separate ways could improve transplant survival. By interrupting the type I interferon receptor (IFNAR) signaling during CoB, fully MHC mismatched BALB/c skin grafted onto C57BL/6J mice survived significantly longer than CoB alone. We determined that IFNAR signaling on recipient hematopoietic cells was the primary contributor to rejection during CoB, and that this treatment regimen showed efficacy in a preliminary pilot study in nonhuman primates. In addition, we explored how modifying the costimulation blockade reagent, by using an anti-CD28 domain antibody (dAb), affects skin graft survival and T cell differentiation in the clinically relevant setting of T cell depletion (TCD) and homeostatic reconstitution. By selectively blocking CD28 signaling, CTLA-4 coinhibition can occur, further inhibiting effector T cell responses and enhancing regulatory T cell function. We found that TCD+anti-CD28dAb improved BALB/c to C57BL/6 skin graft survival compared to anti-CD28dAb alone. Furthermore, we observed reduced CD69 expression on $CD4^+$ and $CD8^+$ T cells in mice that received TCD+anti-CD28dAb compared to TCD alone. In the kidney, the frequencies of tissue resident $CD69^+CD103^+CD4^+$ T cells were reduced in TCD+CD28dAb compared to TCD alone. We also observed an increased frequency of $CD8^+Foxp3^+$ T cells in the blood and kidney of mice given TCD+anti-CD28dAb compared to TCD alone. These findings demonstrate that blocking IFNAR signaling or changing the CoB regimen to selectively target CD28 signaling could lead to improvements critical for the widespread implementation of CoB in the clinic.

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Chapter 1: Introduction

Solid organ transplantation is the primary treatment for end-stage organ failure, with over 42,000 organ transplants performed in the United States in 2022 (1). More than half of these transplanted organs are kidneys, and patients that receive kidney transplants enjoy a better quality of life compared to chronic dialysis treatment (2). Those fortunate enough to receive a kidney transplant must take powerful immunosuppressants in order to prevent rejection. Over the past 45 years, traditional immunosuppressants such as calcineurin inhibitors (CNIs) have been instrumental in reducing the incidence of transplant rejection (3, 4). However, the off-target effects of these therapies result in increased risks of heart attack, stroke, diabetes, and allograft vasculopathy, which contributes to inferior transplant outcomes (5, 6, 7). The demand for more targeted immunosuppressants has led to the development of belatacept, a novel costimulation blockade (CoB) therapy.

Belatacept is the first FDA approved alternative to CNIs and functions by specifically interrupting T cell activation by blocking T cell costimulation. Belatacept is a fusion protein of cytotoxic lymphocyte protein 4 (CTLA-4) and the crystallizable fragment (Fc) portion of immunoglobulin (Ig) that binds to the costimulatory molecules CD80 and CD86 on antigen presenting cells (APCs) with high affinity, blocking CD28 ligation on T cells. A phase III clinical trial evaluating the outcome of renal transplants treated with belatacept compared to cyclosporine revealed that patients treated with CoB live longer and maintain superior kidney function (8, 9). Unfortunately, a significant subset of belatacept-treated patients experience increased rates of acute rejection (10). The reason for this costimulation blockade-resistant rejection (CoB-RR) is not well understood, though it is thought to be mediated by alloreactive memory CD8 T cell responses, as these cells are less reliant on CD28 costimulatory signals (11). The generation and

activation of these alloreactive memory CD8 T cells can be influenced by inflammatory cytokines such as type I interferons (IFNs) and is exacerbated by the fact that CTLA-4, a key inhibitory signal on T cells, is also prevented from binding to its receptors CD80 and CD86 on APCs. Therefore, in the setting of belatacept-mediated costimulation blockade, T cells are susceptible to activation via cytokines and are lacking important negative regulation provided by CTLA-4 signaling.

Type I IFNs are a class of inflammatory cytokines that activate the innate and adaptive immune system by signaling through the interferon-α/β receptor (IFNAR) (12). Plasmacytoid dendritic cells (pDCs) are a major producer of type I IFN, predominantly IFN-α, and are known to play integral roles in response to viral infections and in autoimmunity (13). While pDCs contribute to immune activation, emerging findings have revealed their tolerogenic capacity (14). However, the contribution of pDCs and type I IFN during CoB-RR remains unknown.

Costimulation blockade resistant rejection

Allograft specific T cells are a major contributor to transplant rejection. T cell activation can be broken down into three distinct and sequential signals (15). Signal one is provided by ligation of the T cell receptor (TCR) to its corresponding peptide bound to major histocompatibility complex (MHC) on APCs. This initiates a cascade of signals that leads to the translocation of nuclear factor of activated T cells (NFAT) into the nucleus, which results in the transcription of cytokine genes. CNIs prevent T cell activation by blocking the ability of calcineurin to dephosphorylate NFAT, sequestering it in the cytoplasm (16). Several other cell types utilize this conserved signaling pathway, which explains the off-target toxicity of CNIs (17, 18, 19, 20). Signal two, or costimulation, is best demonstrated by the binding of CD28 on T cells to CD80 and CD86 on APCs (21). Signaling through CD28 leads to the phosphorylation of proteins that propagate

signal one and facilitates the production of cytokines such as interleukin-2 (IL-2) that drive T cell proliferation (21, 22). This second signal serves as a check on the first signal by ensuring that the T cell only becomes activated by APCs that have received danger signals and now express the costimulatory molecules CD80 and CD86 (23). When a T cell receives strong TCR signaling in the absence of signal two it becomes anergic, contributing to peripheral tolerance (24). Another important costimulatory signal is conveyed through the binding of CD40 on APCs to CD154 (CD40L) on T cells. Both the T cell and APC are affected by this interaction, and this signaling pair is particularly important for the initiation of T cell-dependent B cell responses (25). Signal three is provided by a variety of cytokines, most notably IL-2, IL-12, and type I IFN (26). This third signal promotes the survival, proliferation, and differentiation of activated T cells (27, 28, 29). Together, these three signals allow for a productive T cell response.

While multiple drugs have been in development that target costimulatory signals, belatacept is the first (and to this date only) approved by the FDA for use as an alternative to CNIs for kidney transplant. It's precursor, abatacept, was approved in 2005 for the treatment of rheumatoid arthritis but was insufficient to prevent kidney allograft rejection (30). Using a codon-based mutagenesis and surface plasmon resonance screening approach, the CTLA-4 portion of belatacept was modified to include two amino acid changes that gave it a higher binding affinity for CD80 and CD86 than abatacept. Not only does belatacept inhibit T cell costimulation, but it further dampens the immune response by preventing T cell-dependent antibody production. The Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial (BENEFIT) was a large phase III study comparing the effectiveness of kidney transplants treated with a less intense (LI) or more intense (MI) belatacept regime to those treated with cyclosporine. The study revealed that after 7 years there was a 43% reduction in the risk of death or graft loss in

both belatacept groups compared to the cyclosporine group and the mean estimated glomerular filtration rate (eGFR) increased in the belatacept groups but declined in the cyclosporine group (8). While belatacept therapy was associated with improved kidney function, it unfortunately led to a higher rate of acute T cell mediated rejection compared to treatment with cyclosporine. These episodes of acute rejection occurred within 6 months of transplant and biopsies of the rejected organs confirmed that rejection was T cell mediated (31). While the reason for this costimulation independent rejection is not fully understood, it is likely multifaceted and patient-specific. Several studies have investigated the mechanism behind CoB-RR (Table 1). The bulk of these studies identified memory CD8 T cells as the driving force behind CoB-RR.

Table 1. Compilation of experimental studies that investigate the mechanism underlying costimulation blockade resistant rejection. (NHP: nonhuman primate. OVA: ovalbumin)

Transplant Model	**CoB Regimen**	**Cause of CoB-RR**	**Reference**
Murine OVA skin graft	CTLA-4-Ig + anti-CD154	High precursor frequency of antigen specific T cells	Ford et al. 2007. (32)
Murine OVA skin graft	CTLA-4-Ig + anti-CD154	Donor specific memory T cells	Kitchens et al. 2017 (33)
Murine Islet allograft	CTLA-4-Ig	IL-15 signaling on $CD8^+$ T cells	Ferrari-Lacraz et al. 2001 (34)
Murine skin allograft	CTLA-4-Ig + anti-CD154	IL-2 signaling	Jones et al. 2002 (35)
NHP Kidney allograft	CTLA-4-Ig	Virally-induced alloreactive CD8 CM T cells	Adams et al. 2003 (36)
NHP Kidney allograft	CTLA-4-Ig	$CD28^-$ TEMRA	Mathews et al. 2017 (37)
NHP Kidney allograft	CTLA-4-Ig	Secondary lymphoid organs	Mulvihill et al. 2019 (38)
Human Kidney	CTLA-4-Ig	$CD57^+PD1^-$ $CD4^+$ T cells	Espinosa et al. 2016 (39)

Type I interferon signaling

Alick Isaacs and Jean Lindenmann discovered interferon in 1957 by observing its capacity to interfere with viral replication (40). It was later understood that there are three distinct types of interferon, each with multiple subtypes. Type I IFN is the most diverse, with at least 17 subtypes identified in humans (41, 42). The predominant forms of type I IFN produced during an infection are IFN-α and IFN-β, which signal through the interferon-alpha/beta receptor (IFNAR) (43). IFNAR is comprised of a heterodimer of the transmembranal IFNAR1 and IFNAR2 chains, and is expressed on the surface of virtually all cells (44). Likewise, most cell types are capable of producing type I IFN to some degree, typically in response to innate immune signals that detect viral infections (45). The pattern recognition receptors (PRRs) that induce type I IFN are typically located within endosomes or the cytoplasm and canonically signal via a cascade of adaptor proteins that leads to the translocation of interferon regulatory factor 3 (IRF3) or IRF7 into the cell's nucleus, resulting in the transcription of type I IFN genes (46).

Binding of type I IFN to IFNAR initiates signaling through the JAK/STAT pathway, leading to the formation of the transcription factor IFN-stimulated gene factor 3 (ISGF3), which results in the transcription of interferon stimulated genes (ISGs) (47). ISG products include a multitude of proteins that induce an anti-viral state within cells and can also both positively and negatively regulate type I IFN signaling. ISGs induce the production of additional type I IFN along with proteins involved in the IFN signaling pathway, resulting in a positive feedback loop (48, 49). Conversely, in order to avoid aberrant type I IFN production over the course of the immune response, IFNAR signaling has inherent negative feedback mechanisms. Reviewed by Schneider et al., there are three primary methods by which type I IFN signaling is regulated (50). Binding of type I IFN to IFNAR induces the endocytosis and degradation of the receptor, which allows for

immediate desensitization to type I IFN. Another regulatory mechanism occurs early following IFNAR signaling when ISGs produce inhibitory proteins such as suppressor of cytokine signaling, which inhibits JAK/STAT signaling. Lastly, a more sustained mechanism of regulation involves ISG-induced ubiquitination of IFNAR2, which causes a conformational change in the receptor that prevents the lower affinity IFN-α from binding but still allows IFN-β to signal through IFNAR (50). The autocrine and paracrine features of type I IFN, along with the diverse outcomes of IFNAR signaling, result in a variety of potential effects on multiple cell types. Type I interferon is beneficial during acute viral infections but can become aberrantly activated during bacterial infections, cancer, autoimmunity, and potentially transplantation (51).

Type I interferon production during transplantation

While type I interferon can be produced by most cell types, plasmacytoid dendritic cells (pDCs) produce over 100 times more IFN-α during a viral infection than any other cell type. In fact, they were first identified as "interferon producing cells" (52, 53). The name "plasmacytoid" stems from their plasma cell-like morphology, rich in rough endoplasmic reticulum that facilitates the production of copious amounts of IFN-α (54). PDCs develop in the bone marrow from common DC progenitors and traffic to secondary lymphoid organs where they survey the environment for pathogens (55, 56). Though pDCs are not as capable as conventional dendritic cells (cDCs) at directly initiating T cell immune responses, they can still function as APCs. PDCs have been shown to initiate $CD4^+$ T cell responses and can cross-present antigen to $CD8^+$ T cells following TLR activation (57, 58, 59). This facilitates the capability of pDCs to initiate a Th1-polarized immune response against pathogens (60).

PDCs are particularly capable at recognizing nucleic acids due to their high expression of TLR7 and TLR9, which detects single stranded RNA and CpG DNA, respectively (61). Another important signal that pDCs utilize to sense viral DNA and produce type I interferon is the cGAS-STING pathway. The cytosolic DNA receptor cyclic GMP-AMP synthase (cGAS) activates the adaptor protein stimulator of interferon genes (STING) to induce type I IFN production. STING has been demonstrated to provide a positive feedback loop of type I IFN production in pDCs (62).

The inflammatory triggers that initiate type I IFN production during transplantation can originate from an infection or because of sterile inflammation. Immunocompromised transplant recipients are at a greater risk of nosocomial infections such as MRSA or community-acquired infections such as pneumonia. In addition, reactivation of a latent infection such as EBV is serious risk for transplant recipients (63). Sterile inflammation as a result of ischemia reperfusion injury (IRI) could also trigger type I IFN production. Damage from IRI occurs both when the blood supply to an organ is halted and when it is restored. The cell damage that results from IRI has been shown to activate multiple TLRs including TLR3 and TLR9, both of which lead to type I IFN production by pDCs and other cells (64, 65). Castellaneta et al. identified IFN-α produced by pDCs as the primary culprit of IRI damage during murine liver transplants and found that both IFN-α neutralization and pDC depletion decreased hepatocyte apoptosis (66). The underlying trigger of type I IFN production during transplant is multifaceted, as is the impact of type I IFN on other cell subsets.

Impact of type I interferon on cell subsets

$CD8^+$ T cells

CoB resistant memory $CD8^+$ T cells are a primary driving force of costimulation independent kidney allograft rejection, and their generation and reactivation are influenced by type I IFN (67). Studies investigating the role of type I IFN on $CD8^+$ T cells are primarily framed in the context of viral infections, which are a major cause of morbidity and mortality after kidney transplantation (68). It has been demonstrated that type I IFN can enhance the cytolytic and recall response of memory $CD8^+$ T cells during a viral challenge (69). In addition, type I IFN signaling prevents the apoptosis of activated $CD8^+$ T cells and provides crucial signaling to drive the expansion and formation of memory $CD8^+$ T cells (27, 70, 71). Finally, type I IFN can function in a similar manner to IL-12 and IL-15 as a signal 3 cytokine to drive $CD8^+$ T cell effector function and memory formation (72, 73, 74, 75).

Regulatory $CD4^+$ T cells

There is evidence that type I IFN has divergent effects on regulatory $CD4^+$ T cells (Tregs) (76). IFNAR signaling has been shown to negatively regulate Tregs during lymphocytic choriomeningitis virus (LCMV) infection and within tumor microenvironments (77). However, it is also evident that IFNAR signaling promotes Treg development and function under stress conditions (78). Also, in a murine model of arthritis, IFN-α activates Tregs through the activity of indoleamine 2,3-dioxygenase (IDO) during antigen sensitization, which prevents arthritis triggered by antigen rechallenge (79). Whether the blockade of IFNAR during CoB administration will promote or dampen Treg mediated tolerance has yet to be elucidated.

Dendritic cells

Both donor and recipient DCs are significant drivers of acute T cell mediated rejection. Activation of recipient T cells against the graft can occur following recognition of foreign MHC presented by donor APCs (direct antigen presentation) or by recipient APCs that present donor antigen (indirect antigen presentation) (80). Type I IFNs can influence DC maturation by impacting antigen uptake, processing, and presentation, along with promoting the upregulation of costimulatory molecules. Le Bon et al. demonstrated that during LCMV infection, type I IFN induces cross priming of exogenous antigen to $CD8^+$ T cells by DCs (81). Simmons et al. further established that type I IFN primes DCs to be able to continually process and present antigen on MHC II whereas priming with TLR agonist results in only transient antigen processing and presentation on MHC II (82). Additionally, it has been shown that type I IFN drives the production of IL-15 by DCs, providing additional signal 3 cytokine to T cells (83).

B cells

Jejo et al. demonstrated that pDC-derived type I IFN drives the differentiation of B cells into antibody producing plasma cells (84). Another group found that IFNAR signaling lowers the threshold for B cell induction, accelerating B cell activation and antibody production (85). It has also been shown that type I IFN directly influences T cell-dependent B cell antibody responses (86).

pDCs: inflammatory or tolerogenic

The role of pDCs in the immune system has largely been studied in the setting of viral infections, cancer, and autoimmunity. Interestingly, pDCs may impart a dual role in immune homeostasis, both initiating immune responses via IFN production but also dampening them by

stimulating Tregs (87, 88). In fact, this rare cell type might be more complex than initially thought. Björck et al. identified two pDC subsets in mice that are distinct in their expression of CD9 and Siglec-H. The group found that $CD9^{+}$Siglec-H^{low} pDCs secrete IFN-α and induce $CD8^{+}$ T cells whereas $CD9^{-}$Siglec-H^{high} pDCs secrete much less IFN-α and induce $CD4^{+}$ regulatory T cells. They further characterized $CD9^{+}$ pDCs as newly generated from the bone marrow, and as they mature the pDCs lose CD9 expression as well as their capacity to produce type I IFN (89). Similar pDC subset diversity has been characterized in humans. Zhang et al. identified $CD5^{+}CD81^{+}$ pDCs as poor producers of type I IFN, likely due to their diminished expression of IRF7, compared to conventional $CD5^{-}CD81^{-}$ pDCs. They further characterized $CD5^{+}CD81^{+}$ pDCs as strong stimulators of B cells, inducers of T cell proliferation, and mediators of Treg formation (90).

This new development in pDC characterization may influence therapies centered on pDC manipulation. The role of pDCs as a driving force for systemic lupus erythematous (SLE) is of great interest both because the type I IFN produced by pDCs exacerbates disease activity, and the release of nucleic acids from cell death as a result of the disease further drives pDC activation (91). Murine models of SLE confirmed the role of IFN-α produced by pDCs as a driving force for the disease. By depleting pDCs early during disease induction, Rowland et al. demonstrated that pDCs drive disease activity in a type I IFN dependent manner. The beneficial effects of pDC depletion persisted even after pDCs reconstituted (92).

Type I interferon signaling blockade

Therapeutically targeting the type I IFN pathway could aid in preventing alloreactive T cells from initiating CoB-RR. In the field of autoimmunity, blocking this pathway has proven beneficial for patients with SLE. Clinical trials using the anti-IFN-α drugs rontalizumab and

sifalimumab revealed the drugs were well tolerated but did not reduce SLE disease activity significantly enough to warrant further development (93, 94, 95). However, blocking IFNAR directly has proven more efficacious. Anifrolumab is a fully human IgGk1 monoclonal antibody that binds to IFNAR1, sterically inhibiting all subtypes of type I IFN from binding to IFNAR. Also, binding of anifrolumab to IFNAR rabidly induces internalization of the receptor, further amplifying its effect. It was engineered with mutations in the heavy chain to prevent Fc-mediated effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Anifrolumab prevents CpG-A induced type I IFN production from pDCs and inhibits plasma cell differentiation in a dose-dependent manner (96).

As of yet there have been two phase III clinical trials evaluating anifrolumab. The Treatment of Uncontrolled Lupus via the Interferon Pathway-1 (TULIP-1) phase III clinical trial assessed the efficacy and safety of anifrolumab versus control in SLE patients who were receiving standard of care therapy. While the primary end point of the TULIP-1 trial was not reached, secondary end points suggested clinical benefits of anifrolumab versus placebo (97). The TULIP-2 phase III clinical trial used one of these secondary end points as its primary end point and successfully showed a reduction in SLE disease activity after 1 year of monthly anifrolumab administration (97). The encouraging outcome of the TULIP-2 trial suggests the FDA will soon approve anifrolumab for the treatment of SLE, which could pave the way for its use to potentially prevent CoB-RR.

