BEYOND HISTOCOMPATIBILITY – UNDERSTANDING THE NON-MHC DETERMINANTS SHAPING TRANSPLANTATION OUTCOME AND TOLERANCE INDUCTION

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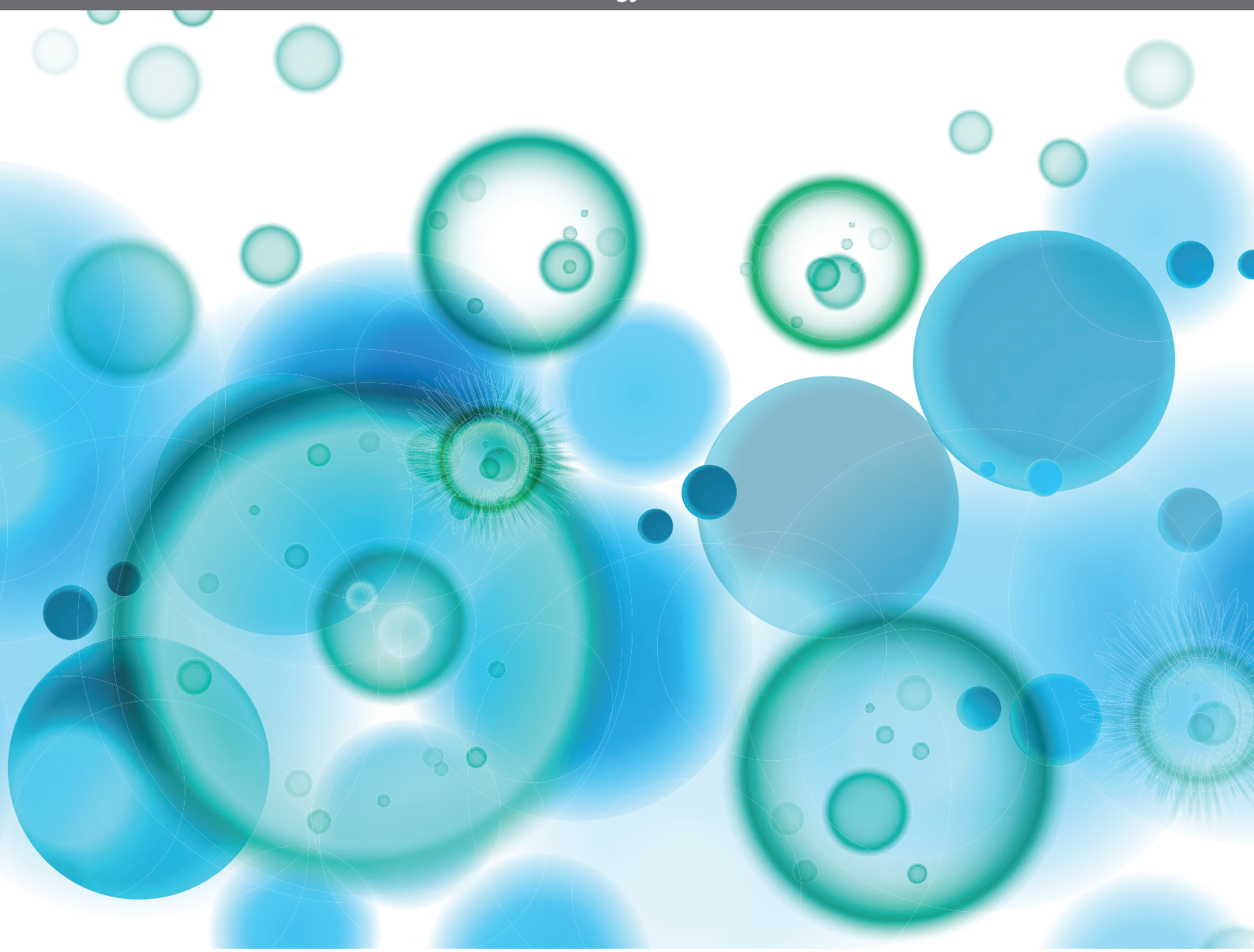