Impact of CTLA-4 deficiency during costimulation blockade

Conventional T cells

While belatacept prevents CD28 mediated T cell costimulation by binding to CD80 and CD86 on APCs, an unintentional consequence of this therapy is that these ligands are also prevented from binding to CTLA-4. CTLA-4 expressed on a variety of cell types (98), but on T cells it provides crucial coinhibitory signals in a cell intrinsic and extrinsic manner (99, 100, 101, 102). On activated T cells, CTLA-4 is recruited to the immunological synapse and binds to CD80 and CD86 on APCs, dampening the immune response to prevent aberrant overactivation (103, 104). Following ligation to CD80 and CD86, the intracellular domain of CTLA-4 interacts with various intracellular proteins including phosphatases that further diminish T cell activation (105, 106, 107, 108, 109). As these processes cannot take place during CTLA-4-Ig mediated CoB, T cells that are less reliant on CD28 signaling, such as memory $CD8^+$ T cells, are missing this important inhibitory signal (11, 37).

Regulatory T cells

CTLA-4 is highly expressed on Tregs and functions in a cell extrinsic manner by providing non-redundant immunosuppressive signals (110). Sakaguchi et al. demonstrated that mice with CTLA-4-deficient Tregs spontaneously develop systemic lymphoproliferation and fatal T cell-mediated autoimmune disease, and these Tregs failed to sufficiently downregulate CD80 and CD86 expression on APCs (111). During CoB with CTLA-4-Ig, endogenous CTLA-4 is unable to provide these key inhibitory signals which may contribute to CoB-RR. Therefore, we sought to investigate the impact of a selective CD28 blocking reagent that leaves CTLA-4 signaling intact, the details of which are covered in chapter three of this dissertation.

Beyond T cells

On B cells, CTLA-4 signaling is inhibitory as determined by experiments utilizing CTLA-$4^{-/-}$ mice that exhibit hypergammaglobulinemia and upregulated B cell CD86 expression (98, 112). On dendritic cells, CTLA-4 is also inhibitory and its ligation on this cell type can lead to a regulatory phenotype. In the setting of human hepatocellular carcinoma, Han et al. found that $CD14^{+}CTLA\text{-}4^{+}$ "regulatory dendritic cells" suppress T cell responses *in vitro* via IL-10 and IDO production in a CTLA-4 dependent manner (113). Another group similarly found that agonistic ligation of CTLA-4 on human monocyte-derived DCs resulted in a shift towards a regulatory phenotype, resulting in enhanced secretion of IL-10 and reduced secretion of proinflammatory cytokines (114). In mice, a group demonstrated that CTLA-4 signaling on $CD11c^{+}$ splenic DCs led to IDO-dependent T cell suppression (115).

Conclusion

Costimulation blockade with belatacept is a promising alternative immunosuppressive strategy for renal transplantation. Patients treated with belatacept live longer with fewer side effects and maintain superior kidney function compared to patients treated with CNIs. Unfortunately, the increased rate of acute rejection that is associated with CoB has hindered the field of transplantation's adoption of this novel therapy as standard of care. The underlying mechanism of CoB-RR is complex and likely involves inflammatory cytokines such as type I IFN that instigate $CD8^{+}$ T cell mediated immune responses that would otherwise not occur. Blockade of type I IFN dampens multiple arms of the immune response, potentially preventing T cell activation during costimulation blockade. In addition, improvement of the current costimulation blockade therapy by directly targeting CD28 while leaving CTLA-4 coinhibitory signaling intact could further improve transplant outcomes. The discovery of a therapy that prevents costimulation

independent rejection could transform the field of transplantation, affording more people the opportunity to live healthier lives.

Chapter 2: Type I Interferon Signaling in Costimulation Blockade Resistant Rejection

Jakob G. Habib, David Mathews, Ying Dong, Allison Stephenson, Andrew Adams

This manuscript is in preparation.

Introduction

Solid organ transplantation is the primary treatment for end-stage organ failure, with almost 40,000 organ transplants performed annually. Traditional non-specific immunosuppressants, such as the calcineurin inhibitors (CNIs) tacrolimus and cyclosporin, have been instrumental in reducing the incidence of early rejection. However, the off-target effects of these therapies can increase the risk of cardiovascular disease, stroke, and diabetes contributing to inferior long-term outcomes. In addition, long-term administration of CNIs can lead to irreversible nephrotoxicity, ultimately resulting in kidney failure in transplant recipients (6).

Belatacept is the first approved alternative to CNIs and functions by specifically interrupting T cell activation. Belatacept is a high affinity CTLA4-Ig fusion protein that binds to the costimulatory molecules CD80/CD86, blocking CD28 ligation on T cells. Patients treated with belatacept live longer and maintain superior kidney function compared to patients treated with CNIs (8). Unfortunately, a sizeable subset of patients treated with belatacept experience increased rates of acute rejection, so far limiting its broader use (10, 116). The underlying mechanisms of costimulation blockade-resistant rejection (CoB-RR) are not well understood, but it is thought to be mediated by alloreactive $CD8^+$ T cell responses, as they are less reliant on CD28 signaling (11, 37). In addition, we and others have shown that inflammatory cytokines can modify the immune response to the transplanted organ and promote transplant rejection, particularly in the setting of costimulation blockade (117, 118).

Type I interferons (IFNs), predominantly IFN-α and IFN-β, are a class of inflammatory cytokines that activate the innate and adaptive immune system by signaling through the interferon-α/β receptor 1 (IFNAR) (12). Type I IFN has been shown to enhance the cytolytic and recall response of memory $CD8^+$ T cells during a viral challenge (69). In addition, type I IFN signaling

prevents the apoptosis of activated $CD8^+$ T cells and provides critical signaling to drive the expansion and formation of memory $CD8^+$ T cells during a viral infection (70, 71). Interestingly, there is substantial data to suggest that type I IFNs may play a role in transplant rejection. For example, administration of type I interferon to treat hepatitis C infection in transplant recipients is known to promote rejection in some patients, suggesting that type I interferon signaling can be a critical signal to support transplant rejection (119, 120, 121, 122, 123, 124). Plasmacytoid dendritic cells (pDCs) are a major producer of type I IFN and their role in viral infection and autoimmunity has been previously described (13, 14, 52). In other scenarios pDCs may provide tolerogenic signals by inducing regulatory T cells (88, 125, 126). The contribution of pDC-produced type I IFN to costimulation blockade resistant rejection is unknown. In this study we seek to understand the role of type I IFN signaling in costimulation blockade-resistant rejection.

Methods

Mice

C57BL/6 and BALB/c mice were obtained from the National Cancer Institute (Frederick, MD). $Ifnar1^-$ ($IFNAR^{-/-}$, IFNAR-KO), CD45.1 (Pepboy), CD4-Cre, LysM-Cre, and $IFNAR^{fl/fl}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). OT-I (127) and OT-II (128) transgenic mice were purchased from Taconic Farms (Germantown, NY) and bred to $Thy1.1^+$ background at Emory University. OVA-transgenic mice (C57BL/6 background) (129) were a generous gift from Dr. Marc Jenkins (University of Minnesota, Minneapolis, MN). IFNAR-KO mOVA, IFNAR-KO BALB/c, IFNAR-KO OTI Thy1.1, IFNAR-KO OTII Thy1.1, CD4-Cre $IFNAR^{fl/fl}$, and LysM-Cre

IFNAR$^{fl/fl}$ mice were bred in-house. Unless otherwise stated in the figure legend and text, mice were 6-8 weeks old at the start of the experiment, experimental hosts were male, and donor animals for adoptive transfers and skin transplantation were male or female. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All surgery was performed under general anesthesia with maximum efforts made to minimize suffering. All animals were housed in specific pathogen-free animal facilities at Emory University.

Murine Skin transplantation and *in vivo* pharmacological treatment

Full-thickness tail and ear skin grafts were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages as previously described (130). Unless otherwise stated in the figure legend, donor skin was obtained from BALB/c mice and recipients were C57BL/6 mice. Where indicated, mice were treated with or without 250µg CTLA-4-Ig, 250µg anti-CD40L, and 250µg anti-IFNAR (all BioXcell) on days 0, 2, 4, and 6 post transplantation. For experiments investigating pDC depletion and inhibition, mice were treated as indicated with or without 250µg CTLA-4-Ig, 250µg anti-CD40L, 250µg anti-PDCA1, and 250µg anti-Siglec-H on days -2, 0, 2, 4, and every 3 days until day 22.

Bone marrow chimera

Bone marrow recipient mice 6-8 weeks old were irradiated with two doses of 550 rads separated by 4 hours using a RadSource RS2000 X-ray irradiator. During the 4-hour waiting period, bone marrow from the tibia and fibula of euthanized WT CD45.1 and IFNAR-KO CD45.2 mice were

flushed with a 21-gauge needle using PBS and homogenized into single cell suspensions, kept on ice or at 4˚C. Following the second round of irradiation, $20x10^6$ cells were administered into the tail vein of recipient mice. Animals were maintained in sterile housing and were provided with sterile food and sterile water adjusted to a pH of 3.0 using HCL (Sigma) containing 12.5µg/ml Polymyxin B (Sigma) and 0.5µg Neomycin (Sigma). Stock of antibiotic water was kept at 4˚C and was refreshed in the cages every two days. Mice were maintained on this diet in sterile housing for three weeks, after which they were returned to a normal diet and housing. The chimeric status of these mice was validated via blood draws on days 28, 35, and 60. Mice were then transplanted with BALB/c skin and treated with 250µg CTLA4-Ig, 250µg anti-CD40L, with or without 250µg anti-IFNAR on days 0, 2, 4, and 6.

Tissue Processing

Blood, spleen, lymph nodes, or bone marrow were harvested from mice at the specified timepoints. Bone marrow from tibia and fibula were flushed with a 21-gauge needle using PBS and homogenized into single cell suspensions. Spleens and lymph nodes were processed into single cell suspensions, and blood, spleen, and bone marrow were lysed with Fixative-Free Lysing Solution following manufacturer's instructions (Invitrogen). Tissues were then washed in FACS buffer and stained with antibodies for flow cytometry.

Flow Cytometry

For analysis of T cells, spleen samples were surface stained with CD3-AF700, CD4-APC-Cy7, CD8a-BV711, CD44-PE-Cy5, IFNAR-PE, MHC-I ($H2\text{-}K^b$)-Pacific Blue (BioLegend). For intracellular cytokine staining, cells were cultured with or without Cell Activation Cocktail (with

Brefeldin A) following the manufacturer's instructions (BioLegend, Cat# 423303). Cells were subsequently surface stained with CD3-PE-Dazzle, CD4-APC-Cy7, and CD8a-BV711 (BioLegend) and then washed in FACS buffer and permeabilized using Cytofix/Cytoperm following manufacturer's instructions (BD, Cat# 554722). Cells were then intracellularly stained with TNF-α-PE-Cy7 and IFN-ɣ-APC and then washed in FACS buffer. For analysis of dendritic cells (DCs), spleen samples were surface stained with CD3-PE-Dazzle, CD40-PerCP-Cy5.5, MHC-I (H2-K^b)-Pacific Blue, MHC-II (I-A^b)-FITC, IFNAR-PE (Biolegend), CD19-BV650, CD11b-APC-Cy7, and CD11c-PE-Cy7 (BD). For analysis of pDCs, cells were surface stained with CD3-PE-Dazzle, CD8a-BV711, B220-BV786, Siglec-H-FITC, PDCA1-PerCP-Cy5.5, MHC-I (H2-K^b)-Pacific Blue, MHC-II (I-A^b)-BV605, IFNAR-PE (Biolegend), CD19-BV650, CD11b-APC-Cy7, CD11c-PE-Cy7 (BD). For analysis of neutrophils, cells were stained with CD3-PE-Dazzle, MHC-I (H2-K^b)-Pacific Blue, Ly-6G-FITC, F4/80-PerCP-Cy5.5, IFNAR-PE (BioLegend), CD19-BV650, CD11b-APC-Cy7, CD11c-PE-Cy7 (BD). For analysis of BALB/c IFNAR-KO mice, cells were stained with H-2K^b-APC and H-2K^d-Pacific Blue. For analysis of bone marrow chimera mice, cells were stained with CD45.1-FITC and CD45.2- PE-Dazzle. Absolute numbers were calculated using CountBright Absolute Counting Beads according to the manufacturer's instructions (ThermoFisher). When more than one Brilliant Violet (BV) antibody was used at a time, Brilliant Stain Buffer Plus was used per the manufacturer's instructions (BD, Cat# 566385). Samples were analyzed on an LSR II flow cytometer (BD). Data were analyzed using FlowJo software (Tree Star).

Detection of IFN-α via ELISA

The bone marrow from tibia and fibula from C57BL/6 mice was flushed with a 21-gauge needle using PBS and homogenized into single cell suspensions prior to lysing with Fixative-Free Lysing Solution following manufacturer's instructions (Invitrogen). Bone marrow was washed in FACS buffer and pDCs were isolated using the Plasmacytoid Dendritic Cell Isolation Kit following the manufacturer's instructions (Miltenyi Biotec, Cat# 130-107-093) but with the addition of CD11c microbeads following the manufacturer's instructions (Miltenyi Biotec, Cat# 130-125-835). Non-pDCs were collected from the magnetically bound fraction and were brought to the same concentration as the unbound pDC fraction in R10 (RPMI with 10% FBS). Cells (pDCs or non-pDCs) were added to a 96 well plate ($1x10^5$ cells/well) that had first been coated overnight at 4˚C with 10ug/well anti-Siglec-H (BioXCell, Cat# BE0202) or 10ug/well IgG2b isotype control (BioXCell, Cat# BE0090), washed twice with PBS. CpG type A (0.6µg/well, Enzo Life Sciences, Cat# 746-003-C100) or R10 was added to half of the wells and the cells were incubated overnight at 37˚C, 5% CO_2. Interferon-α was detected via enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocol (Invitrogen cat# 50-246-672).

Detection of pSTAT1 via ELISA

Human and rhesus macaque whole blood was collected in Vacutainer CPT tubes (BD, Cat# 362753) and peripheral blood mononuclear cells (PBMCs) were isolated following the manufacturer's instructions. Cells were washed in R10 and added to a 6 well plate, $5x10^6$ cells/well. Anti-IFNAR (10µg/well) or R10 was added to each well and the plate was incubated for 30 minutes at 37˚C, 5% CO_2. Recombinant human IFN-α2 (1000IU/well, BioLegend, 592702) or R10 was added to each well and the plate was incubated for 30 minutes at 37˚C, 5% CO_2.

Phosphorylated STAT1 (pSTAT1) was then detected via ELISA following the manufacturer's protocol (abcam ab126456).

Non-human primate kidney transplantation

Outbred rhesus monkeys (Macaca mulatta) ranging between 3 and 5 years old were obtained from AlphaGenesis, Inc., and Emory National Primate Research Center. Donor-recipient pairs were chosen to maximize genetic disparity at both MHC class I and class II alleles based on 454 deep-sequencing analysis (University of Wisconsin, Madison, WI). Animals were heparinized (100 U/kg) during organ procurement and implantation. Left native nephrectomy was performed at least 3 weeks prior to transplantation, and a completion right native nephrectomy was performed at the time of transplantation. Animals were treated with belatacept (20mg/kg) with or without anti-IFNAR (20mg/kg) on D0, 5, 7, 14, 28, 42, 56, and 70. All transplanted animals were monitored with daily clinical assessment and serial laboratory evaluations, including complete blood count and serum chemistry. Animals demonstrated excellent graft function postoperatively and were sacrificed at the time of allograft rejection, defined by graft dysfunction. Depressed renal function pursuant to allograft rejection was determined by 2 consecutive measurements of Cr greater than 5 or BUN greater than 120.

Results

Combined IFNAR and costimulation blockade improves skin graft survival and reduces $CD8^+$ T cell and dendritic cell activation

In order to investigate the role of IFNAR signaling during CoB-RR, full-thickness MHC-mismatched skin grafts were performed as described and graft survival was monitored (Figure 1A). While skin grafts treated with costimulation blockade alone exhibited increased survival compared to no treatment (median survival time [MST] 23 days vs 11 days, $p<0.0001$), the combination of CoB+anti-IFNAR further improved survival compared to CoB alone (MST> 66 days, $p<0.0001$, Figure 2A). To investigate the combined impact of CoB and anti-IFNAR on immune activation, the proportion of $CD44^+$ T cells among $CD4^+$ and $CD8^+$ T cell populations and the proportion of $TNF\alpha^+IFNy^+$ $CD8^+$ T cells following *in vitro* stimulation was investigated in the spleen of mice 10 days post skin transplantation (Figure 1C). Results demonstrated that all groups treated with CoB had reduced frequencies of $CD44^+CD4^+$ T cells compared to no treatment ($p<0.0001$). In CD8 T cells, CoB alone reduced the frequency of $CD44^+CD8^+$ T cells compared to no treatment ($p<0.0001$). The addition of anti-IFNAR reduced the frequency of CoB+anti-IFNAR $CD44^+CD8^+$ T cells compared to CoB alone, whereas IFNAR-KO recipients treated with CoB exhibited a further reduction in the frequency of $CD44^+CD8^+$ T cells compared to wildtype recipients treated CoB alone ($p=0.0136$, $p<0.0001$ respectively, Figure 1C). We further investigated the frequency of effector CD8+ T cells following *in vitro* stimulation with PMA and Ionomycin and found that CoB+anti-IFNAR reduced the frequency of $TNF\alpha^+IFNy^+$ $CD8^+$ T cells compared to CoB alone ($p=0.0374$).

We next investigated the effect that CoB and anti-IFNAR have on the expression of dendritic cell activation markers. In the spleen 10 days post skin transplantation, we found elevated expression of MHC-I in CoB alone mice compared to no treatment ($p=.0009$, Figure 1D). We observed that WT recipients treated with CoB+anti-IFNAR and IFNAR-KO recipients treated with CoB expressed reduced MHC-I on DCs compared to WT recipients treated with CoB alone ($p<0.0001$). The expression of MHC-II and CD40 was unaffected by CoB alone administration compared to no treatment ($p=.239$, $p=.189$, respectively, Figure 1D). However, WT recipients treated with CoB+anti-IFNAR and IFNAR-KO recipients treated with CoB exhibited reduced MHC-II and CD40 expression compared to WT recipients treated with CoB alone ($p<0.001$, Figure 1D). These results indicate that combined administration of CoB+anti-IFNAR following skin graft transplantation result in reduced CD8+ T cell and DC activation.

IFNAR signaling on the recipient is the primary contributor to costimulation blockade-resistant rejection

Next, we sought to determine whether IFNAR signaling on the donor or on the recipient contributes to rejection in the setting of CoB. In order to investigate this, we grafted BALB/c skin onto WT or IFNAR-KO recipients and treated with CoB or Cob+anti-IFNAR (Figure 2B). The MST for WT recipients treated with CoB was 17 days compared to 47.5 days for recipients treated with CoB+anti-IFNAR ($p<0.0001$), and compared to IFNAR-KO mice treated with CoB which had an MST >60 days ($p<0.0001$, Figure 2B). This data suggests that IFNAR signaling on donor skin does not contribute to CoB-RR, but to confirm this, we bred IFNAR-KO BALB/c mice to use as donors. These donor mice are MHC-I H-2K$^{d/d}$ (Figure 2C) and IFNAR-KO (validated by flow

cytometry and PCR, data not shown). We tracked the survival of WT BALB/c skin and IFNAR-KO BALB/c skin on C57BL/6 mice treated with CoB (250μg CTLA4-Ig and 250μg CD40L) on days 0, 2, 4, and 6, and found that WT skin had an MST of 31 days and IFNAR-KO skin had an MST of 58.5 days, though this was not significantly different ($p=0.11$, Figure 2D). These data support the hypothesis that IFNAR signaling on the donor does not impact graft survival during CoB.

We further investigated the role of IFNAR signaling on donor skin by utilizing an antigen specific model system where TCR transgenic Thy1.1 OT-I ($CD8^+$) and OTII ($CD4^+$) T cells were adoptively transferred into naïve C57BL/6 recipients prior to engraftment with ACT-mOVA skin and treated with CTLA4-Ig with or without anti-IFNAR. These skin grafts constitutively express full-length membrane-bound chicken ovalbumin (mOVA) protein under control of the β-actin promoter (129). We bred this mOVA genotype to IFNAR-KO mice and investigated the impact of IFNAR signaling on the donor (Figure 2E). We found that WT mOVA skin treated with CTLA4-Ig had an MST of 22 days, IFNAR-KO mOVA skin treated with CTLA4-Ig had an MST of 22.5 days, and WT mOVA skin treated with CTLA4-Ig+anti-IFNAR had an MST >95 days (Figure 2F). This finding further supports our hypothesis that IFNAR signaling on the donor does not impact graft survival during CoB.