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BEYOND HISTOCOMPATIBILITY – UNDERSTANDING THE NON-MHC DETERMINANTS SHAPING TRANSPLANTATION OUTCOME AND TOLERANCE INDUCTION

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EDITORIAL
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Editorial: Beyond Histocompatibility – Understanding the Non-MHC Determinants Shaping Transplantation Outcome and Tolerance Induction

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Editorial on the Research Topic

Beyond Histocompatibility – Understanding the Non-MHC Determinants Shaping Transplantation Outcome and Tolerance Induction

In seventy years, solid organ transplantation (SOTx) has progressed from a high-risk experimental procedure, with few recipients surviving into the 2nd year post-transplant, to becoming the standard of care for many end-stage organ diseases (1–3). During a similar period, allogeneic stem cell transplantation (AlloHCT) became a central treatment for malignancy and to correct life-threatening lymphohematopoietic disorders (4). This success is due to the development of potent immunosuppressants that blunt T cell responses to major and minor histocompatibility complex (MHC) antigens, the dominant T cell targets after transplantation. Yet, numerous hurdles remain. Toxicities and side effects associated with general immunosuppression are well appreciated. Furthermore, while immunosuppression is a necessary evil after life sustaining SOTx, it is a roadblock for progress in the application of composite tissue allografts (CTA). Here, the side effects from the high doses of immunosuppression needed may outweigh the benefits to life quality provided. In both CTA and SOTx, even toxic levels of immunosuppressants are ineffective against the development of vasculopathy and fibrosis in solid organs over time (5). Finally, the combination of donor AlloHCT and SOTx has provided evidence that tolerance to donor antigens can be induced, yet the risk of graft-versus-host disease (GVHD) and unidentified barriers to routine tolerance induction with these protocols remain (6).

Articles in this Research Topic encompass Original Research and Reviews from transplant researchers seeking to understand the mechanisms controlling transplant outcomes beyond T cell recognition of donor MHC. It is widely accepted that allograft rejection results from coordinated interactions between the innate and the adaptive immune systems, where activated myeloid antigen

presenting cells (APCs), such as dendritic cells (DC) or macrophages, stimulate alloreactive T cell responses. A number of the articles received focused on how innate immune cell can also act as direct effectors of transplant outcomes. In one review, we focused on the consolidating knowledge that damage associated molecular patterns (DAMPs) coordinate the function of innate immune cells to shape alloimmunity and rejection, but also direct graft tissue repair (Dwyer and Turnquist). Here, we also made the case that poor outcomes after SOTx may result not only from abundant DAMP-driven inflammation supporting rejection, but also from inadequate or dysregulated DAMP-mediated tissue repair (Dwyer and Turnquist). Similarly, Ordikhani et al. make a convincing argument that macrophages have a dual role in allograft transplantation and can both trigger inflammatory responses or induce tolerogenic environments. They highlight the role of monocytes and macrophages in SOTx, summarize macrophage heterogeneity, and describe the role of macrophages in rejection versus tolerance. In particular, they highlight monocyte "trained immunity" that is associated with augmented immune responses and retained by epigenetic and metabolic changes. They further suggest that therapeutic targeting of trained immunity represents a novel paradigm to prevent allograft rejection. A review by Zhao et al. furthers similar considerations, first by making a strong case for innate immune cell involvement in allograft rejection, followed by a detailed review of how donor polymorphisms in SIRPα promote allorecognition by CD47 signaling on monocytes. This cascade causes accumulation of monocyte-derived DCs and initiation/maintenance of T cell responses. The authors then describe an innate memory response to MHC class I that is driven by paired immunoglobulin-like receptor A (PIR-A) molecules sensing allogeneic MHCI and contributing to SOTx chronic rejection. The authors close their review by contextualizing the impact of innate allorecognition and innate memory in relevant clinical scenarios.