IFNAR signaling on hematopoietic cells are the primary contributors to costimulation blockade-resistant rejection

Next, we sought to determine whether IFNAR signaling on hematopoietic or on non-hematopoietic cells contributes to rejection in the setting of CoB. To investigate this, we generated bone marrow chimeric mice that had either wildtype CD45.1 or IFNAR-KO CD45.2 hematopoietic or non-hematopoietic cells, as indicated in Figure 3A. The wildtype mice used in this experiment expressed the congenic marker CD45.1, which allows us to delineate between hematopoietic vs non-hematopoietic cells. We validated the chimerism of these mice in the blood via flow cytometry, confirming the expression of IFNAR on CD45.1 and CD45.2 cells (Figure 3B). After 60 days of bone marrow reconstitution, we grafted these mice with BALB/c skin and treated with CoB with or without anti-IFNAR as indicated in Figure 3A. The group with IFNAR-KO hematopoietic cells and wildtype non-hematopoietic cells treated with CoB alone (MST of 29.5 days) and the group with WT hematopoietic cells and IFNAR-KO non-hematopoietic cells treated with CoB+anti-IFNAR (MST of 31 days) survived longer than the group with WT hematopoietic cells and IFNAR-KO non-hematopoietic cells treated with CoB alone (MST 24 days, $p=0.0016$, $p=0.0015$, respectively, Figure 3C). We observed no survival difference between the group with IFNAR-KO hematopoietic cells and wildtype non-hematopoietic cells treated with CoB alone and the group with WT hematopoietic cells and IFNAR-KO non-hematopoietic cells treated with CoB+anti-IFNAR (Figure 3C). These results indicate that IFNAR signaling on hematopoietic cells contributes more strongly to CoB-RR than IFNAR signaling on non-hematopoietic cells.

IFNAR signaling solely on T cells or Myeloid cells does not drive costimulation blockade-resistant rejection

We next sought to investigate which hematopoietic cell subset was contributing to CoB-RR. We hypothesized that IFNAR signaling on T cells or on myeloid cells would drive rejection, as IFNAR signaling on these cell subsets is important to effectively mount an immune response to a viral infection (69, 70, 71, 131, 132). We first investigated whether IFNAR signaling on antigen specific T cells mediates CoB-RR. Utilizing the mOVA system, we adoptively transferred WT or IFNAR-KO OTI and OTII cells into C57BL/6 mice which were then grafted with mOVA expressing skin and were subsequently treated with CTLA4-Ig with or without anti-IFNAR (Figure 4A). Mice that received WT OTI and WT OTII cells treated with CTLA4-Ig+anti-IFNAR exhibited improved graft survival compared to mice that received the same cells treated with CTLA4-Ig alone ($p=0.0018$, Figure 4B). However, no other permutation of adoptive transfer treated with CTLA4-Ig alone showed improved survival, suggesting IFNAR signaling solely on antigen specific T cells does not drive CoB-RR.

We next used the Cre-Lox system to excise IFNAR specifically on T cells by generating CD4-Cre $IFNAR^{fl/fl}$ mice. Importantly, because $CD8^+$ T cells express CD4 during development, IFNAR is excised on both $CD4^+$ and $CD8^+$ T cells in CD4-Cre $IFNAR^{fl/fl}$ mice. The MFI of IFNAR on WT T cells is significantly greater than on IFNAR-KO T cells ($p<0.0001$) and on CD4-Cre $IFNAR^{fl/fl}$ T cells ($p<0.0001$, Figure 4C). We further validated the non-responsiveness of these cells to IFN-α stimulation by culturing T cells from CD4-Cre $IFNAR^{fl/fl}$ mice overnight with or without IFN-α. We found that compared to unstimulated cells, IFN-α-stimulated wildtype T cells upregulated MHC-I ($p<0.0001$) whereas IFNAR-KO T cells and T cells from CD4-Cre $IFNAR^{fl/fl}$ mice did not upregulate MHC-I (Figure 4C). Upon generation of these mice, we sought to investigate whether IFNAR signaling on T cells contributes to CoB-RR. We grafted BALB/c skin onto WT or CD4-

Cre IFNAR$^{fl/fl}$ mice and treated with CoB with or without anti-IFNAR on days 0, 2, 4, and 6. The median survival time for WT mice treated with CoB was 21 days, for WT mice treated with CoB+anti-IFNAR was 57.5 days, for CD4-Cre IFNAR$^{fl/fl}$ treated with CoB was 19 days, and for CD4-Cre IFNAR$^{fl/fl}$ treated with CoB+anti-IFNAR was 59 days (Figure 4D). We found that the addition of anti-IFNAR improved graft survival compared to CoB alone in WT mice ($p=0.0004$) and in CD4- Cre IFNAR$^{fl/fl}$ mice ($p=0.0011$). However, there was no survival benefit in CD4-Cre IFNAR$^{fl/fl}$ mice compared to WT mice when either group was treated with CoB alone (Figure 4D). These findings further suggest that IFNAR signaling specifically on T cells does not drive CoB-RR.

To investigate whether IFNAR signaling on myeloid cells contributes to CoB-RR we generated LysM-Cre IFNAR$^{fl/fl}$ mice that do not express IFNAR on cells of myeloid origin. The expression of IFNAR on neutrophils from WT mice is significantly greater than on IFNAR-KO neutrophils ($p<0.0001$) and on LysM-Cre IFNAR$^{fl/fl}$ neutrophils ($p<0.0001$). We further validated the non-responsiveness of these cells to IFN-α stimulation by culturing neutrophils from LysM-Cre IFNAR$^{fl/fl}$ mice overnight with or without IFN-α. We found that compared to unstimulated cells, IFN-α-stimulated wildtype neutrophils upregulated MHC-I ($p=0.0003$) whereas IFNAR-KO cells and cells from LysM-Cre IFNAR$^{fl/fl}$ mice did not upregulate MHC-I (Figure 4E). To investigate whether IFNAR signaling on myeloid cells contributes to CoB-RR we grafted BALB/c skin onto WT or LysM-Cre IFNAR$^{fl/fl}$ mice and treated with CoB with or without anti-IFNAR on days 0, 2, 4, and 6. The median survival time for WT mice treated with CoB was 22 days, for WT mice treated with CoB+anti-IFNAR was 66 days, and for LysM-Cre IFNAR$^{fl/fl}$ treated with CoB was 25 days (Figure 4F). We found that the addition of anti-IFNAR improved graft survival compared

to CoB alone in WT mice (p=0.0002) and in LysM-Cre IFNAR$^{fl/fl}$ mice (p<0.0001). However, there was no survival benefit in LysM-Cre IFNAR$^{fl/fl}$ mice compared to WT mice when either group was treated with CoB alone (Figure 4F). These findings suggest that IFNAR signaling specifically on myeloid does not drive CoB-RR.

Depletion or inhibition of pDCs is insufficient to prevent costimulation blockade-resistant rejection

We next sought to investigate how pDCs influence CoB-RR as pDCs are known to produce large quantities of IFN, particularly during a viral infection. In the spleen 10 days post skin transplantation, we found that CoB alone reduced the expression of MHC-I on pDCs compared to no treatment (p=0.0161, Figure 5A). Treatment with CoB+anti-IFNAR reduced MHC-I on pDCs compared to CoB alone (p=0.0002) and CoB administration in IFNAR-KO recipients further reduced MHC-I expression on pDCs compared to WT recipients that received CoB alone (p<0.0001) or CoB+anti-IFNAR (p=0.0097, Figure 5A). In addition, the longitudinal expression of MHC-I one-, two-, and three-weeks post engraftment revealed that MHC-I on pDCs in mice treated with CoB+anti-IFNAR was consistently reduced compared to CoB alone in the spleen (p=0.0021, p=0.0008, p=0.0122 respectively) and in the lymph node (p=0.0004, p<0.0001, p=0.0172 respectively, Figure 5A). In the spleen 10 days post engraftment, administration of CoB alone or CoB+anti-IFNAR in WT mice did not reduce the expression of MHC-II compared to no treatment. However, IFNAR-KO mice treated with CoB showed reduced MHC-II expression compared to no treatment (p=0.001), CoB alone (p=0.0043), and CoB+anti-IFNAR (p=0.021, Figure 5A). We also measured the expression of IFNAR on pDCs and show in Figure 5B that they

express IFNAR to a greater extent than on T cells or cDCs, suggesting that pDCs might therefore by disproportionally affected by anti-IFNAR antibody treatment. This is corroborated by other studies which found that pDCs became far more efficient producers of IFN-α after IFNAR ligation in an auto-amplification step, a process that was interrupted with anti-IFNAR therapy (133).

In order to investigate the role that pDCs have during CoB-RR we utilized an antibody against PDCA1 that depletes pDCs as well as an antibody against Siglec-H that abrogates the production of IFN-α by pDCs. We validated the efficacy of anti-PDCA1 by administering 250μg i.p. *in vivo* and then measuring the absolute count of pDCs over time in the spleen, bone marrow, and lymph node (Figure 5C). We found a sharp drop in the absolute count of pDCs after one day in the spleen ($p=0.0067$), bone marrow ($p=0.0002$) and lymph node ($p=0.0409$, Figure 5C) and did not observe substantial pDC numbers in these compartments until day 7.

Next, we validated the efficacy of anti-Siglec-H *in-vitro* by culturing pDCs or non-pDCs with or without CpG-A, which stimulates IFN-α production, with or without anti-Siglec-H (Figure 5D). We found that CpG-A strongly stimulated the production of IFN-α when cultured with an isotype control antibody compared to unstimulated ($p<0.0001$), but this was substantially abrogated when pDCs were cultured with CpG-A and anti-Siglec-H ($p<0.0001$), though even in the presence of anti-Siglec-H we did detect a small about of IFN-α produced when pDCs were stimulated with CpG-A compared to unstimulated ($p=0.0021$, Figure 5D). We did not detect any substantial amount of IFN-α produced by non-pDCs (Figure 5D). To test how pDC depletion or inhibition affects CoB-RR, we performed skin grafts as indicated in Figure 5E. We observed improved skin graft survival in mice treated with CoB+anti-IFNAR compared to CoB alone ($p=0.0060$),

CoB+anti-PDCA1 (p=0.0085), and CoB+anti-Siglec-H (p=0.0497, Figure 5F). These findings indicate that the depletion of pDCs or the abrogation of their production of IFN-α is insufficient to prevent CoB-RR.

Combined costimulation and IFNAR blockade provides long-term survival in a life-sustaining nonhuman primate renal allotransplantation model

In order to translate these findings into the clinic, we utilized a fully human anti-IFNAR antibody. This reagent has been shown to be well tolerated in humans and was efficacious in a phase-III clinical trial of SLE (97, 134). We validated the functionality of this reagent in rhesus macaque and human peripheral blood mononuclear cells (PBMCs) by measuring the phosphorylation of STAT1 (pSTAT1) when stimulated with IFN-α2 with or without anti-IFNAR. For both rhesus macaque and human PBMCs, IFN-α2 alone upregulated pSTAT1 compared to no treatment ($p<0.001$ for both) while IFN-α2 combined with anti-IFNAR prevented the upregulation of pSTAT1 ($p<0.001$ for both, Figure 6A). In order to investigate how CoB combined with anti-IFNAR affects renal transplantation survival in nonhuman primates, we double nephrectomized a rhesus macaque and transplanted a life sustaining kidney from a donor rhesus macaque. We treated the animal with 20mg/kg Belatacept and 20mg/kg anti-IFNAR on days 0, 5, 7, 14, and every other week until day 70 as indicated in Figure 6B. This pilot animal survived until day 241, well beyond the MST of belatacept-alone treated animals (50 days, Figure 6C). Promisingly, we did not detect an emergence of cytomegalovirus (CMV), and creatinine levels remained stable until the time of rejection (Figure 6C). As this is a limited sample consisting of one experimental subject, we are

unable to make definitive conclusions regarding the effectiveness of this treatment, but future nonhuman primate transplantations will elucidate the efficacy of this therapy.

Discussion

The aim of this study was to understand how IFNAR signaling influences costimulation blockade resistant rejection. We found that IFNAR signaling on recipient hematopoietic cells is a major contributor to CoB-RR, and that abrogating IFNAR signaling specifically on T cells or on myeloid cells is insufficient to prevent CoB-RR. In addition, modulation of pDCs by depletion or inhibition does not prevent costimulation independent rejection. Interestingly, Lakkis and colleagues previously demonstrated that type I IFNs were not critical for skin allograft rejection, nor did they interfere with donor-specific T cell development (135). One important note is that these experiments were performed in otherwise untreated mice without interruption of T cell costimulation or other T cell activating signals. Here, we observe that in the setting of costimulatory blockade, IFNAR signaling is a crucial component of costimulation independent allograft rejection (Figure 1B). In support of our data, we and others have previously shown that viral infection is a potent stimulant that provokes costimulation blockade resistant rejection (136, 137). In fact, additional studies using costimulation blockade suggested a key role for type I interferon in bridging the innate and adaptive immune responses, resulting in the abrogation of transplant tolerance (138). Others have shown that a type I interferon signature characterizes chronic antibody mediated rejection in kidney transplantation (139). Taken together, these studies illustrate that in the context of reduced T cell costimulation, the type I interferon pathway may play a key role in supporting alloimmune responses.

Several signals and cell types have been implicated in costimulation blockade resistant rejection. One likely explanation suggests that the increased rejection is mediated by alloreactive $CD8^+$ T cells that are less reliant on CD28 costimulatory signals (11, 37). Type I IFN signaling directly enhances the proliferation, differentiation, and lifespan of activated $CD8^+$ T cells (70, 71, 73, 118, 140, 141). However, our study revealed that knocking out IFNAR specifically on T cells or myeloid cells is insufficient to prevent costimulation blockade resistant rejection. It is possible that due to the ubiquitous expression of IFNAR, different recipient cell types signaling through this pathway produce downstream cytokine signals that induce T cells to drive rejection during costimulation blockade. This could explain why we only observe improved skin graft survival during costimulation blockade when IFNAR is fully knocked out on recipient hematopoietic cells or blocked pharmacologically. In addition, signaling through IFNAR promotes dendritic cell maturation, which in turn enhances presentation of peptide-MHC and costimulatory signals to T cells (142). IFNAR signaling on DCs also enhances the cross-presentation of exogenous antigen to CD8 T cells, which are an important contributor to graft rejection (81, 143). In support of these studies, our findings reveal that combined costimulatory and IFNAR blockade during transplantation results in reduced MHC-I, MHC-II, and CD40 expression on dendritic cells, which might in part explain the improved graft survival in these mice.

A growing body of evidence reveals that type 1 interferons have a complex immunomodulatory role which is dependent on the context, cell-type, timing, and presence of associated signals, such as costimulation. Administration of IFN-α to transplant patients for treatment of Hepatitis C is known to precipitate transplant rejection and graft loss (119, 120, 121, 122, 123, 124). Conversely, a recent study by Fueyo-González et al. revealed a tolerogenic role for IFN-β in transplantation.

The group found that IFN-β enhanced heterotopic heart graft survival in a $CD4^{+}FoxP3^{+}$ regulatory T cell dependent manner (144). Similarly, mounting evidence has revealed that pDCs serve a dual function in the immune system. This rare subset of cells has been associated with systemic lupus erythematous (SLE) and more recently organ transplant rejection (13, 88, 145, 146, 147). However, pDCs can also express proteins involved in the induction of T cell tolerance (148, 149, 150, 151). Ochando et al. detailed a prominent role for pDCs in mediating tolerance to vascularized heart allografts via a regulatory T cell dependent mechanism (88, 125). While we hypothesized that the depletion or inhibition of pDCs would influence IFNAR-mediated costimulation blockade-resistant rejection, we did not observe improved skin graft survival in mice treated costimulation blockade and anti-PDCA1 or anti-Siglec-H compared to anti-IFNAR. It is possible that pDCs are not essential to IFNAR-mediated costimulation blockade-resistant rejection, which might explain why modulation of this subset had no impact on graft survival.

In the context of clinical application, Anifrolumab, a fully human monoclonal antibody specific for IFNAR, has shown promise. In a phase III trial in patients with active SLE, treatment with anifrolumab resulted in a significant reduction of disease activity compared to placebo (96, 152, 153). The effect of anti-IFNAR is not restricted to autoimmunity, as noted in a previously reported preclinical study testing another monoclonal antibody to the human type I interferon receptor, resulting in prolonged transplant/allograft survival in cynomolgus monkeys when combined with low dose cyclosporine (154). Collectively, the results of our murine data and our NHP pilot study suggests that the interruption of type I interferon signaling holds promise as a therapy to prevent costimulation blockade resistant rejection.

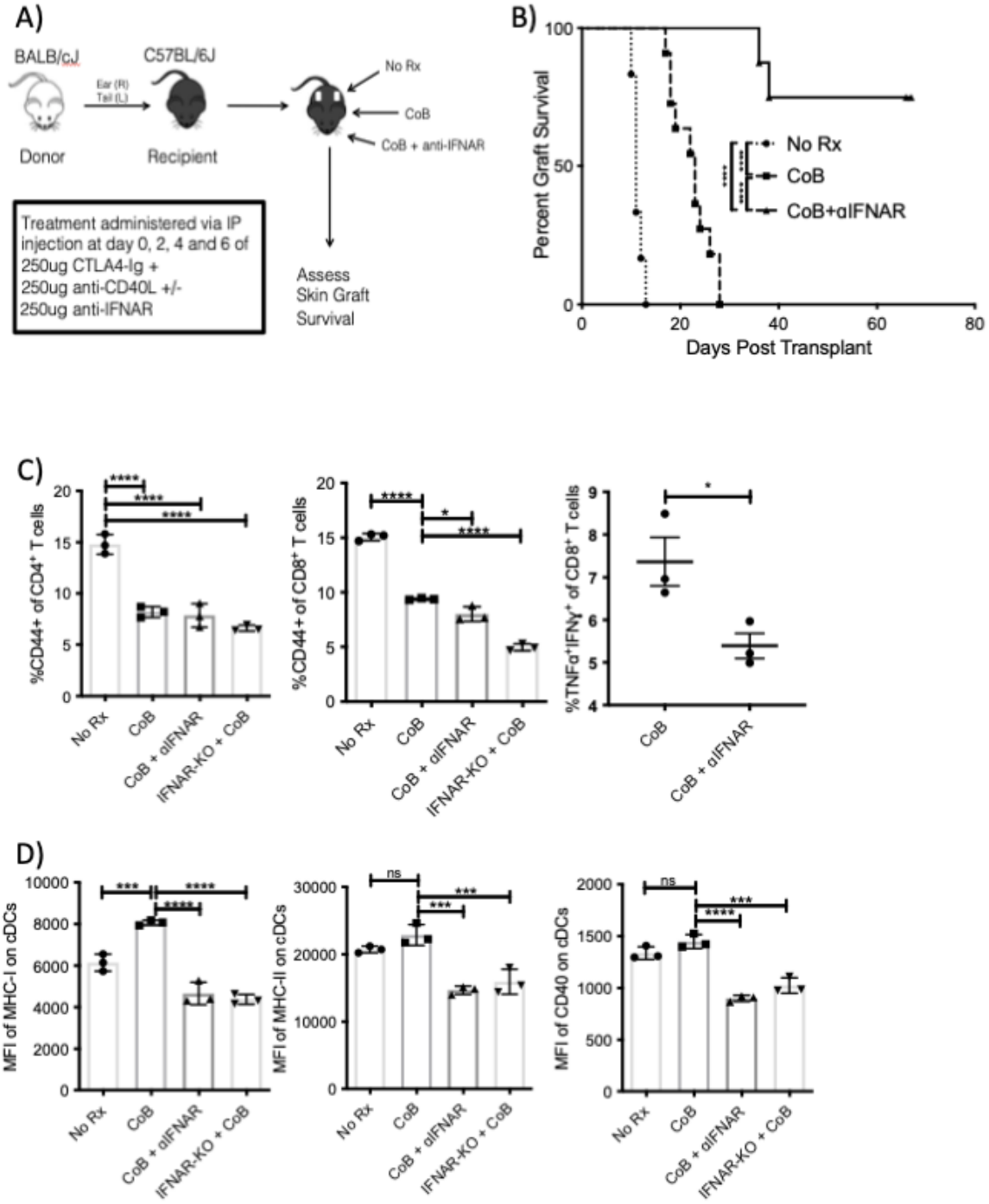
A)
BALB/cJ
C57BL/6J
Ear (R)
Tail (L)
Donor
Recipient
No Rx
CoB
CoB + anti-IFNAR
Treatment administered via IP injection at day 0, 2, 4 and 6 of 250ug CTLA4-Ig + 250ug anti-CD40L +/- 250ug anti-IFNAR
Assess Skin Graft Survival
B)
Percent Graft Survival
Days Post Transplant
No Rx
CoB
CoB+αIFNAR
C)
%CD44+ of CD4+ T cells
%CD44+ of CD8+ T cells
%TNFα+IFNγ+ of CD8+ T cells
No Rx
CoB
CoB + αIFNAR
IFNAR-KO + CoB
D)
MFI of MHC-I on cDCs
MFI of MHC-II on cDCs
MFI of CD40 on cDCs
ns

Figure 1: Combined IFNAR and costimulation blockade improves skin graft survival and reduces $CD8^+$ T cell and dendritic cell activation. A) Skin from BALB/c donors were grafted onto C57BL/6 recipients. Recipients were untreated or treated with 250μg CTLA4-Ig & 250μg anti-CD40L with or without 250μg anti-IFNAR on day 0, 2, 4, and 6. B) The median survival time of no treatment was 11 days, for CoB was 23 days, and for CoB+aIFNAR was >66 days. ****$p < 0.0001$ by Mantel-Cox log-rank test C) Frequency of $CD44^+$ $CD4^+$ T cells, $CD44^+$ $CD8^+$ T cells, and $TNF\alpha^+IFN\gamma^+$ $CD8^+$ T cells in the spleen 10 days post skin transplantation. D) MFI of MHC-I, MHC-II, and CD40 on cDCs from the spleen 10 days post skin transplantation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$P < 0.0001$ by one way ANOVA correcting for multiple comparisons.

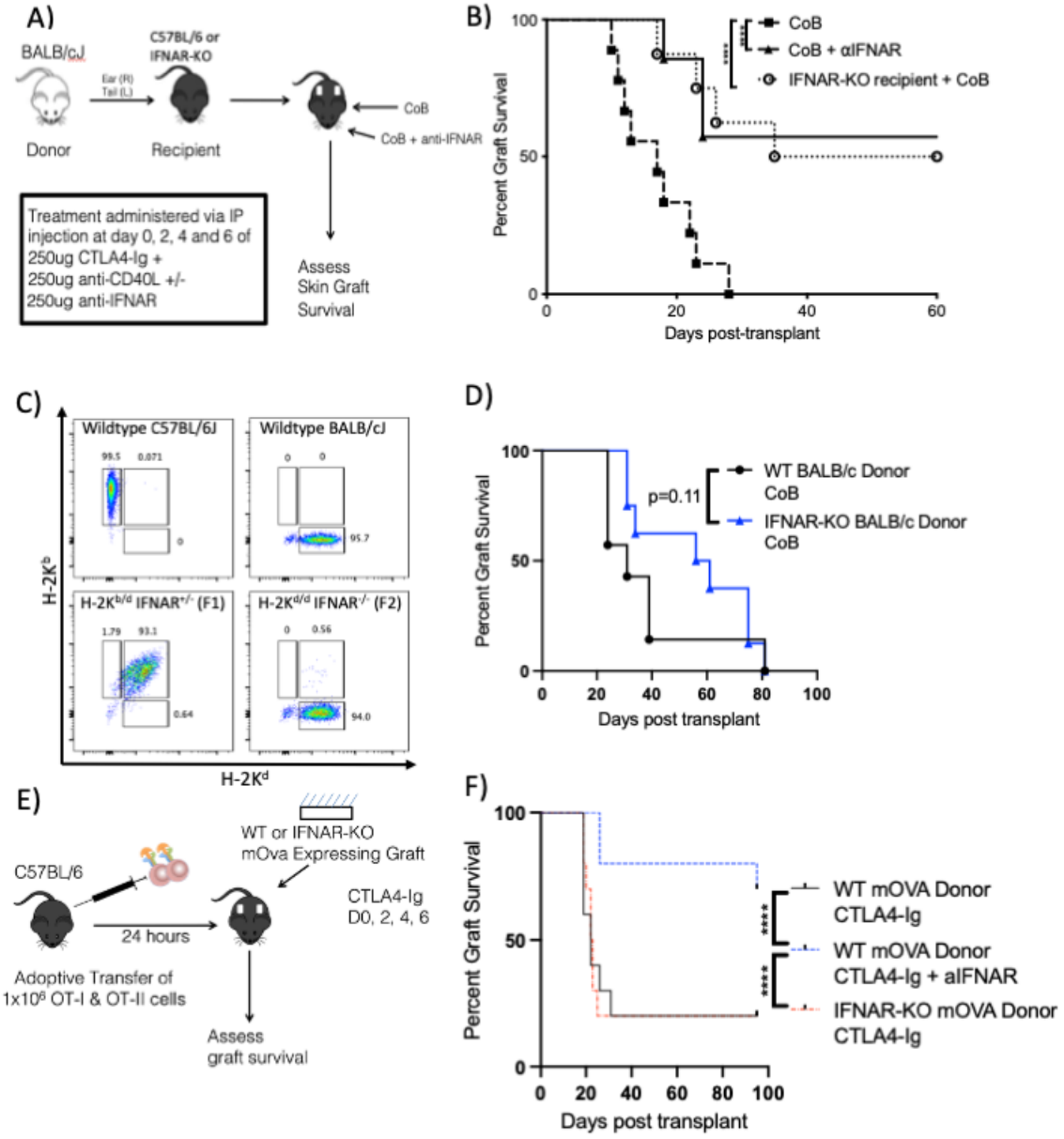

Figure 2: IFNAR signaling on the recipient is the primary contributor to costimulation blockade-resistant rejection. A) Skin from BALB/c donors were grafted onto C57BL/6 or IFNAR-KO recipients. Recipients were treated with 250µg CTLA4-Ig & 250µg anti-CD40L, with or without 250µg anti-IFNAR on day 0, 2, 4, and 6. B) The median survival time for WT mice treated with CoB was 17 days, for WT mice treated with CoB+aIFNAR was 47.5 days, and for IFNAR-KO mice treated with CoB was undefined. C) Representative flow cytometric plots showing the

expression of H-2K^{b} and H-2K^{d} from the blood of wildtype C57BL/6J, wildtype BALB/cJ, H-2K$^{b/d}$ IFNAR$^{-/+}$, and H-2K$^{d/d}$ IFNAR$^{-/-}$ mice. D) The median survival time of WT BALB/c skin grafts for mice treated with CoB was 31 days and for IFNAR-KO BALB/c skin grafts for mice treated with CoB was 58.5 days. E) One day prior to skin transplantation, recipient C57BL/6 mice received an adoptive transfer of 1x10^{6} OTI and 1x10^{6} OTII cells. Mice were then grafted with WT or IFNAR-KO mOVA expressing skin and treated with 250µg CTLA4-Ig on day 0, 2, 4, and 6. F) The median survival time for WT mOVA skin grafts for mice treated with CTLA4-Ig was 22 days, for WT mOVA skin grafts treated with CTLA4-Ig+anti-IFNAR was undefined, and for IFNAR-KO mOVA skin grafts treated with CTLA4-Ig was 22.5 days. ****$p < 0.0001$ by Mantel-Cox log-rank test.

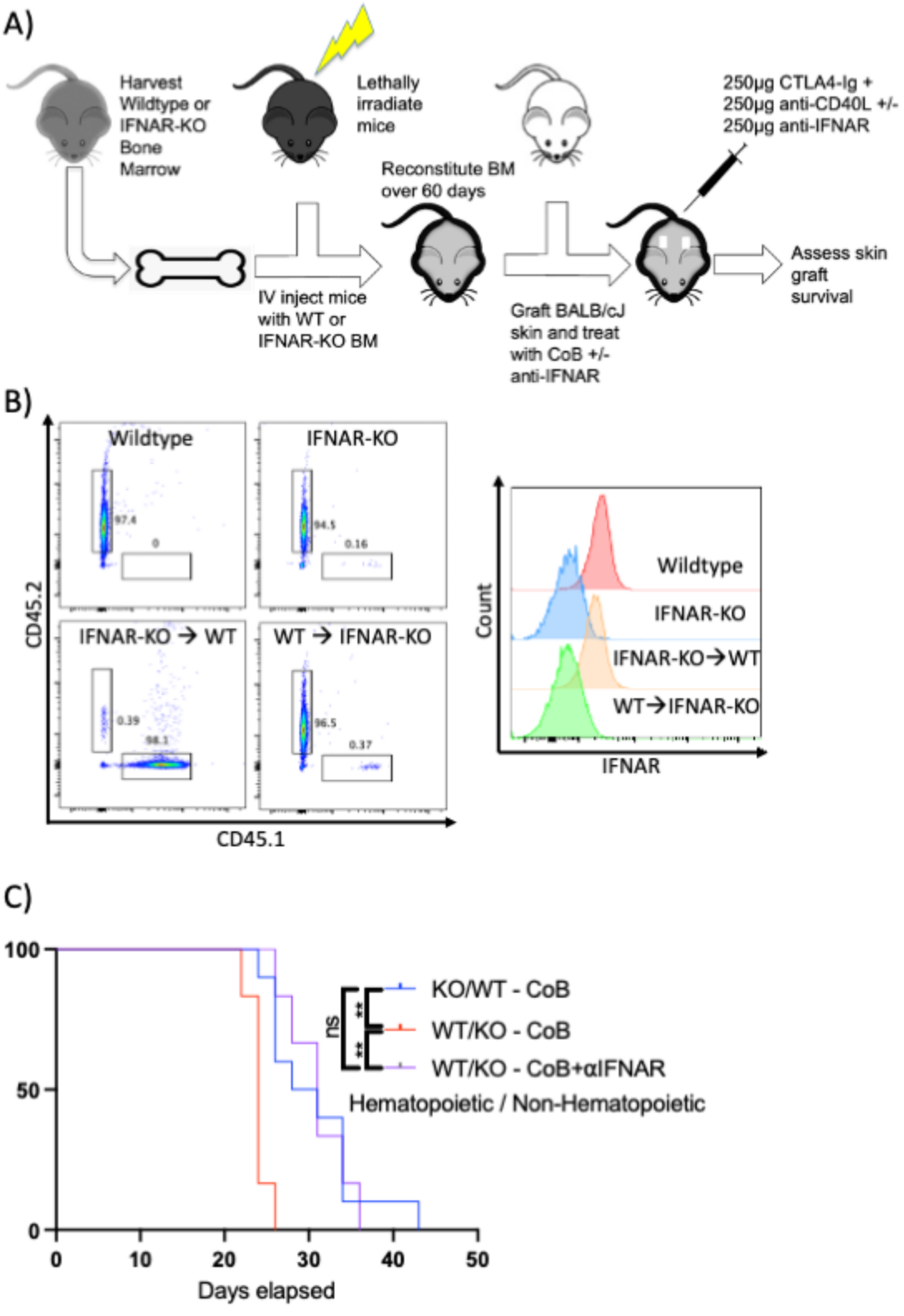
A)
Harvest Wildtype or IFNAR-KO Bone Marrow
Lethally irradiate mice
Reconstitute BM over 60 days
250µg CTLA4-Ig + 250µg anti-CD40L +/- 250µg anti-IFNAR
IV inject mice with WT or IFNAR-KO BM
Graft BALB/cJ skin and treat with CoB +/- anti-IFNAR
Assess skin graft survival
B)
Wildtype
IFNAR-KO
IFNAR-KO → WT
WT → IFNAR-KO
97.4
0
94.5
0.16
0.39
98.1
96.5
0.37
CD45.2
CD45.1
Count
IFNAR
Wildtype
IFNAR-KO
IFNAR-KO→WT
WT→IFNAR-KO
C)
Percent Graft Survival
Days elapsed
100
50
0
0
10
20
30
40
50
ns
**
**
KO/WT - CoB
WT/KO - CoB
WT/KO - CoB+αIFNAR
Hematopoietic / Non-Hematopoietic

Figure 3. IFNAR signaling on hematopoietic cells are the primary contributors to costimulation blockade-resistant rejection. A) WT or IFNAR-KO mice were lethally irradiated with two doses of 550 rads separated by 4 hours. WT or IFNAR-KO BM was IV injected into these mice and reconstituted over 60 days. Mice were then grafted with BALB/c skin and treated with 250μg CTLA4-Ig & 250μg anti-CD40L, with or without 250μg anti-IFNAR on day 0, 2, 4, and 6. B) Representative flow cytometric plots validating the expression of CD45.1 and CD45.2 from the blood of wildtype, IFNAR-KO, and chimeric mice. Histogram of IFNAR expression on wildtype, IFNAR-KO, and chimeric mice. C) The median survival time for mice with IFNAR-KO hematopoietic cells and WT non-hematopoietic cells treated with CoB was 29.5 days, for mice with WT hematopoietic cells and IFNAR-KO non-hematopoietic cells treated with CoB was 24 days, and for mice with WT hematopoietic cells and IFNAR-KO non-hematopoietic cells treated with CoB + anti-IFNAR was 31 days. $^{**}p < 0.01$ by Mantel-Cox log-rank test.

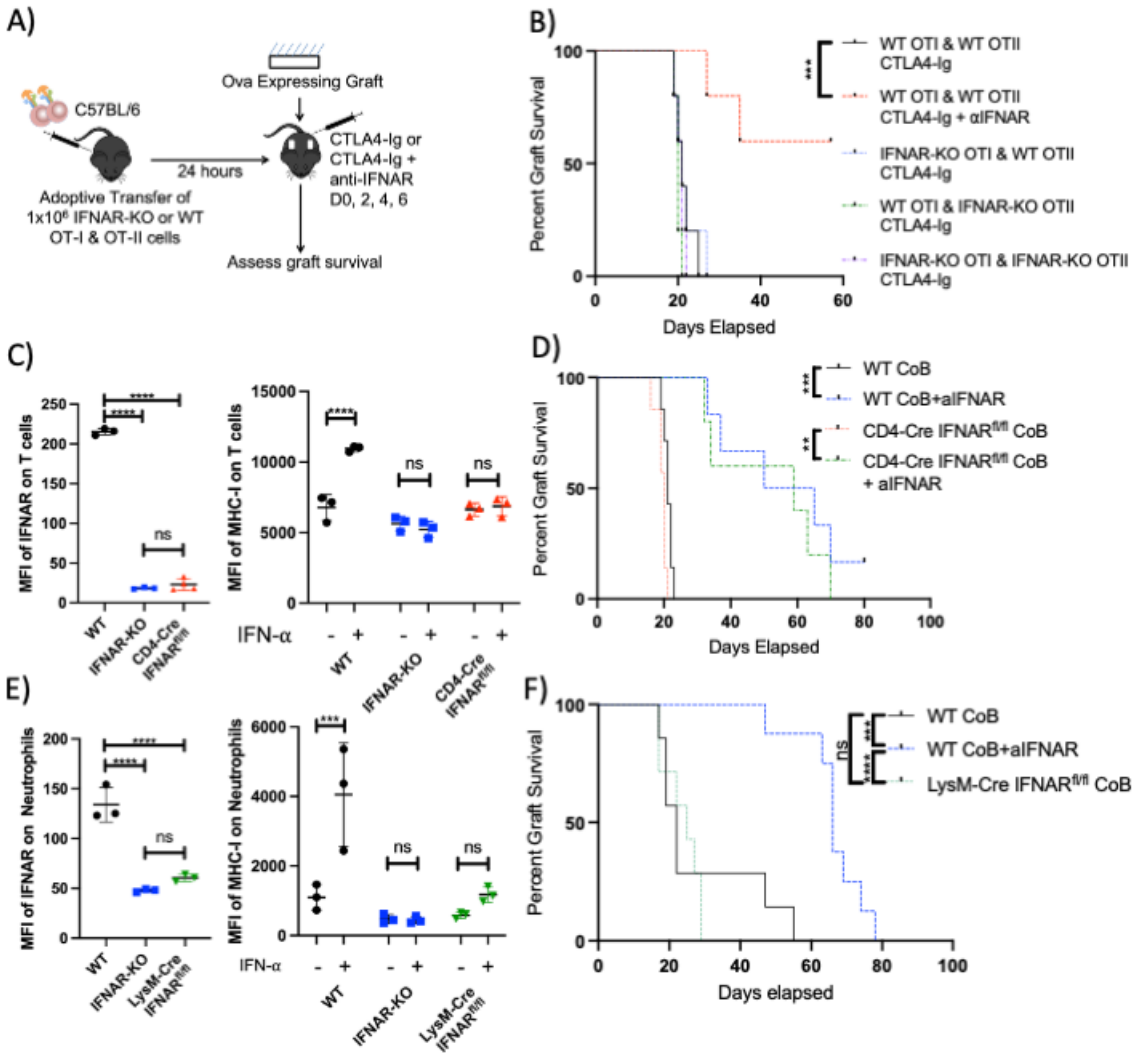

Figure 4: IFNAR signaling solely on T cells or Myeloid cells does not drive costimulation blockade resistant rejection. A) One day prior to skin transplantation, recipient C57BL/6 mice received an adoptive transfer of $1x10^6$ OTI and $1x10^6$ OTII WT or IFNAR-KO cells. Mice were then grafted with mOVA expressing skin and treated with 250µg CTLA4-Ig with or without 250µg anti-IFNAR on day 0, 2, 4, and 6. B) The median survival time of mice that received an adoptive transfer of WT OTI and OTII cells treated with CTLA4-Ig was 21 days, of mice that received an adoptive transfer of WT OTI and OTII cells treated with CTLA4-Ig+anti-IFNAR was >57 days, of mice that received an adoptive transfer of IFNAR-KO OTI and WT OTII cells treated with CTLA4-Ig

was 21 days, of mice that received an adoptive transfer of WT OTI and IFNAR-KO OTII cells treated with CTLA4-Ig was 20 days, and of mice that received an adoptive transfer of IFNAR-KO OTI and IFNAR-KO OTII cells treated with CTLA4-Ig was 21 days. C) MFI of IFNAR on $CD3^+$ T cells from WT, IFNAR-KO, or CD4-Cre $IFNAR^{fl/fl}$ mice. MFI of MHC-I on T cells from WT, IFNAR-KO, or CD4-Cre $IFNAR^{fl/fl}$ mice cultured overnight with or without IFN-α. D) The median survival time for WT mice treated with CoB was 21 days, for WT mice treated with CoB+anti-IFNAR was 57.5 days, for CD4-Cre IFNAR-$^{fl/fl}$ treated with CoB was 19 days, and for CD4-Cre IFNAR-$^{fl/fl}$ treated with CoB+anti-IFNAR was 59 days. E) MFI of IFNAR on $CD3^-CD19^-CD11c^-CD11b^+F4/80^-Ly6G^+$ neutrophils from WT, IFNAR-KO, or LysM-Cre $IFNAR^{fl/fl}$ mice. MFI of MHC-I on neutrophils from WT, IFNAR-KO, or LysM-Cre $IFNAR^{fl/fl}$ mice cultured overnight with or without IFN-α. F) The median survival time of WT mice treated with CoB was 22 days, of WT mice treated with CoB+anti-IFNAR was 66 days, and of LysM-Cre $IFNAR^{fl/fl}$ mice treated with CoB was 25 days. ****$p < 0.0001$ by one way ANOVA correcting for multiple comparisons for IFNAR expression. ***$p < 0.001$, ****$P < 0.0001$ by two way ANOVA correcting for multiple comparisons for MHC-I expression. ***$p < 0.001$, ****$p < 0.0001$ by Mantel-Cox log-rank test for skin graft survival.