DCs are increasingly part of therapeutic strategies to achieve allograft tolerance. Schroth et al. provide a timely review of emerging roles different DC subsets and their molecular protagonists play during allograft rejection and tolerance after cardiac transplantation. They highlight differential roles for DC subsets, describe the part innate DCs play in cardiac transplantation, and describe the prominence of DCs in tolerance induction therapies using apoptotic donor cells and costimulatory blockade, and the relation of DC immunometabolism to effector phenotype. Rouselle et al. explore this topic in a series of studies testing if DCs propagated *ex vivo* in the presence of FTY720 (FTY), a Sphingosine 1-phosphate receptor (S1PR) agonist, could protect against kidney ischemia reperfusion injury (IRI). Adoptive transfer of FTY-DCs significantly protected kidneys from IRI, a result dependent upon a recipient spleen, DC expression of S1P1, and functional viability of DC-associated mitochondria. Their report further implicates a mechanism involving transfer of mitochondria to splenic macrophages as an underlying mechanism and support this supposition by demonstrating the transfer of mitochondria from bone marrow-derived DCs to cultured macrophages. Molina et al. investigate DC biology in the context of GVHD by demonstrating that pre-transplant conditioning with bendamustine plus total body irradiation increased CD8α cDC1 cell number and percentage, a subset known to ameliorate GVHD (7, 8), and promoted commitment of DC progenitors to the cDC1 lineage pre-transplant, where expression of CD24 allowed enhanced DAMP sensing.

Metabolic reprogramming is critical to T cell activation, differentiation, and function (9). Cheng et al. build on their past work in this space and demonstrate that costimulatory blockade in combination with targeting T cell metabolism can promote skin allograft survival and long-term cardiac allograft acceptance in the absence of maintenance immunosuppression. Interestingly, metabolic inhibition appeared to play more of a role during acute rejection, while addition of CTLA4-Ig demonstrated a synergistic effect on acute and memory T cell responses. Our review (Brown and Byersdorfer) summarizes the current understanding of the metabolic pathways available to alloreactive T cells and highlights key metabolic proteins and pathways linking T cell metabolism to effector function. A current picture of alloreactive T cell metabolism during AlloHSCT is provided, with roles for glycolysis, fat oxidation, and glutamine metabolism as well as a potential explanation for how presumably contradictory metabolic findings might be reconciled. Finally, the caveats and challenges of assigning causality using the current metabolic toolbox, as well as future directions in the field, are summarized.

Other papers in the Research Topic provide novel insights into mechanisms shaping T cell functions after transplantation. Activation of GVHD-causing alloreactive T cells relies on TCR engagement (Signal 1) and coordinated co-stimulation (Signal 2), in concert with signals from a network of secreted cytokines (Signal 3). The review by Kim and Reddy describes current approaches targeting Signal 3 in clinical GVHD and reviews extracellular cytokine blockade, therapies that target intracellular cytokine synthesis, and pathways which impact cytokine transport, the latter of which represents a novel starting point for rational design of GVHD therapies (Kim and Reddy). In their work, Mammadli et al. demonstrate that as well as the intracellular signaling protein Interleukin-2-inducible T cell Kinase (ITK) is necessary in T cells for the development of GVHD but dispensable for graft-versus-leukemia (GVL) effects. Mechanistically, investigators noted a cell intrinsic decrease in proinflammatory cytokine expression and cell extrinsic decrease in CD8 T cell proliferation, as well as impaired chemokine expression in ITK knock-out cells, resulting in decreased migration to target organs. In their review, Gill and Burrack describe two concepts that expand the commonly held view of how memory cells contribute to transplantation immunity and tolerance disruption. First, they stress that autoimmune T cells may interact with graft-derived autoantigens in addition to cross-reactive, heterologous alloimmune MHC molecules. Additionally, they posit that a common APC may license naïve alloreactive T cells if a vaccine- or pathogen-directed memory cells recognizes the same APC *in vivo*. Indeed, this speculation is

reminiscent of CD4 helper T cells licensing CD8 cytotoxicity and suggest that assessing only anti-donor MHC reactivity pre-transplant may insufficiently predict success in tolerance-promoting therapies. Understanding transplantation tolerance also requires knowledge of the crosstalk between pathogenic T cells and their tissue resident counterparts. In their review, Lei et al. focus on interactions between alloreactive T cell and cells of the liver microenvironment, paying particular attention to adhesion molecule, chemokine expression, cytokine secretion by immune cells, and a role for regulatory T cells in promotion of transplant-specific tolerance. The authors close with a review of recent and ongoing clinical trials that seek to influence post-transplant tolerance using cell mediated approaches.

The studies presented in this Research Topic show how our knowledge of transplant biology continues to not only increase, but also broaden into new roles for long studied immune cells and immunological mechanisms. There is clearly a rapidly advancing understanding of innate immunity, improved manipulation of immune cell metabolism, and functional elucidation of the pathways controlling pathogenic alloreactive immune responses cells in SOTx and AlloHCT. There has been a sustained history of observation, collaboration, and knowledge assimilation between research discoveries and clinician scientists in the fields of SOTx and AlloHCT. It is encouraging to see investigators in both areas in this Research Topic, and we hope that efforts like this help the diverse groups of transplant scientists learn from each other as we aim to provide transplant recipients with the best hope for long-term resolution with the least amount of toxicity.

AUTHOR CONTRIBUTIONS

Both authors generated and edited the editorial. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mechanisms of Immune Tolerance in Liver Transplantation-Crosstalk Between Alloreactive T Cells and Liver Cells With Therapeutic Prospects

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Liver transplantation (LTx) is currently the most powerful treatment for end-stage liver disease. Although liver allograft is more tolerogenic compared to other solid organs, the majority of LTx recipients still require long-term immune suppression (IS) to control the undesired alloimmune responses, which can lead to severe side effects. Thus, understanding the mechanism of liver transplant tolerance and crosstalk between immune cells, especially alloreactive T cells and liver cells, can shed light on more specific tolerance induction strategies for future clinical translation. In this review, we focus on alloreactive T cell mediated immune responses and their crosstalk with liver sinusoidal endothelial cells (LSECs), hepatocytes, hepatic stellate cells (HSCs), and cholangiocytes in transplant setting. Liver cells mainly serve as antigen presenting cells (APCs) to T cells, but with low expression of co-stimulatory molecules. Crosstalk between them largely depends on the different expression of adhesion molecules and chemokine receptors. Inflammatory cytokines secreted by immune cells further elaborate this crosstalk and regulate the fate of naïve T cells differentiation within the liver graft. On the other hand, regulatory T cells (Tregs) play an essential role in inducing and keeping immune tolerance in LTx. Tregs based adoptive cell therapy provides an excellent therapeutic option for clinical transplant tolerance induction. However, many questions regarding cell therapy still need to be solved. Here we also address the current clinical trials of adoptive Tregs therapy and other tolerance induction strategies in LTx, together with future challenges for clinical translation from bench to bedside.

Keywords: liver transplantation, alloreactive T cells, crosstalk, liver cells, tolerance induction

INTRODUCTION

Liver transplantation (LTx) is currently the most powerful treatment for end-stage liver disease. Benefitting from advances in surgical techniques, remarkable improvements in transplant recipient survival have been achieved in the last decades since Dr. Starzl conducted the first human LTx in 1963 (1). As an immunoregulatory organ, liver allograft in the transplant setting is more

tolerogenic compared to other organs such as the kidney, heart, and intestine. It is reported that almost 20% of stable and carefully selected liver transplant recipients can be weaned safely off all immunosuppression (IS) (2). However, the majority of liver transplant recipients still require open-ended, or even lifelong IS to control the unwanted alloimmune responses, which is dominantly mediated by long-term, high magnitude CD8 T cells with the help of secondary lymph nodes and CD 4 T cells. Long-term or overdose IS treatment can lead to serious side effects such as severe infections and malignancy recurrence post transplantation (3–5). Therefore, understanding the mechanism of liver transplant tolerance and crosstalk between immune cells, especially alloreactive T cells and liver cells, can shed light on more specific tolerance induction strategies for clinical translation.