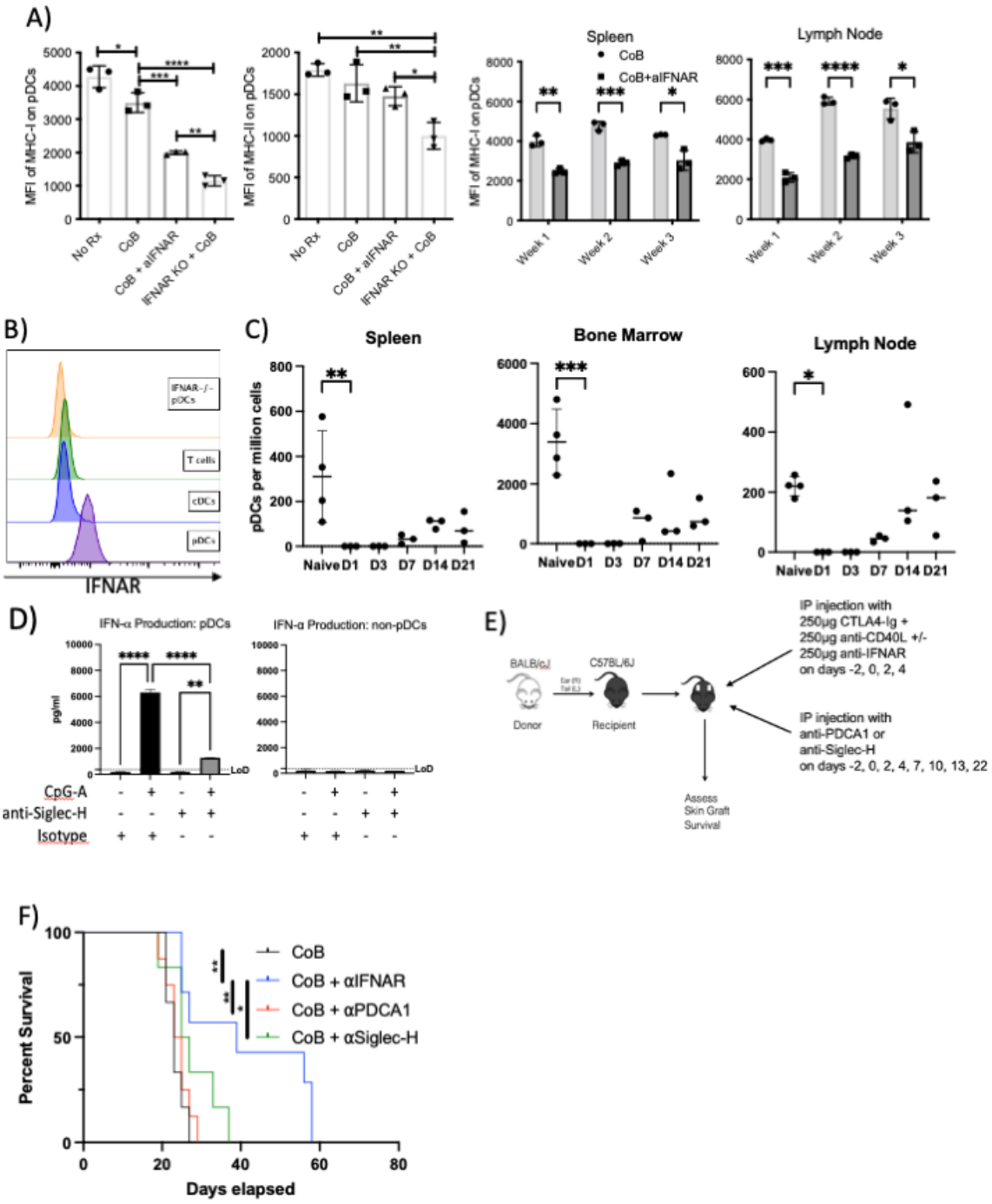

Figure 5: Depletion or inhibition of pDCs is insufficient to prevent costimulation blockade-resistant rejection. A) MFI of MHC-I and MHC-II on $CD3^{-}CD19^{-}CD8a^{-}CD11b^{-}B220^{+}Siglec\text{-}H^{+}PDCA1^{+}$ pDCs in the spleen 10 days post skin transplantation and MFI of MHC-I in the spleen and lymph node 1, 2, & 3 weeks post skin transplantation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,

****P < 0.0001 by one way ANOVA correcting for multiple comparisons. B) Absolute count of pDCs in the spleen, bone marrow, and lymph node in mice 0, 1, 3, 7, 14, or 21 days after administration of anti-pDCA1. *p < 0.05, **p < 0.01, ***p < 0.001 unpaired t-test. C) Production of IFN-α from pDCs (left) and non-pDCs (right) cultured overnight with or without CpG-A, anti-Siglec H, and Isotype control. **p < 0.01, ****P < 0.0001 by one way ANOVA correcting for multiple comparisons. D) pDC depletion experimental design. E) The median survival time of skin grafts for mice treated with CoB was 23 days, for CoB+anti-IFNAR was 39 days, for CoB+anti-PDCA1 was 24 days, and for CoB+anti-Siglec-H was 26 days. *p < 0.05, **p < 0.01 by Mantel-Cox log-rank test.

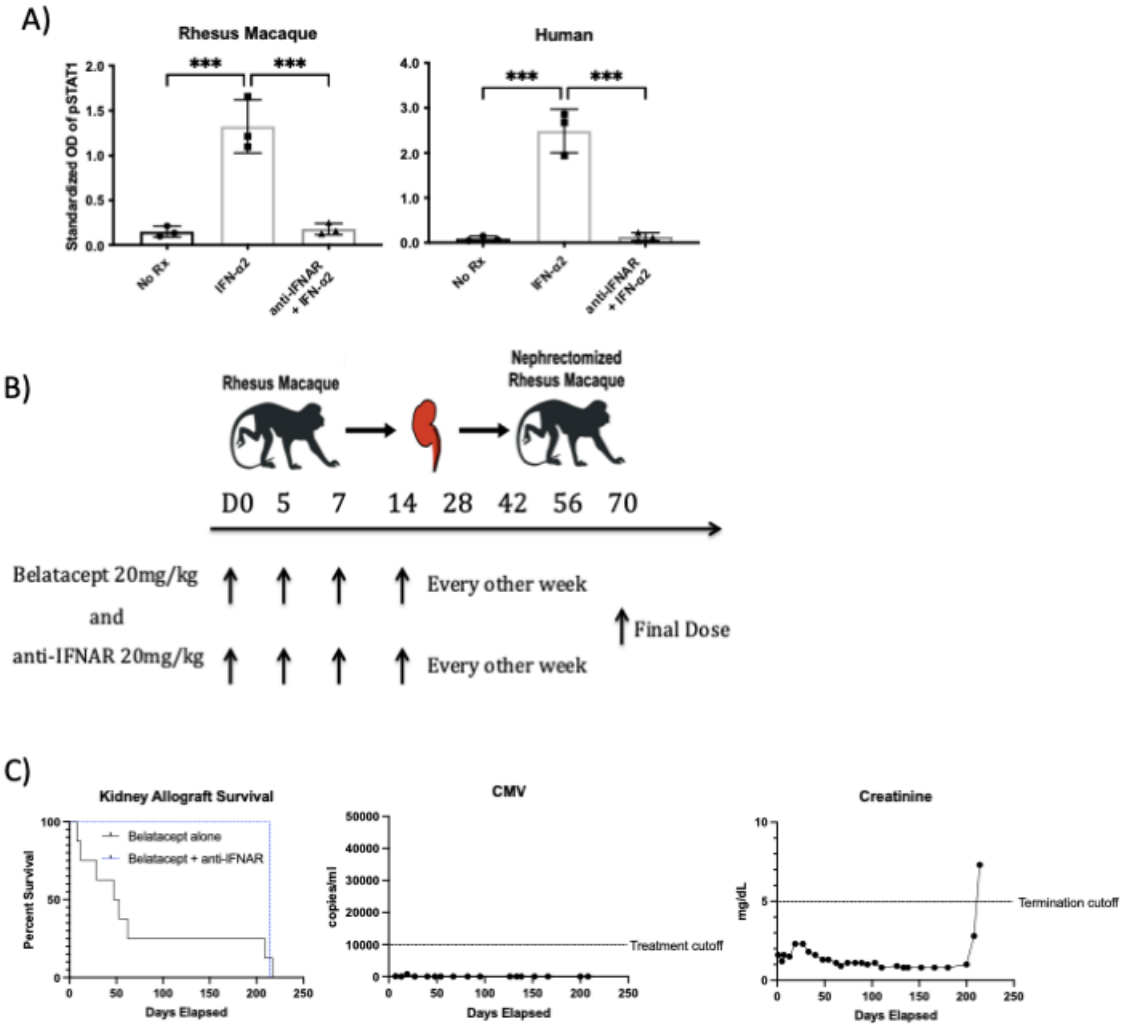

Figure 6: Combined costimulation and IFNAR blockade provides long-term survival in a life-sustaining nonhuman primate renal allotransplantation. A) Quantification of pSTAT1 in rhesus macaque and human PBMCs that were untreated, cultured with IFN-α2, or cultured with IFN-α2 + anti-IFNAR. OD = Optical Density. B) A nephrectomized rhesus macaque received a life-sustaining kidney treated with belatacept (20mg/kg) and anti-IFNAR (20mg/kg) on day 0, 5, 7, 14, and every other week up to day 70. C) The median survival time of Belatacept alone was 50 days and for belatacept + anti-IFNAR was 214 days. The CMV and creatinine levels are reported for the renal transplanted rhesus macaque treated with belatacept + anti-IFNAR. ***$p < 0.001$ by one way ANOVA correcting for multiple comparisons.

Chapter 3:

Selective CD28 Blockade Impacts T Cell Differentiation During Homeostatic Reconstitution Following Lymphodepletion

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Introduction

Belatacept is the first costimulation blockade therapy approved for use in the clinic for renal transplantation (155, 156). It is comprised of a CTLA-4-Ig fusion protein that binds to CD80 and CD86 on antigen presenting cells (APCs), preventing CD28 costimulation on T cells. Costimulation blockade with belatacept results in less nephrotoxicity, better kidney function, and significantly improved long-term graft survival compared to calcineurin inhibitors (157). However, treatment with belatacept is also associated with increased rates and severity of acute rejection (158). One potential cause for this may be due to the lack of CTLA-4 coinhibition, as CTLA-4 on T cells also binds to CD80 and CD86 on APCs but is blocked by belatacept. CTLA-4 functions to suppress T cell responses in both a cell intrinsic and extrinsic manner and is differentially expressed on different T cell subsets (99, 100, 101, 102). CTLA-4 suppresses alloreactive responses in a cell-intrinsic manner on $CD8^+$ memory T cells (159) and Th17 cells, both of which have been shown to drive costimulation blockade resistant rejection (11, 37, 130, 160). On regulatory T cells (Tregs), CTLA-4 provides non-redundant immunosuppressive signals in a cell-extrinsic manner (110). Treg-specific CTLA-4 deficiency in mice leads to spontaneous development of systemic lymphoproliferation and fatal T cell-mediated autoimmune disease (111). Therefore, selectively blocking CD28 while leaving CTLA-4 signaling intact may prove beneficial in a transplant setting.

Recently, selective CD28 blockers such as the anti-CD28 domain antibody (dAb) lulizumab (BMS-931699) and anti-CD28 Fab' FR104 (VEL-101) have been developed (161). These selective CD28 blockers have been shown to be equally able to inhibit CD80-elicited T cell proliferation and five times more potent at inhibiting CD86-elicited T cell proliferation as

compared to belatacept (162). Importantly, Fc-silent non-crosslinking reagents do not elicit cytokine release, unlike the earlier Fc-containing anti-CD28 TGN1412 (163, 164). These reagents may also be more potent against alloimmune responses than belatacept, yet are comparable to belatacept in terms of their impact on $CD8^+$ T cell protective immunity in a murine EBV homolog model (165). Moreover, selective CD28 blockade attenuates $CD8^+$ memory T cell effector function in a CTLA-4-dependent manner during murine skin transplantation (166). In a model of murine cardiac allotransplantation, Zhang et al. (167) demonstrated that selective CD28 blockade attenuated acute and chronic graft rejection which was dependent on the preservation of CTLA-4 signaling. Using a single chain monovalent non-activating reagent to block CD28 signaling (sc28AT), Poirier et al. (168) showed that in nonhuman primate models of renal and cardiac transplantation, selective CD28 blockade synergized with calcineurin inhibitors to improve the frequency and function of Tregs and prolong graft survival. Further, Zhang et al. (169) demonstrated improved cardiac allograft survival and reduced T cell activation in nonhuman primates treated with sc28AT induction followed by anti-CD154 administration. Additionally, FR-104) was shown to prevent acute rejection in nonhuman primate renal allografts, and to promote the accumulation of Tregs in the blood and graft without the need for steroids (161). This reagent has also been shown to be safe for administration in humans (170).

Future clinical trials investigating the efficacy of selective CD28 blockers during renal transplantation will likely include induction therapy via administration of anti-thymocyte globulin (ATG) (171, 172). The impact of selective CD28 blockade on T cell depletion (TCD) and subsequent homeostatic reconstitution of T cells is not known. Prlic et al. (173) found that CD28 signaling was dispensable for the bulk reconstitution of $CD4^+$ and $CD8^+$ T cells as measured by

CFSE proliferation. While informative, this study used CD28-knockout mice as opposed to the selective anti-CD28dAb that is now available. In addition, the impact on specific T cell subsets beyond bulk $CD4^+$ and $CD8^+$ T cell populations, such as regulatory T cells and memory T cell subsets, was not assessed. Thus, here we investigated the impact of selective CD28 blockade on T cell phenotype following TCD and homeostatic reconstitution in a murine model of skin transplantation. We demonstrated that CD28 signaling impacts the differentiation of both $CD4^+$ and $CD8^+$ T cells during homeostatic reconstitution following lymphodepletion, resulting in a shift towards fewer activated memory T cells in lymphoid organs and more $CD8^+Foxp3^+$ T cells in the blood and kidney. This favorable immune profile is accompanied by improved skin graft survival and suggests that selective CD28 blockers could be efficacious at controlling allograft rejection in patients in the context of T cell depletion.

Materials and Methods

Mice

C57BL/6J and BALB/cJ mice were obtained from the National Cancer Institute (Frederick, MD). All animals were maintained in accordance with Emory University Institutional Animal Care and Use Committee guidelines. All animals were housed in specific pathogen-free animal facilities at Emory University.

Skin transplantation and in vivo co-stimulatory molecule blockade

Full-thickness BALB/cJ tail and ear skin grafts were transplanted onto the dorsal thorax of recipient C57BL/6J mice and secured with adhesive bandages as previously described (130). Where indicated, mice were injected with 100μg anti-CD28 dAb (Bristol-Myers Squibb) on days 0, 2, 4, 6, and weekly thereafter until the mice were sacrificed on day 42. For T cell depletion experimental groups, 0.2mg of anti-CD4 (clone GK1.5) and 10μg of anti-CD8 (clone 2.43; both from BioXcell) were administered one day prior to skin transplantation.

Intravascular labeling and tissue processing

Blood was collected weekly to assess T cell phenotype and absolute count. On day 42 prior to organ harvest, mice were intravenously injected with 1.5μg of anti-CD4-BV650 and anti-CD8a-BV650 antibody 2 minutes before euthanasia to label circulating blood cells. The spleen, axillary lymph nodes, bone marrow, kidneys, liver, lungs, and blood were then procured. Kidney and lung samples were chopped and digested for 30 minutes at 37˚C with 2mg/ml Collagenase (type 4, Sigma-Aldrich) and 50μg/ml DNAse (ThermoFisher) in HBSS. Digested lungs were then homogenized, filtered, and washed in FACS buffer (PBS with 2% FBS). Livers were homogenized

manually, filtered through 40μm strainers, and spun lightly at 300rpm to pellet the hepatocytes. The liver supernatant and digested kidneys were resuspended in a 40% Percoll solution, overlaid on 70% Percoll, and spun at 2000rpm for 20 minutes with the brake off. The buffy coats were isolated and washed in FACS buffer. Bone marrow from tibia and fibula were flushed with a 21-gauge needle using PBS and homogenized into single cell suspensions. Spleens and lymph nodes were processed into single cell suspensions, and blood, spleen, and bone marrow were lysed with Fixative-Free Lysing Solution following manufacturer's instructions (Invitrogen). Each tissue was then washed in FACS buffer and stained with antibodies for flow cytometry.

Flow Cytometry

For phenotypic analysis, cells were surface-stained with: TIGIT-BV421, CD103-BV605, CD69-BV711, PD-1-BV786, CD28-FITC, CD49d-PE, CD122-PE-Cy7, DNAM-1 (CD226)-APC, CX3CR1-Alexa700, CD27-APC-Cy7, CTLA-4 (CD152)-BV421, TIM3-BV605, OX40-BV711, CD8-BV786, 2B4 (CD244)-FITC, CD62L-PerCP-Cy5.5, ICOS-PE-Dazzle, CD43-PE-Cy7, CD44-Alexa700, CD25-APC-Cy7 (BioLegend), CD8-PacOrange, CD127-APC (ThermoFisher), CD4-BUV395, CXCR3-BV510 (BD). For Fc staining, cells were incubated with biotinylated CD16/CD32 (2.4G2, BD Biosciences), washed twice, and stained with streptavidin-PerCP-Cy5.5 (BioLegend). For transcription factor staining, cells were permeabilized using a FoxP3/transcription factor kit (Invitrogen) and stained with FoxP3-PE (ThermoFisher). Absolute numbers were calculated using CountBright Absolute Counting Beads according to the manufacturer's instructions (ThermoFisher). Samples were analyzed on an LSRFortessa flow cytometer (BD). Data were analyzed using FlowJo software (Tree Star).

Statistical Analysis

Two-way ANOVA with multiple comparisons was performed when comparing multiple groups. Survival data were plotted on Kaplan-Meier curves, and a log-rank (Mantel-Cox) test was performed. All analyses were done using GraphPad Prism version 9.4.1 for Mac, GraphPad Software, San Diego, California USA. In all legends and figures, mean ± SD is shown, and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.`

Results

Selective CD28 blockade reverses lymphopenia-induced differentiation of memory CD4$^+$ T cells in the spleen and lymph node

To investigate the impact of selective CD28 blockade on immune reconstitution following T cell depletion (TCD) in the setting of transplantation, C57BL/6 mice were randomized to receive either vehicle controls or anti-CD4 and anti-CD8 depleting antibody one day prior to receiving a fully MHC-mismatched BALB/c skin graft. Following transplantation, mice in each group were further randomized to receive either no further treatment or anti-CD28dAb (Figure 1A). Flow cytometric analysis of peripheral blood was used to monitor T cell depletion on the day of transplantation and blood was drawn weekly to monitor the kinetics of T cell reconstitution (Figures 1A-G). Results indicated that across the majority of timepoints, the frequencies of reconstituted CD4$^+$ and CD8$^+$ T cells among CD3$^+$ cells were largely unaffected by CD28 blockade (Figure 1C). The exception to this was the fact that the absolute count of CD4$^+$ T cells was reduced on day 21 post-transplantation in the TCD+anti-CD28dAb group as compared to TCD alone ($p<0.05$), but was no different by day 42, indicating that CD4$^+$ T cell reconstitution is delayed in the absence of CD28 signaling but ultimately not inhibited (Figure 1D). No differences in the reconstitution kinetics of CD8$^+$ T cells between the TCD alone and the TCD+anti-CD28dAb groups were observed (Figure 1E). Interestingly, the absolute count of both CD4$^+$ and CD8$^+$ T cells remained lower in the TCD groups compared to non-depleted groups, regardless of CD28 blockade status (Figures 1D, 1E).

While no differences in the magnitude of the CD4$^+$ (Figure 1D) and CD8$^+$ T cell compartments (Figure 1E) were observed following T cell reconstitution in the presence or absence of selective

CD28 blockade, we next sought to determine if selective CD28 blockade impacted memory T cell phenotype under these conditions. Results indicated that at the earlier timepoint (day 21), TCD resulted in increased frequencies of $CD44^+$ effector/memory T cells within both $CD4^+$ and $CD8^+$ T cell compartments ($p<0.0001$ for both) (Figure 1G). At day 21 for $CD8^+$ T cells, selective CD28 blockade of TCD-treated animals resulted in a significant reduction in the frequency of $CD44^+$ effector/ memory T cells ($p<0.0001$). This finding was also observed within the $CD4^+$ T cell compartment but at the later timepoint (day 42), in that selective CD28 blockade of TCD-treated animals resulted in a significant reduction in the frequency of $CD44^+$ effector/ memory $CD4^+$ T cells in both the spleen ($p=0.021$) and lymph node ($p=0.042$)(Figure 1G). Overall, these results indicate that T cell depletion results in an increased frequency of effector/memory $CD44^+$ T cells within both the $CD4^+$ and $CD8^+$ T cell compartments, an effect which is mitigated by the addition of selective CD28 blockade at d21 in the blood for $CD8^+$ T cells and at d42 in secondary lymphoid organs for $CD4^+$ T cells.