The liver receives 75% of the blood from the portal vein, which is rich in antigens and microbial products originated from the stomach, gut and spleen, and 25% of the blood is oxygenated from the hepatic artery (6). Thus, the hepatic immune system is tightly controlled and regulated under physiological conditions. In addition to the leukocytes from the blood flow through the liver, the liver itself consists of hepatocytes, hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs), cholangiocytes, and a diverse array of immune cells residing within or trafficking to the liver (7). Crosstalk between liver cells and immune cells plays a central role in keeping the balance of immunity and tolerance. In general, innate immune cells such as dendritic cells (DC) and liver-resident DCs (Kupffer cells) serve as professional APCs to T cells, thereby mediating hepatic immunity. Interaction of innate immune cells and liver cells has been reviewed intensively by others (8–10). As alloreactive T cells or memory T cells mediated rejection represents a major hurdle to successful transplant tolerance induction, in this review, we mainly focus on the crosstalk between alloreactive T cells and liver cells in the transplant setting together with potential therapeutic prospects for tolerance induction.

T CELL MEDIATED REJECTION WITH ALLOANTIGEN RECOGNITION PATHWAYS

When a liver is transplanted from the donor to the recipient, the alloantigen—mainly the allogenic major histocompatibility complex (MHC), or human leukocytes antigens (HLA) in humans—is ubiquitous, persists probably for life, and can be presented by both professional and unprofessional antigen presenting cells (APCs) at numerous sites. Thus, transplant rejection is mainly caused by the mismatch of MHCs or HLAs even in LTx. Alloantigen activated helper T cells (Th) secrete cytokines including TNFα, IFNγ, and IL-2 to further enhance the innate immune responses upon alloantigen challenge; on the other hand, they also stimulate effector CD4 T cells and cytotoxic CD8 T cells to express granzyme and perforin, thereby attacking the liver graft. In addition to the cell-mediated acute rejection, donor (graft) specific antibody (DSA) mediated humoral immune response is another important reason for hyper-acute rejection and chronic rejections. DSA mediated rejection is initiated by and in conjunction with T cell mediated alloimmunity (11–13). Several groups have shown that increased memory T cells or stem-like memory T cells correlate to allograft rejection or graft vs. host disease (GvHD) in human and animals. Stem-like memory T cells have the capacity to not only reconstitute the full diversity of memory and effector T cell population, but also maintain their own pool size through self-renewal (14–16). Therefore, memory T cells, especially donor-antigen specific memory T cells, are a major obstacle for successful tolerance induction. Moreover, as the counterpart of conventional T cells, regulatory T cells (Tregs), which are a specialized CD4 T cell subpopulation with the key transcription factor FoxP3 expression, are found to play an essential role in operational tolerance post solid organ transplantation. We showed previously that memory Tregs had superior capacity compared with naïve Tregs through higher expression of CD25 (IL-2 receptor α chain), CD39, CTLA-4 and other important molecules (17, 18). Nevertheless, the formation of immune memory initiate through alloantigen recognition and alloreactive T cells response is the backbone of adaptive immunity to allograft in the transplant setting (19). Notably, the alloimmune response is distinct from the immune response to classically pathogenic antigens because the alloreactive repertoire is highly diverse, especially in the naïve T cells subpopulation, as we showed before with next-generation sequencing (NGS) technology (18). The T cell receptor (TCR) provides a unique identity for each cell clone with around $2.5*10^7$ TCRs for human naive T cells in each individual; the TCR repertoire against a given allogenic MHC haplotype is believed to be <10% of the entire TCR repertoire (19, 20). Therefore, recognition of the alloantigen is the first critical step for the following immune response or tolerance induction in the transplant setting.

To recognize the alloantigen by host TCRs, there are mainly 3 pathways: (i) direct way, (ii) indirect way, and (iii) semi-direct way through cross-dressing of graft MHC by host dendritic cells (DC) (19). Firstly, as shown in **Figure 1A**, through the direct recognition way, allograft APCs present the alloantigen with their own MHC-I molecules to the host CD8 T cells and allograft MHC-II to the host CD4 T cells. The intact antigen (protein) is recognized directly without the processing procedure. Direct recognition of the alloantigen is believed to be the dominant pathway of transplant rejection, which also includes the passenger leukocytes theory. "Passenger leukocytes" refer broadly to all the graft-derived immune cells that are transferred to the host secondary lymphoid tissue and trigger allograft rejection by direct recognition of the alloantigen (21–23). However, the contribution of passenger leukocytes to allograft rejection or tolerance induction is still not clearly understood. Irradiation of the allograft before surgery in rodent models results in killing of the graft lymphocytes and transplant rejection in otherwise tolerant recipients, suggesting the tolerance induction role of donor-derived graft-resident lymphocytes (24–26). On the other hand, the majority of donor lymphocytes are replaced by recipient bone marrow derived hematolymphoid cells within months post LTx (27–29). Nevertheless, direct recognition of the alloantigen by CD4 T cells was considered to persist at early time points after transplantation

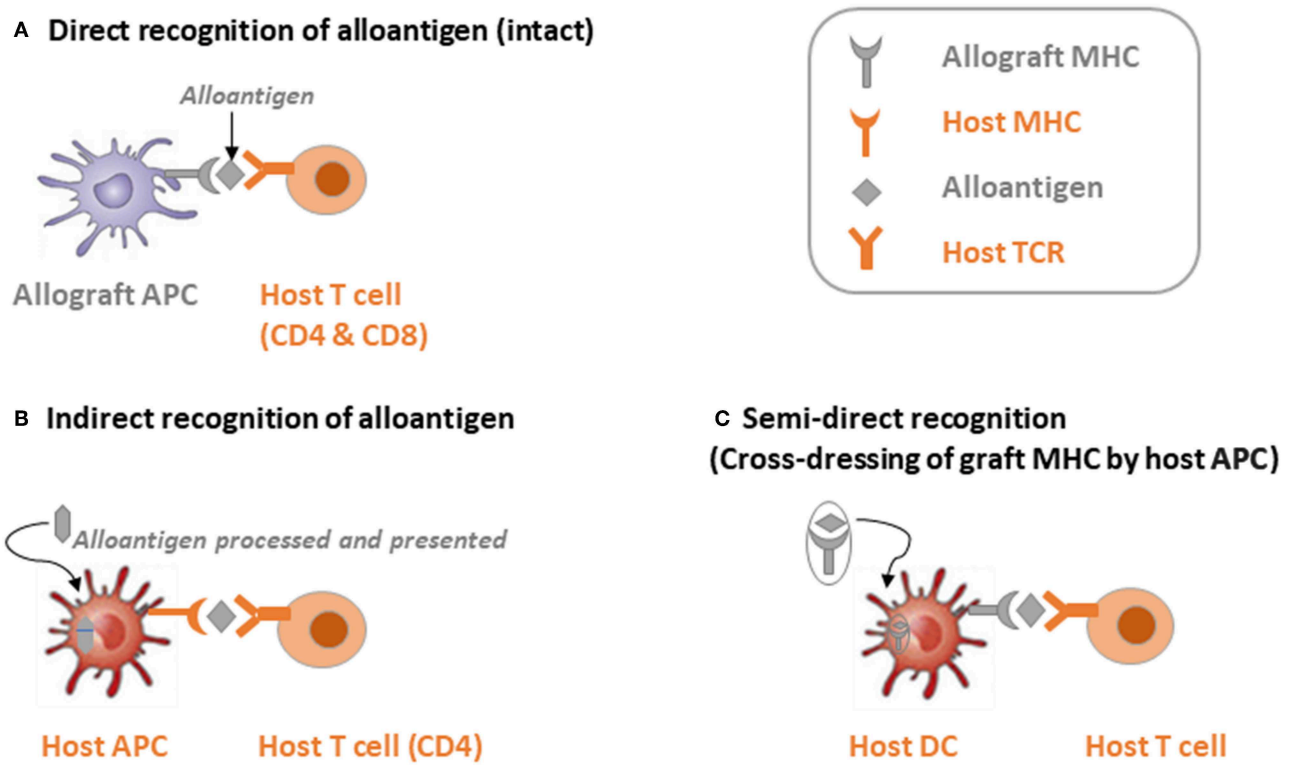

FIGURE 1 | Schematic illustration of three alloantigen recognition pathways. **(A)** Direct recognition of alloantigen: allograft antigen-presenting cells (APCs) present intact alloantigen directly to host T cells. **(B)** Indirect recognition of alloantigen: host APCs process and present the allograft-derived peptides to host T cells, mainly CD4 T cells. **(C)** Semi-direct recognition way: host APCs, mainly dendritic cells (DCs), acquire graft MHC molecules, which is called cross-dressing, and present the peptide directly to host T cells.