Selective CD28 blockade during T cell lymphodepletion and reconstitution improves skin graft survival and reduces expression of $CD4^+$ T cell activation and senescence markers

To assess the impact of TCD on anti-CD28dab mediated graft survival, full-thickness MHC-mismatched skin grafts were performed as described above and graft survival was monitored for six weeks. While skin grafts in groups treated solely with anti-CD28dAb exhibited increased survival relative to untreated animals (median survival time [MST] 26 days vs. 19 days, $p=0.016$), the combination of TCD+anti-CD28dAb further improved skin graft survival compared to no treatment (MST 37 days, $p=0.006$, Figure 2A). To investigate the combined impact of anti-

CD28dAb and TCD on immune activation, the proportion of $CD69^+$ T cells among $CD4^+$ and $CD8^+$ T cell populations in the spleen and lymph node was examined (Figures 2B, 2C). Results demonstrated a reduced frequency of CD69-expressing $CD4^+$ T cells in the spleen and lymph node in animals treated with anti-CD28dAb versus animals that did not receive the blockade, both in the presence and absence of TCD (Figure 2C). A similar trend was observed within the $CD8^+$ T cell compartment, though in the spleen no differences in CD69-expressing $CD8^+$ T cells were observed between the no treatment group and the anti-CD28dAb treated group (Figure 2C).

Next, to investigate the impact of TCD and selective CD28 blockade on T cell senescence, the expression of the coinhibitory receptors PD-1 and TIGIT were investigated (Figure 2D). In general, anti-CD28dAb treatment reduced the expression of both PD-1 and TIGIT on $CD4^+$ T cells, both in the presence and absence of T cell depletion (Figure 2D). No difference was observed in the expression of PD-1 or TIGIT on $CD8^+$ T cells between any of the groups (data not shown). Taken together, these results suggest that TCD combined with CD28 blockade improves skin graft survival, and that anti-CD28dAb reduces $CD4^+$ T cell activation and expression of the coinhibitory receptors PD-1 and TIGIT.

Selective CD28 blockade reduces the frequency of $CD4^+$ and $CD8^+$ T_{RM} in the kidney in the absence of T cell lymphopenia-induced reconstitution

Given the above results which demonstrate the impact of selective CD28 blockade on the homeostatic reconstitution of circulating memory $CD4^+$ T cells following TCD, the impact of selective CD28 blockade on the tissue resident memory T cell (T_{RM}) compartment was

subsequently investigated, as this subset has been recently identified as a mediator of allograft rejection (174). To distinguish between circulating and tissue resident T cells, 1.5μg of fluorescently-labeled anti-CD4 and anti-CD8 were injected into the tail vein of each animal 2-3 minutes prior to euthanasia. Upon flow cytometric analysis of homogenized kidney, circulating $CD4^+$ and $CD8^+$ T cells stain positively for this IV label, while tissue resident cells are negative (Figure 3A).

Tissue resident memory T cells can be identified by their expression of CD69 with additional subsets, particularly $CD8^+$ T cells, co-expressing CD103 (175, 176, 177, 178, 179, 180, 181). In contrast to the clear impact of TCD and homeostatic reconstitution on the frequencies of circulating memory T cells (Figure 1G), TCD did not significantly impact the frequency of $CD69^+CD103^+$ T_{RM} within either the $CD4^+$ or $CD8^+$ T cell compartment in the kidney. Administration of anti-CD28dAb in the absence of TCD resulted in a significant reduction in the frequency of $CD69^+CD103^+$ $CD4^+$ ($p=0.001$) and $CD8^+$ ($p=0.005$) T cells in the kidney (Figure 3B). In groups that received anti-CD28dAb in the setting of T cell homeostatic reconstitution, there was no difference in the frequency of $CD69^+CD103^+$ among $CD4^+$ or $CD8^+$ T cells compared to groups that received TCD alone (Figure 3B). As $CD69^+CD103^-$ T_{RM} have been described, particularly among $CD4^+$ T cells (182, 183, 184, 185), the frequency of $CD69^+CD103^-$ T_{RM} was further investigated. In the kidney, the frequency of $CD4^+CD69^+CD103^-$ T cells was elevated ($p=0.0103$) in the group that received anti-CD28dAb in the setting of T cell homeostatic reconstitution compared to the group that received TCD alone (Figure 3C). There were no differences in the frequencies of $CD8^+CD69^+CD103^-$ T cells among any of the groups in the kidney (Figure 3C). Moreover, the frequency of PD-1 expressing $CD4^+$ and $CD8^+$ T cells in the kidney

(p=0.0002, p=0.047) were reduced in the groups that received anti-CD28dAb compared to the no treatment groups (Figure 3D).

Selective CD28 blockade reduces the frequency of FoxP3$^+$ CD4$^+$ T cells but increases the frequency of FoxP3$^+$ CD8$^+$ T cells following T cell homeostatic reconstitution

Seminal studies have shown that CD4$^+$FoxP3$^+$ Tregs require CD28 signaling for homeostatic maintenance and survival (186). As such, we sought to determine the impact of selective CD28 blockade on CD4$^+$FoxP3$^+$ Tregs during homeostatic reconstitution following transplantation. Animals treated with anti-CD28dAb exhibited reduced frequencies of FoxP3$^+$ cells among CD4$^+$ T cells in the blood, lymph node, spleen, kidney, liver, and lung (Figure 4A, B), confirming the requirement of CD28 signaling for Treg maintenance.

There has been growing interest in CD8$^+$ FoxP3$^+$ T cells as potential mediators of immunological tolerance (187, 188, 189, 190). Thus, the impact of selective CD28 blockade on this subset was investigated in the setting of transplantation and immune reconstitution. It was found that in contrast to the effect of selective CD28 blockade on CD4$^+$Foxp3$^+$ Tregs, animals treated with anti-CD28dAb in the setting of TCD actually exhibited an increased frequency of CD8$^+$ Foxp3$^+$ T cells in the blood and kidney (Figure 4C, D).

CD8$^+$ FoxP3$^+$T cells exhibit distinct cell surface expression profiles compared to CD4$^+$FoxP3$^+$ T cells in the blood and kidney

The phenotype of $FoxP3^+$ T cells in groups that received TCD and received anti-CD28dAb were further explored by comparing the expression of several cell surface markers on $FoxP3^-$ versus $FoxP3^+$ cells within both the $CD4^+$ and $CD8^+$ T cell compartments (Figure 5A, 5B). In the blood, $CD4^+$ and $CD8^+$ $FoxP3^+$ T cells expressed higher levels of CD25, ICOS, and OX40 compared with $CD4^+$ and $CD8^+$ $FoxP3^-$ T cells. Additionally, CXCR3, CD44, TIM3, and CD43 were more highly expressed on $CD8^+FoxP3^+$ T cells than on $CD8^+FoxP3^-$ T cells or on $CD4^+FoxP3^+$ T cells (Figure 5A). In kidney resident cells, CD25 was more highly expressed on $CD8^+FoxP3^+$ T cells compared to $CD8^+FoxP3^-$ and $CD4^+FoxP3^+$ T cells. ICOS was elevated on $CD4^+FoxP3^+$ and $CD8^+FoxP3^+$ T cells compared to $CD4^+FoxP3^-$ and $CD8^+FoxP3^-$ T cells. CXCR3 was elevated on $CD8^+$ $FoxP3^+$ T cells compared to $CD8^+FoxP3^-$ T cells ($p=0.020$). CD44 expression was decreased on $CD4^+FoxP3^+$ T cells compared to $CD4^+FoxP3^-$ T cells ($p=0.020$) and was further decreased on $CD8^+$ $FoxP3^-$ T cells compared to $CD4^+FoxP3^-$ T cells ($p=0.001$). There was no difference in the expression of OX40, TIM3, or CD43 on $FoxP3^-$ vs $FoxP3^+$ T cells in the kidney. CTLA-4 was elevated on $CD4^+$ $FoxP3^+$ and $CD8^+$ $FoxP3^+$ T cells compared to $CD4^+$ $FoxP3^-$ and $CD8^+$ $FoxP3^-$ T cells. (Figure 5B).

Discussion

The aim of this study was to understand the effect of CD28 blockade on the immune system during TCD and immune reconstitution in the setting of transplantation. The role of CD28 on T cell homeostatic proliferation has been investigated in the past by Prlic et al. (34). Using CD28 knockoout mice, their group revealed that CD28 costimulation was dispensible for homeostatic expansion of $CD4^+$ and $CD8^+$ T cells (173). Results from our study are largely consistant with this finding, though we did observe a small impact of CD28 blockade on the early kinetics of CD4 T cell reconstitution which may be due to the fact that we tracked the absolute count of T cells rather than the number of divisions (Figure 1D). It has also been established that TCD induces a transient memory-like phenotype in naïve T cells, marked by upregulation of CD44 (191, 192, 193). Our study reveals that CD28 blockade prevents homeostatically proliferated T cells from acquring this $CD44^+$ memory phenotype, which might help to prevent allorejection (194).

ATG induction therapy is routinely used in the clinic to deplete donor-reactive immune cells. While in this study we utilize antibodies directed against $CD4^+$ and $CD8^+$ T cells to investigate the effect of TCD and homeostatic reconstitution, ATG is known to have more widespread effects on the immune system. Beyond TCD, ATG induces the apoptosis of B cells, modulates surface molecules that mediate leukocyte interactions, and induces the expansion of Tregs *ex vivo* (195). In mice, naïve T cells are more susceptible to ATG-mediated depletion than memory and effector T cells, with $CD4^+$ T cells being more resistant to depletion than $CD8^+$ T cells (196, 197). Therefore, it is possible that ATG induction therapy could influence the immune landscape during CD28 blockade in a different manner than anti-CD4/ anti-CD8-mediated depletion.

It has been well established that $CD4^+FoxP3^+$ Tregs require CD28 signaling for thymic development and homeostatic maintenance. While we found that the frequency of $CD4^+FoxP3^+$ Tregs was reduced in the anti-CD28dAb treated groups, it has been previously demonstrated that these Tregs are still able to effectively function through preserved CTLA-4 signaling (110, 198). We show that in the kidney, $CD4^+$ and $CD8^+$ $FoxP3^+$ T cells express greater amounts of CTLA-4 than $CD4^+$ and $CD8^+$ $FoxP3^-$ T cells, so it may follow that they are also governed by CD28 signaling. Nonobese diabetic (NOD) mice that lack CD28 signaling are deficient in $CD4^+$ Tregs which results in exacerbated onset of spontaneous diabetes (199). Tang et al. (200) demonstrated that CD28 signaling affects $CD4^+$ Treg homeostasis both directly by upregulating CD25 expression on these cells and indirectly by inducing IL-2 production by conventional T cells , which in turn provides survival signals to Tregs. Furthermore, studies utilizing CTLA-4-Ig (abatacept) to treat type 1 diabetes and rheumatoid arthritis have shown a reduction in the frequency of $CD4^+$ Tregs in these patients (201, 202). Our findings in the $CD4^+Foxp3^+$ Treg compartment are consistent with these results. However, the requirement for CD28 signaling on $CD8^+Foxp3^+$ T cells has not been well studied, and our results suggest that in contrast to $CD4^+$ Tregs, $CD8^+$ $Foxp3^+$ T cells exhibit a reduced requirement for CD28 signaling and are therefore not negatively impacted by CD28 blockade in vivo in the setting of transplantation.

Not only did we find that CD28 blockade did not inhibit the survival of $CD8^+$ Tregs, instead, in the context of TCD, CD28 blockade actually promoted the expansion or accumulation of this population. Previous studies using RNA-silencing experiments have shown that $CD8^+Foxp3^+$ T cells exhibit suppressive function; specifically that FOXP3-knock down on $CD8^+$ T cells significantly reduces their ability to suppress both $CD4^+$ T cell proliferation and the production of

autoantibodies (203). Moreover, previous reports have shown that $CD8^+FoxP3^+$ T cells express CD25, CTLA-4, and ICOS (188, 204, 205, 206, 207), findings which are supported by our data in the context of TCD and transplantation. These surface receptors have been shown to contribute to the functional suppressive activity of both $CD4^+$ and $CD8^+$ regulatory T cells (188, 204, 208, 209, 210). For example, one study found that $CD8^+FoxP3^+$ T cells inhibit the upregulation of costimulatory molecules on dendritic cells, suppress $CD4^+$ and $CD8^+$ T cell proliferation, and, following adoptive transfer, protect full MHC mismatched skin allografts from rejection (188). Moreover, $CD8^+FoxP3^+$ T cells have been shown to be induced to differentiate from $CD8^+FoxP3^-$ T cells via exposure to TGF-β and TCR stimulation (206). While $CD8^+FoxP3^+$ cells are typically present at frequencies much lower than their $CD4^+FoxP3^+$ counterparts, a previous study reported that frequencies of $CD8^+FoxP3^+$ T cells were increased in RA patients treated with T cell depletional therapy (204), a finding which is in line with our results. Whether the increased frequency of $CD8^+FoxP3^+$ T cells that we observe in the blood and kidney during CD28 blockade following TCD and homeostatic reconstitution are induced pharmacologically or are naturally occurring, and the specific suppressive capacity of these cells in this setting, remains to be elucidated.

One population of T cells that may be differently impacted by TCD during selective CD28 blockade is T_{RM}. These cells are characterized by their expression of CD69 and CD103 (175, 176), and it has been shown that lymphopenia-induced naïve T cells can traffic to tissues and differentiate into memory T cells (191, 211). T_{RM} have been identified as key mediators of allograft rejection (197) and it has been shown in a murine kidney transplant model that treatment with cyclosporine does not prevent $CD8^+$ T cells from acquiring a T_{RM} phenotype (174). In human

transplant nephrectomies, both donor and recipient derived T_{RM} have been found in transplanted kidneys, with donor derived T_{RM} found in early acutely rejected allografts and recipient derived T_{RM} found in later chronically rejected kidney allografts (212). Another study found the PD-1/PD-L1 pathway to be important for regulating the effector function of T_{RM} in the human pancreas (213). In the kidney we found that anti-CD28dAb alone reduced the frequency of resident PD-1^+ $CD4^+$ and $CD8^+$ T cells compared to no treatment, but not following homeostatic reconstitution. This preserved PD-1 expression suggests that kidney resident $CD4^+$ and $CD8^+$ T cells may be more susceptible to immune regulation via PD-L1 during TCD+CD28dAb. While we observed that selective CD28 blockade alone reduced the frequency of $CD4^+$ and $CD8^+$ T_{RM} in the kidney compared to no treatment, the fact that this was not seen following T cell reconstitution may indicate a reduced susceptibility of this compartment to the effects of CD28dAb.

Taken together, these data support the utilization of combined TCD and selective CD28 blockade to prevent allograft rejection. The emergence of a population of $CD8^+FoxP3^+$ T cells with a cell surface expression profile distinct from $CD4^+FoxP3^+$ T cells might have implications in transplantation and warrants further investigation.

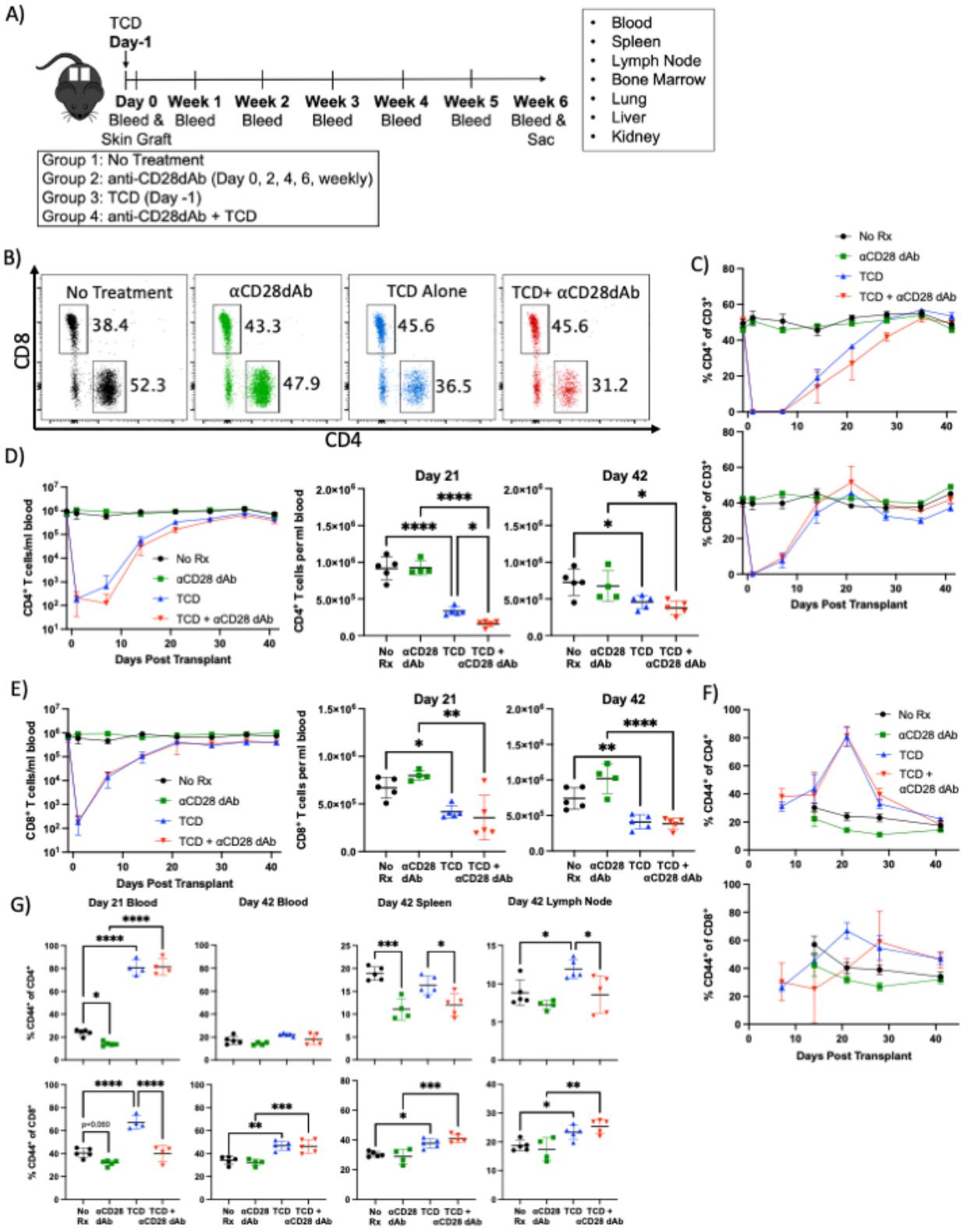
A)
TCD
Day-1
Day 0 Bleed & Skin Graft
Week 1 Bleed
Week 2 Bleed
Week 3 Bleed
Week 4 Bleed
Week 5 Bleed
Week 6 Bleed & Sac
Blood
Spleen
Lymph Node
Bone Marrow
Lung
Liver
Kidney
Group 1: No Treatment
Group 2: anti-CD28dAb (Day 0, 2, 4, 6, weekly)
Group 3: TCD (Day -1)
Group 4: anti-CD28dAb + TCD
B)
No Treatment
αCD28dAb
TCD Alone
TCD+ αCD28dAb
CD8
CD4
38.4
52.3
43.3
47.9
45.6
36.5
45.6
31.2
C)
No Rx
αCD28 dAb
TCD
TCD + αCD28 dAb
% CD4+ of CD3+
% CD8+ of CD3+
Days Post Transplant
D)
CD4+ T cells/ml blood
Days Post Transplant
CD4+ T cells per ml blood
Day 21
Day 42
E)
CD8+ T cells/ml blood
Days Post Transplant
CD8+ T cells per ml blood
Day 21
Day 42
F)
% CD44+ of CD4+
% CD44+ of CD8+
Days Post Transplant
G)
Day 21 Blood
Day 42 Blood
Day 42 Spleen
Day 42 Lymph Node
% CD44+ of CD4+
% CD44+ of CD8+
p=0.060

Figure 1. Selective CD28 blockade reverses lymphopenia-induced differentiation of memory CD4$^+$ T cells in the lymph node. A) Fully MHC-mismatched skin from BALB/c donors were grafted onto C57BL/6 recipients. Recipients were treated with or without 0.2mg anti-CD4 and 10μg anti-CD8 one day prior to transplant and with or without 100μg anti-CD28dAb on days 0, 2, 4, 6, and weekly thereafter. Mice were euthanized six weeks post-transplant and lymphoid cells from the blood, lymph node, spleen, bone marrow, kidney, liver, and lung were analyzed by flow cytometry. Immediately prior to euthanasia fluorescently labeled anti-CD4 and anti-CD8 antibody were administered IV to label circulating T cells. B) Representative flow cytometry plots of CD4$^+$ and CD8$^+$ T cells on day 21 in the blood. C) Frequency of CD4$^+$ and CD8$^+$ T cells in the blood over time. D) Left, absolute count of CD4$^+$ T cells per mL of blood over time. Right, absolute count of CD4$^+$ T cells at day 21 and day 42. E) Left, absolute count of CD8$^+$ T cells per mL of blood over time. Right, absolute count of CD8$^+$ T cells at day 21 and Day 42. F) Proportion of CD44 expressing CD4$^+$ and CD8$^+$ T cells in the blood over time. G) Proportion of CD44 expressing CD4$^+$ and CD8$^+$ T cells in the blood at day 21 and in the blood, spleen, and lymph node at day 42. Experiment shown is representative of 2 independent experiments with a total of 8-10 mice per group. ***$p < 0.001$, by one-way ANOVA correcting for multiple comparisons.

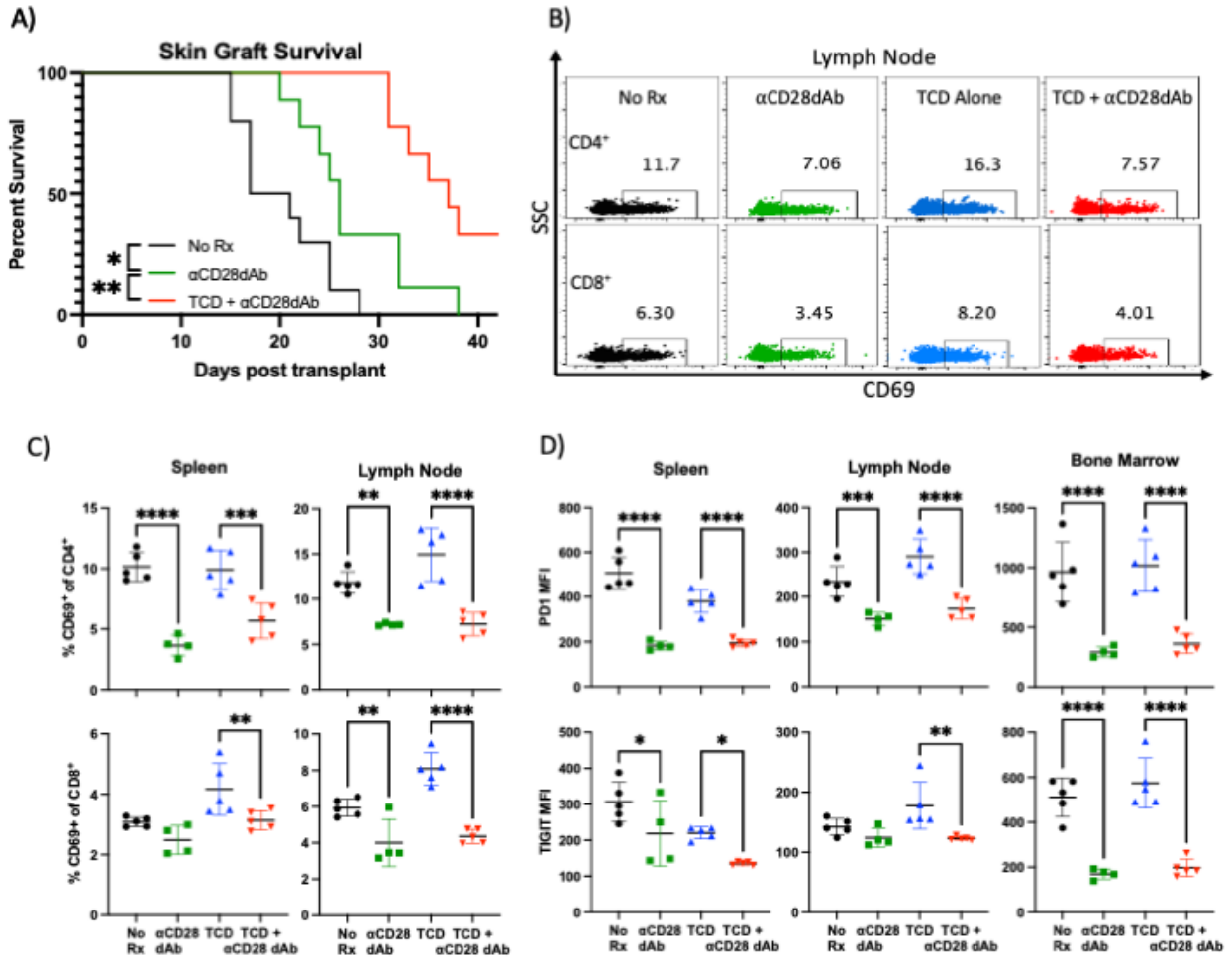

Figure 2. Selective CD28 blockade during T cell lymphodepletion and reconstitution improves skin graft survival and reduces expression of CD4$^+$ T cell activation and senescence markers. A) Median survival time of skin grafts was 19 days with no treatment (No Rx), 26 days for CD28dAb alone, and 37 days for CD28dAb+TCD. *p<0.05, **p < 0.01 by Mantel-Cox log-rank test. B) Representative flow cytometric data depicting the frequency of CD69$^+$CD4$^+$ and CD69$^+$CD8$^+$ T cells in the lymph node at day 42. C) Frequency of CD69$^+$ CD4$^+$ and CD8$^+$ T cells in the spleen and lymph node at day 42. D) MFI of PD-1 and TIGIT on CD4$^+$ T cells in the spleen, lymph node, and bone marrow at day 42. Experiment shown is representative of 2 independent

experiments with a total of 8-10 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by one way ANOVA correcting for multiple comparisons.

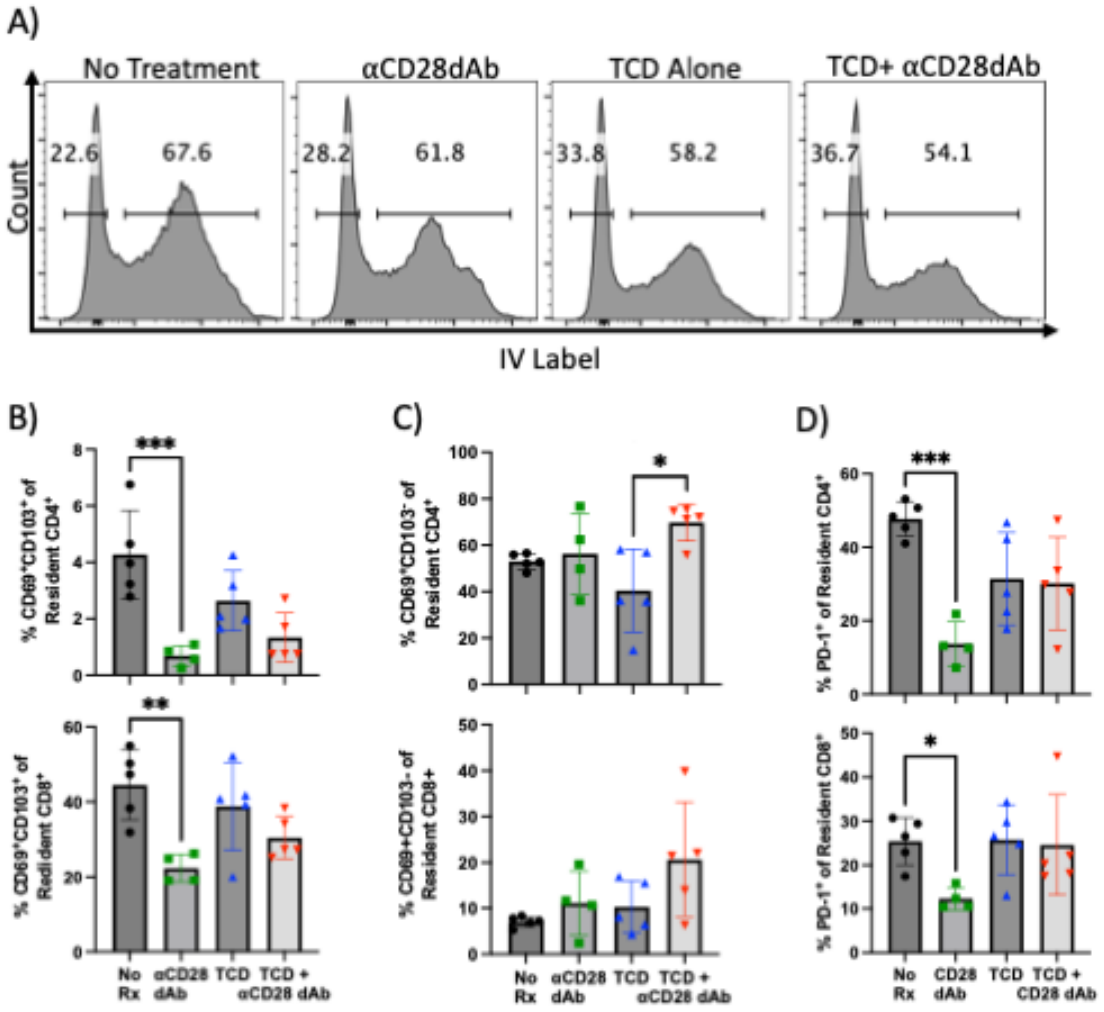

Figure 3. Selective CD28 blockade reduces the frequency of $CD4^+$ and $CD8^+$ T_{RM} in the kidney but not during T cell lymphopenia-induced reconstitution. A) Flow cytometric gating strategy to identify tissue resident T cells in the kidney. B) Frequency of tissue resident $CD69^+CD103^+$ $CD4^+$ and $CD8^+$ T cells in the kidney at day 42. C) Frequency of tissue resident $CD69^+CD103^-$ $CD4^+$ and $CD8^+$ T cells in the kidney at day 42. D) Frequency of $PD\text{-}1^+$ resident $CD4^+$ and $CD8^+$ T cells in the kidney day 42. N=3-5 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, by one way ANOVA correcting for multiple comparisons.

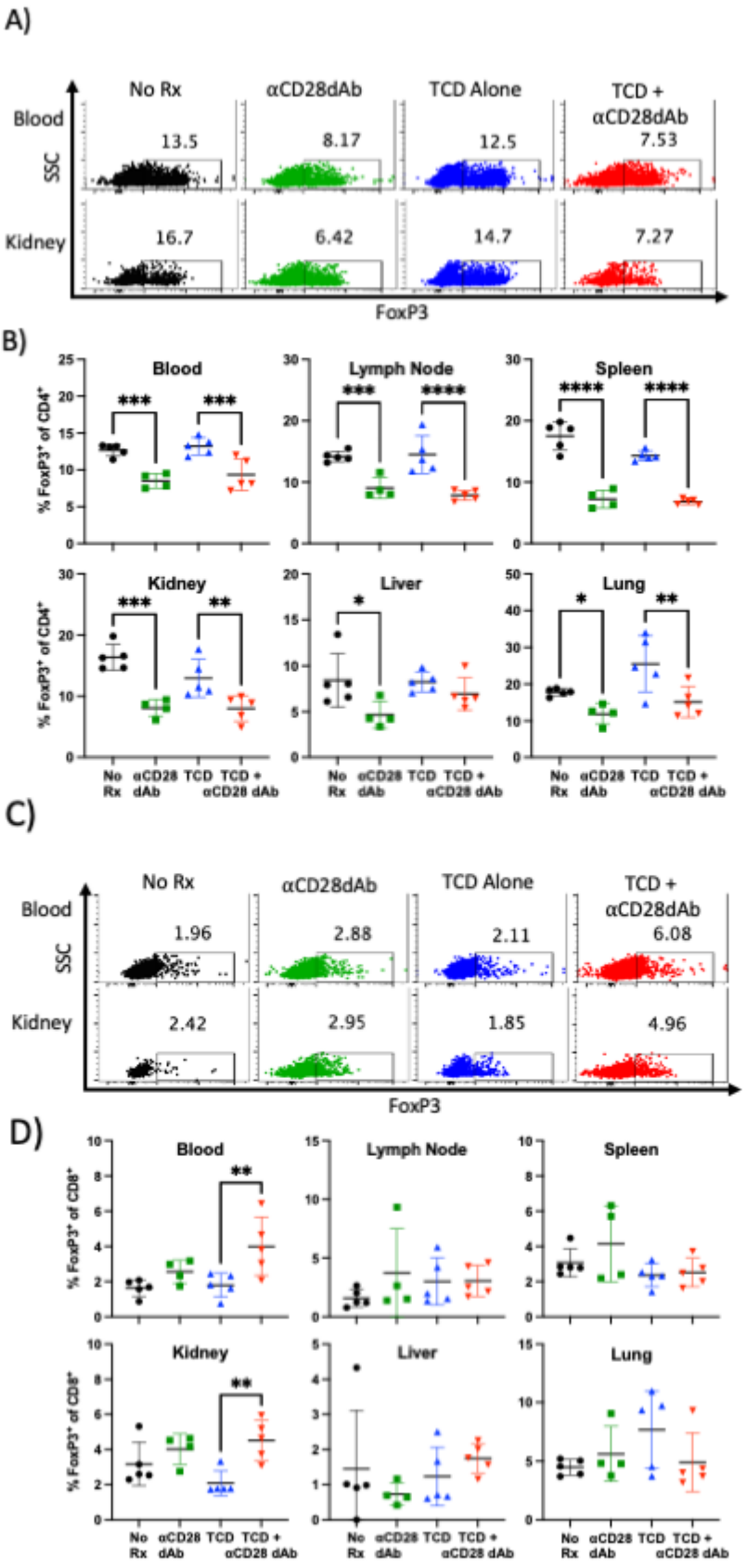
A)
No Rx
αCD28dAb
TCD Alone
TCD + αCD28dAb
Blood
Kidney
SSC
13.5
8.17
12.5
7.53
16.7
6.42
14.7
7.27
FoxP3
B)
Blood
Lymph Node
Spleen
Kidney
Liver
Lung
% FoxP3+ of CD4+
No Rx
αCD28 dAb
TCD
TCD + αCD28 dAb
C)
1.96
2.88
2.11
6.08
2.42
2.95
1.85
4.96
D)
% FoxP3+ of CD8+

Figure 4. Selective CD28 blockade reduces the frequency of FoxP3$^+$ CD4$^+$ T cells but increases the frequency of FoxP3$^+$ CD8$^+$ T cells following T cell homeostatic reconstitution. A) Representative flow cytometric data depicting the frequency of FoxP3$^+$CD4$^+$ T cells in the blood and kidney at day 42 B) Frequency of FoxP3$^+$CD4$^+$ T cells in the blood, lymph node, spleen, kidney, liver, and lung at day 42. C) Representative flow cytometric data depicting the frequency of FoxP3$^+$CD8$^+$ T cells in the blood and kidney at day 42. C) Frequency of FoxP3$^+$CD8$^+$ T cells in the blood, lymph node, spleen, kidney, liver, and lung at day 42. Experiment shown is representative of 2 independent experiments with a total of 8-10 mice per group. *p< 0.05, **p < 0.01, ***p< 0.001, by one way ANOVA correcting for multiple comparisons.

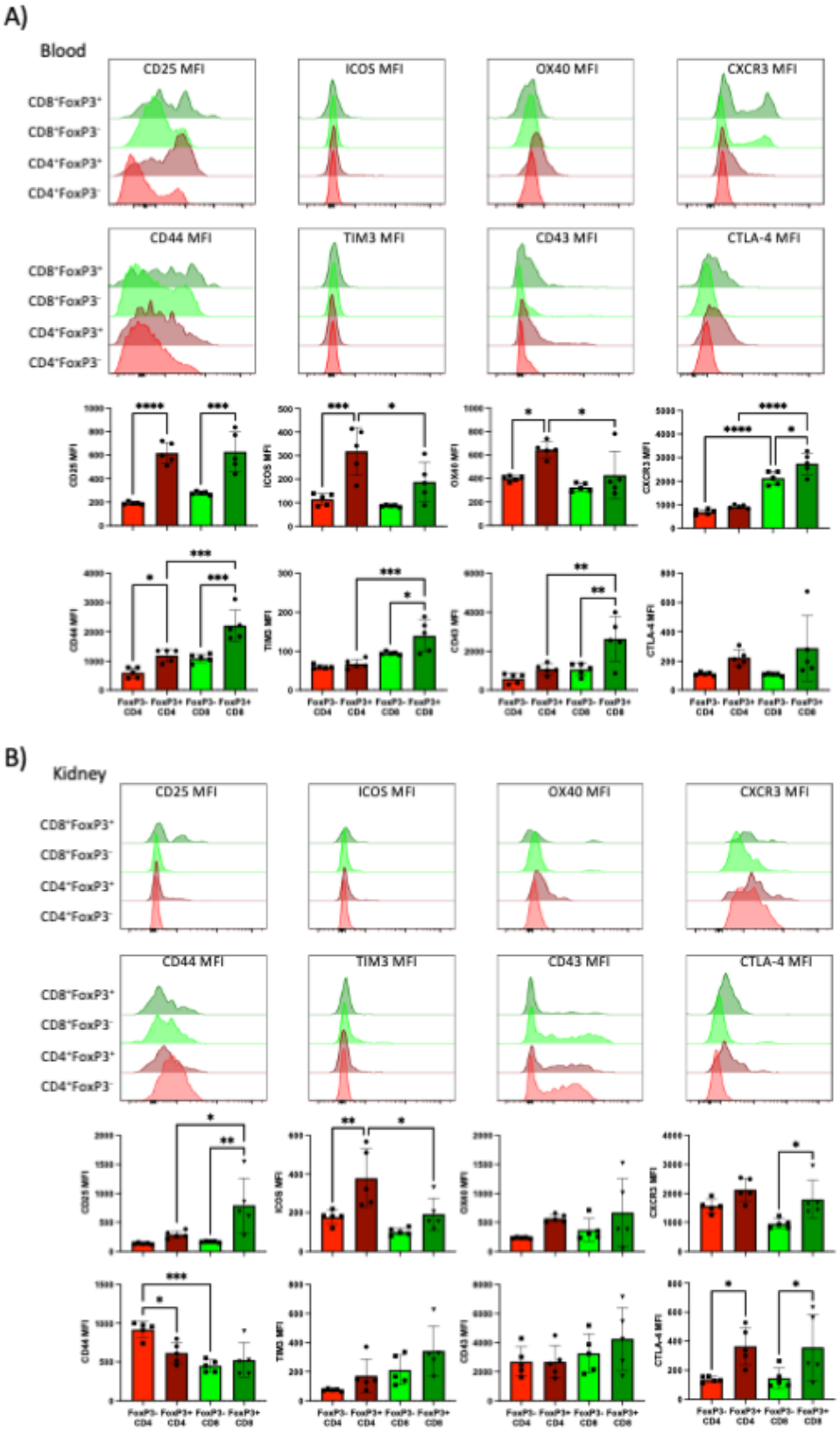
A)
Blood
CD25 MFI
ICOS MFI
OX40 MFI
CXCR3 MFI
CD44 MFI
TIM3 MFI
CD43 MFI
CTLA-4 MFI
CD8+FoxP3+
CD8+FoxP3-
CD4+FoxP3+
CD4+FoxP3-
FoxP3- CD4
FoxP3+ CD4
FoxP3- CD8
FoxP3+ CD8
B)
Kidney

Figure 5. FoxP3$^+$CD8$^+$ T cells exhibit distinct cell surface expression profiles compared to FoxP3$^+$CD4$^+$ T cells in the blood and kidney. A) Representative flow cytometric data of the indicated cell surface proteins expressed on total CD4$^+$ and CD8$^+$ T cells in the blood stratified by FoxP3 expression. B) Representative flow cytometric data of the indicated cell surface proteins expressed on tissue resident CD4$^+$ and CD8$^+$ T cells in the kidney stratified by FoxP3 expression. Experiment shown is representative of 2 independent experiments with a total of 8-10 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****P < 0.0001 by one way ANOVA correcting for multiple comparisons.

Chapter 4: Discussion

Costimulation blockade has the potential to be a gamechanger in the field of transplantation. Renal transplant patients receiving this therapy experience improved graft survival and renal function with fewer adverse side effects compared to therapy with standard of care medicine such as cyclosporine (9). However, the greater incidence of acute rejection in belatacept patients has tempered widespread adoption of this therapy (8, 10). While many groups have investigated the cause behind costimulation blockade-resistant rejection, in this dissertation we propose two mechanisms by which CoB-RR may be alleviated. In Chapter Two we identify IFNAR signaling as a contributor to CoB-RR and investigate how blocking it pharmacologically or genetically modulating it on specific cell subsets in mice impacts graft survival. In Chapter Three we utilize a selective CD28 blocking antibody in the setting of T cell depletion in order to investigate the impact that preserved CTLA-4 signaling has on skin graft survival during homeostatic reconstitution. While we have demonstrated the efficacy of these therapies administered independently in animal models, in this discussion we will hypothesize how these therapies could be further adapted for use in the clinic.

Comparing the effects of IFN-α and IFN-β on the immune system

It has been over sixty years since Isaacs and Lindenmann first characterized interferons (40, 42), and since then, twelve IFN-α subtypes but only one IFN-β subtype has been identified in humans (214, 215). Interestingly, the multiple IFN-α subtypes likely evolved from IFN-β over 100 million years ago as a result of gene duplication and gene conversion (216). The genes encoding IFN-α subtypes share over 70% homology with each other but only 35% with IFN-β (217). Both subtypes of IFNs signal through IFNAR, but IFN-β binds with a higher affinity, approximately 50

times stronger, than any IFN-α subtype (215, 218, 219). Despite differences in amino acid composition, IFN-α and IFN-β are structurally quite similar, consisting of five alpha helices joined together by loops with specific helices binding to particular sites on IFNAR1 and IFNAR2 (215). Mutational analysis revealed that inserting a charged residue into two positions of one of the helices in IFN-β resulted in a mutated IFN-β that was more similar in activity to IFN-α (220). In general, IFN-α is produced by hematopoietic cells, in particular pDCs, whereas IFN-β can be produced by most cell types in response to PRR binding, such as during a viral infection (221). The reagent used in Chapter 2 binds to IFNAR1 and prevents all downstream type I IFN signaling. While these experiments did not determine which subtype of type I IFN was contributing to CoB-RR, several groups have identified markedly different immunological effects between IFN-α and IFN-β signaling. Therefore, elucidating the differences between IFN-α and IFN-β could help to improve our proposed treatment regimen.

Both IFN-α and IFN-β can exert a wide range of effects on the immune system in a temporal and location dependent manner. Deconvoluting the specific impact of either subtype on the immune system is challenging as many investigators do not differentiate between different IFN types, grouping them simply as type I IFNs. Additionally, a large portion of the literature surrounding type I IFNs is centered on their role in particular diseases, further complicating the task of determining how IFN-α signaling differs from IFN-β, especially in the context of transplantation treated with CoB. During a chronic LVMC infection, Ng et al. found that IFNAR blockade promoted viral clearance (222). They determined that IFN-α controls early viral dissemination whereas IFN-β promotes viral persistence, and that blocking IFN-β improved T cell responses and hastened viral clearance (222). Type I IFN can promote DC licensing and cross-presentation of extracellular antigen to $CD8^+$ T cells (41, 223, 224), and Le Bon et al. demonstrated

that IFN-α administration prolonged the duration of the proliferation and expansion of antigen-specific $CD8^+$ T cells in mice (225). Conversely, IFN-β appears to have a more regulatory effect on T cells. Using IFN-$\beta^{-/-}$ mice, Deonarain et al. found that T cells lacking IFN-β exhibited enhanced proliferation compared to wildtype mice (226). These mice also exhibited underdeveloped splenic architecture, suggesting a role for IFN-β during the development of secondary lymphoid organs (226). On human glioma cells cultured *in vitro*, IFN-β inhibited growth by 50% but no grown inhibition was observed following IFN-α administration (227).

Regulatory T cells are also differently affected by IFN-β compared to IFN-α. Bacher et al. found that IFN-α inhibited the suppressive function of Tregs via downregulation of cAMP (228) and Yan et al. identified IFN-α as a mediator of Treg dysfunction in SLE (229). On the other hand, IFN-β has been shown to induce the proliferation of Tregs (230). Recently in the field of transplantation, IFN-β has been shown to synergize with CTLA-4-Ig to induce Treg differentiation and promote murine heart allograft survival (144).

In multiple sclerosis (MS), IFN-β was the first available therapy to treat the disease and is still used to this day (231), whereas IFN-α has been implicated as a potential driver of autoimmunity (232). MS is complex and the specific etiology is not fully understood, varying from patient to patient. It is mediated by autoreactive lymphocytes that chronically damage the central nervous system, resulting in demyelination (233). The disease pathogenesis has been linked to both T and B cells (234), with regulatory T cells (235) and even regulatory B cells (236, 237) potentially playing a role in disease suppression. One way that IFN-β administration therapeutically functions in MS is by the downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines, likely mediated by regulatory T cells (230, 232, 238, 239).

In the field of transplantation, IFN-α was identified as a mediator of acute renal allograft rejection as early as 1984 (240), and multiple studies since have further confirmed this finding (120, 241). A similar effect was observed in liver transplant recipients administered IFN to treat hepatitis C (121). More recently, Herr et al. found that the proliferation of belatacept resistant $CD4^{+}CD57^{+}PD1^{-}$ T cells requires early IFN-α pathway activation as determined by IRF7 expression (242). Lastly, as mentioned above, Fueyo-González et al. demonstrated that IFN-β promotes allograft survival during treatment with CTLA-4-Ig via the induction of Tregs (144). With these ideas in mind, the question arises as to how we could improve our current experimental therapy by taking advantage of the different effect that IFN-β has on the immune system compared to IFN-α.

Shifting IFNAR signaling from IFN-α to IFN-β

A strategy by which anti-IFNAR therapy could be improved such that IFN-α signaling is inhibited but IFN-β signaling could still happen entails utilizing an antibody directed specifically against IFN-α. Two reagents have been developed that could be utilized in this regard: sifalimumab and rontalizumab. Both are monoclonal antibodies that bind to most IFN-α subtypes with high affinity, but not IFN-β or other IFN subtypes (243, 244). Studies into the safety and efficacy of sifalimumab reported minimal adverse events compared to placebo and found a significant reduction in IFN gene signatures (IFNGS) in patients with SLE treated with this drug (245). Another study evaluating the effect of sifalimumab in dermatomyositis and polymyositis patients found reduced IFNGS in blood and muscle samples post-treatment (246), and a follow up study in patients with these diseases showed reduced T cell infiltration in the muscle when treated with sifalimumab (247). Similarly, rontalizumab has been shown to be safe for use in humans and

reduced the expression of IFN-regulated genes in patients with SLE (248). However, results from clinical trials utilizing these reagents were generally lackluster (249), resulting in the discontinuation of both therapies in favor of anifrolumab, which is the anti-IFNAR reagent used in the NHP pilot experiment in Chapter 2. By antagonizing IFNAR, anifrolumab prevents all type I IFN signaling and has proven more efficacious than anti-IFN-α reagents for the treatment of SLE (249, 250). However, as our goal is not to prevent all IFN signaling but rather shift the axis of IFN signaling from alpha towards beta, we propose utilizing an anti-IFN-α antibody in combination with the administration of IFN-β to prevent CoB-RR.

Augmentation of pDCs to prevent IFN-α production

A further method to modulate IFN signaling could be to diminish the ability of pDCs to produce IFN-α. This cell subset is well known for producing large amounts of IFN-α following activation via recognition of viral PRRs but has also been found to be tolerogenic by multiple groups (125, 251, 252). The mechanism behind pDC tolerogenicity is not fully understood but Ochando et al. determined that in mice, tolerizing pDCs acquire alloantigen from the vascularized cardiac allograft and then induce the generation of Tregs within peripheral lymph nodes (253). In order to maintain pDC tolerogenicity but prevent their ability to produce IFN-α in humans, an antibody directed against BDCA2 could be utilized. This monoclonal antibody has a similar effect on human pDCs as anti-Siglec-H has in murine pDCs, as described in Chapter 2, whereby its engagement inhibits the production of type I IFN by pDCs (254, 255, 256, 257). In humans, BDCA2 is exclusively expressed on pDCs and the ligation of this receptor rapidly induces its internalization which subsequently inhibits TLR7 and TLR9-induced type I IFN production without depleting pDCs (258). Two phase II clinical trials have recently concluded that tested the

safety and efficacy of an anti-BDCA2 antibody called litifilimab in lupus patients. Both trials found that the drug was superior to placebo and was largely well tolerated, though a few adverse viral infections did occur in both studies (259, 260). Inhibiting the production of IFN-α by pDCs while avoiding their depletion could shift the overall balance of this cell subset to provide more tolerogenic signals and might synergize with additional immunomodulatory therapies to prevent CoB-RR, discussed further below.

Hypothetical effect of combined therapies

Taking everything into account, we will now hypothesize how these combined therapies might impact human renal transplantation. We will do this by piecing together the impact of ATG induction followed by anti-BDCA2, anti-IFN-α, and IFN-β administration during anti-CD28dAb costimulation blockade. As discussed in chapter 3, ATG administration is a powerful tool to diminish the frequency of alloreactive or cross-reactive lymphocytes that have the potential to induce acute rejection (261, 262, 263). ATG not only induces the apoptosis of T cells and B cells but can also deplete neutrophils, monocytes, and NK cells depending on its composition (264). $CD4^+$ T cells are more resistant to depletion by ATG than $CD8^+$ T cells (196, 197) and this therapy may even induce the expansion of Tregs (195). While lymphopenia-mediated homeostatic proliferation has been shown to induce a transient memory-like phenotype in naïve T cells (191, 192, 193), our study revealed that selective CD28 blockade prevents T cells from acquiring this phenotype to some degree, as measured by CD44 expression, which might help to prevent CoB-RR (194).

The impact of our proposed treatment regimen on Tregs is an important factor to consider. Our results in Chapter 3, as well as other groups, have demonstrated that Tregs require CD28

signaling for thymic development and homeostatic maintenance (199, 200, 201, 202). While we observe a reduced frequency of Tregs in anti-CD28dAb treated mice, the preserved CTLA-4 signaling provided by this reagent as opposed to CTLA-4-Ig potentially enhances the suppressive function of these cells (110, 198). We anticipate this to translate into the clinic and to synergize with IFN-β administration, which has been demonstrated to induce the generation of Tregs, as described above. In addition, maintaining an inhibited population of pDCs that cannot produce IFN-α via anti-BDCA2 and neutralizing any IFN-α produced by other cell types would be expected to further induce Treg differentiation and enhance Treg function (125, 228, 229). The increased frequency of $CD8^{+}FoxP3^{+}$ T cells in the blood and kidney of TCD + anti-CD28dAb treated mice might also translate to the clinic and impact graft survival. However, this effect was observed in the recipient's kidney during skin transplantation and it has not been explored whether this cell subset would also emerge in a transplanted kidney. The impact that IFN-β administration might have on $CD8^{+}FoxP3^{+}$ T cells is also unknown.

Memory T cells also warrant discussion since these cells have been implicated as mediators of CoB-RR (197, 265). Compared to naïve T cells, memory T cells generally possess a reduced requirement for CD28 signaling and are therefore more susceptible to activation in the setting of CoB (266). By administering anti-CD28dAb instead of CTLA-4-Ig, these cells would be able to receive coinhibitory signals through CTLA-4 which has been shown by our group to reduce the effector function of memory $CD8^{+}$ T cells (166). The specific impact that IFN-α versus IFN-β has on memory T cell generation and activation is not clear, as most studies investigating this utilize $IFNAR^{-/-}$ mice and as such all type I IFN signaling is prevented. While it is clear that type I IFN is critical for the generation of memory $CD8^{+}$ T cells during a viral infection (71), Zafranskaya et al. found that IFN-β administration in MS patients resulted in a reduced frequency of autoreactive,

proinflammatory circulating memory $CD8^+$ T cells (267). This suggests that during our hypothetical treatment regimen, IFN-β administration and IFN-α neutralization may reduce the frequency of memory $CD8^+$ T cells and positively impact graft survival.

Overimmunosuppression and off-target side effects are a critical concern surrounding the design and implementation of transplantation medication. The development of belatacept was largely driven by the need for less toxic immunosuppressants, as CNIs cause systemic organ damage that contributes to inferior transplant outcomes (5, 6, 7). While it is impossible to predict the exact effect that our proposed treatment regimen might have in humans, increased susceptibility to infections and the development of cancer are important factors to consider. Latent viral infections such as CMV, Epstein-Barr virus, and hepatitis B & C have a history of re-emerging following transplantation (268, 269). In addition, opportunistic bacterial and fungal infections are a threat, especially following viral infections (268). Mitigating the risk of donor-derived infections by screening for latent viruses is a common strategy to prevent transmission. In addition, administering prophylactics while closely monitoring the recipient for infections is standard procedure. If a threatening viral infection does occur, antivirals such as ganciclovir are typically administered (268). In the long-term, cancer is a major risk following transplantation due to the suppressed immune system. Following renal transplantation, the rate of cancer has been reported to be 3-5 times higher than the general population, but this can vary depending on immunosuppression regime and cancer type (270).

With these risks in mind, we could modify the proposed treatment regimen to be less immunosuppressive. For example, IFN-α neutralization may not be required in the context of IFN-β administration since IFN-β has the highest affinity for IFNAR and ligation of IFNAR induces its internalization. Similarly, IFN-α neutralization combined with anti-BDCA2 may prove to be

redundant and might result in otherwise avoidable viral infections. Another strategy could be to stagger the immunosuppression regimen. IFN-β should be administered early to facilitate the induction of Tregs, followed by either IFN-α neutralization or anti-BDCA2, depending on the efficacy of either therapy, to prevent IFN-α-induced CoB-RR. This proposed strategy would also entail ATG induction therapy and long-term anti-CD28dAb administration to maintain graft tolerance. The efficacy of such a therapy would need to be extensively validated in animal models, the results of which could shape clinical trials and potentially the future of transplantation. The hypothetical effect that this immunomodulatory strategy has on alloimmune responses is diagramed in Figure 1.

Conclusion

In summary, there are two axes of the immune system that can be augmented to preferentially alter the immune landscape during transplantation. In chapter 2, we identified IFNAR signaling on recipient hematopoietic cells as a mediator of transplant rejection during costimulation blockade with CTLA-4-Ig. In chapter 3, we demonstrated the efficacy of selective CD28 blockade following T cell depletion as an alternative costimulation blockade strategy. We predict that shifting IFNAR signaling away from IFN-α and towards IFN-β could improve graft survival by reducing the quantity of proinflammatory cytokines and inducing the generation of Tregs. Concurrently, selective CD28 blockade allows for CTLA-4 coinhibitory signaling to take place on conventional T cells while simultaneously improving the quality of Tregs. It is possible that the combination of these therapies would result in a synergistic effect whereby both the quantity and quality of Tregs are improved, enhancing their suppressive capacities, and promoting transplant acceptance.

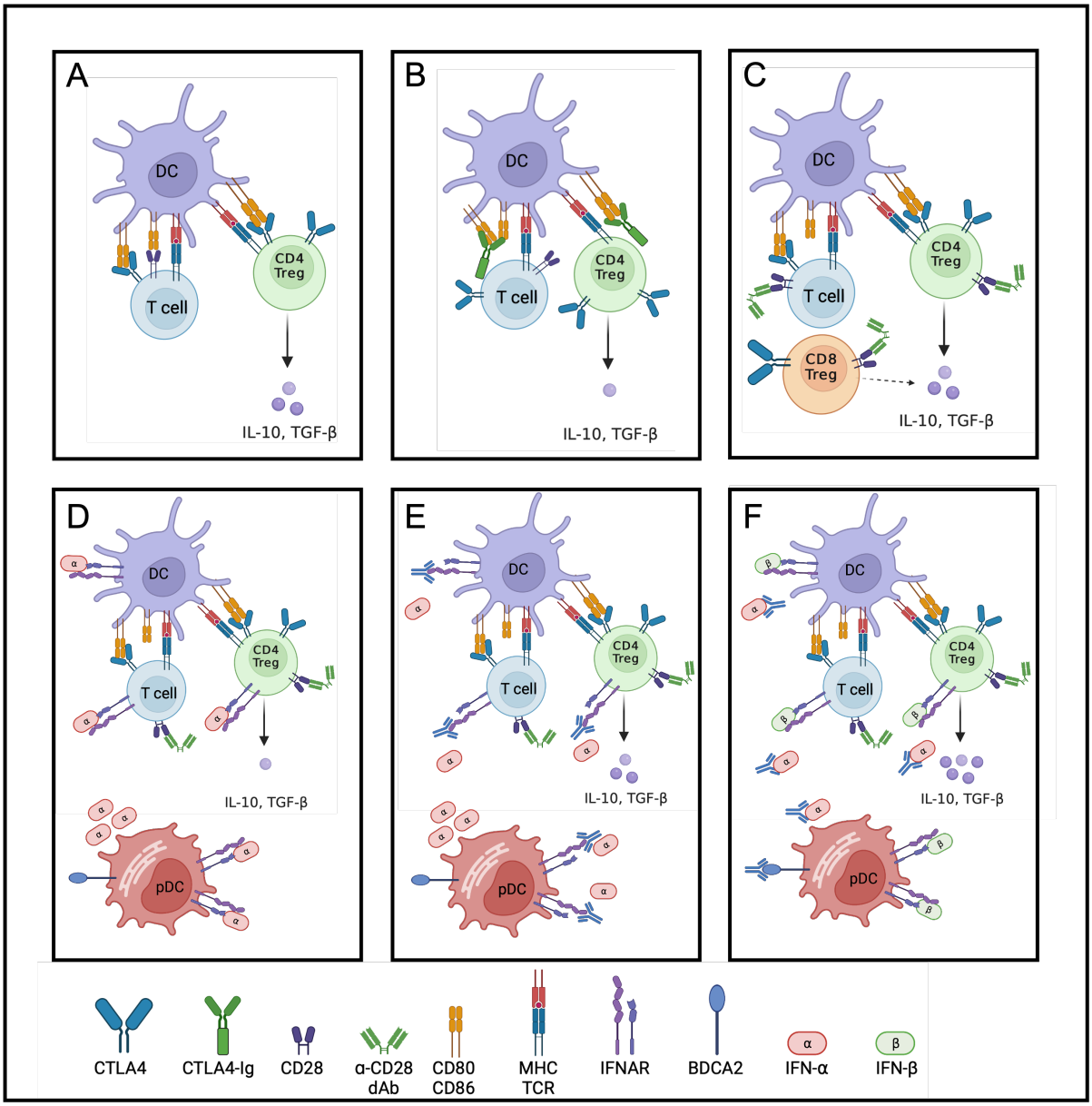

Figure 1: Hypothetical effect of combined immunomodulation. A) Normal immune response mediated by conventional T cells and suppressed by Tregs. B) CTLA-4-Ig blocks the ligands for CTLA-4, CD80 and CD86, preventing coinhibition on conventional T cells and dampening Treg responses. C) Selective CD28 blockade blocks costimulation but allows for CTLA-4 coinhibition on convention T cells and enhanced Treg function. Selective CD28 blockade also results in elevated frequencies of $CD8^+$ Treg, the suppressive capacity of which is uncertain, as indicated by the dashed arrow. D) Interferon alpha produced by pDCs signals through IFNAR and leads to a

pro-inflammatory environment with reduced $CD4^+$ Treg suppressive function. E) Anti-IFNAR antibody prevents all type I IFN signaling. F) Anti-BDCA2 prevents the production of IFN-α by pDCs and neutralization of IFN-α, combined with IFN-β administration, further enhances Treg function.

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