and was highly correlated with the lifespan of graft DCs (30). Whereas, indirect recognition of alloantigen is considered to be related with both acute and chronic transplant rejection. By this way, alloantigen is internalized and processed by host APCs into peptide antigens, which are further presented with host MHC molecules and thereby recognized by the TCR repertoire of host T cells (**Figure 1B**). CD4 T cells response from the indirect recognition way is believed to be more relevant with the allograft rejection than CD8 T cells in solid organ transplantation due to the relatively low expression of host MHC-I antigen epitopes in the vascularized allografts (31, 32). Last but not least, through the semi-direct recognition way, the host DCs acquire expression of the graft MHC molecule, which is also called cross-dressing of the host DCs, then re-present the graft MHC-antigen complex as intact alloantigen to the host T cells without further processing (**Figure 1C**). This phenomenon was also observed by Ono et al. (33) that in a mice LTx model, graft interstitial DCs decreased rapidly post LTx, then they were replaced by host DCs, which peaked at day 7 and persisted indefinitely. Around 60% of the host DCs in the liver graft expressed graft MHC-I, suggesting cross-dressing, and controlled the proliferation of anti-graft host T cells. On the other hand, non-cross-dressed DCs failed to suppress the anti-graft T cell response (34–38). The mechanism behind cross-dressing is believed to be related with cell-cell contact or extracellular exosomes (39–44).

Through collaboration of different alloantigen recognition pathways, host CD4 T cells are activated by continued TCR stimulation with graft MHC-II alloantigen, which are expressed either on the surface of graft APCs or re-presented by host DCs through semi-direct recognition within secondary lymphoid tissue. The principle role of the indirect pathway in CD4 T cell response, which mainly focuses on self-restricted, processed alloantigen, is likely at the late phase of transplant rejection through providing help for cytotoxic T cells and humoral immunity (45–48). The semi-direct pathway allows linked help to be delivered by indirect pathway recognition of CD4 T cells to alloreactive CD8 T cells, which target the MHC-I alloantigen expressing cells within the graft after activation and thereby exhibit cytotoxic activity through expression and secretion of granzyme and perforin (36, 42, 49). Alloantigen recognition by Tregs with different pathways, however, regulates the hepatic

immune "balance" substantially more favorable for "tolerance" (50). Therefore, interaction of alloreactive T cells and APCs will be the first and key step in regulating transplant outcome in LTx.

CROSSTALK BETWEEN LSECS AND ALLOREACTIVE T CELLS

Within the liver allograft, there are many professional APCs such as DCs expressing low amounts of MHC antigens with co-stimulatory molecules and Kupffer cells (KCs) phagocytosing pathogens and secreting cytokines together with antigen processing and presenting (9, 51). Additionally, a large amount of non-professional APCs such as liver cells also interact with alloreactive T cells and contribute a lot to the liver transplant outcome. Composed of 50% of liver non-parenchymal cells, LSECs constitute a unique vascular bed with fenestrae organized in sieve plates without basal membrane in the liver. They interact directly with the immune cells and antigens in the blood flow, benefiting from the rich blood supply to the liver and the special liver sinusoid structure. Therefore, LSECs are also called "gatekeepers" of the hepatic immunity (52). Together with Kupffer cells, LSECs constitute the most powerful scavenger system in the body by the expression of pattern recognition receptors (PRRs) such as Toll-like receptors (TLR), scavenger receptors, and the potent endocytic capacity with their special fenestrae and loosely organized cell junctions (53, 54).

In addition to the potent endocytosis capacity, LSECs are also the unique liver-resident APCs by expressing both MHC-I and MHC-II molecules, which take up, process and present many antigens, including alloantigens to both CD8 and CD4 T cells within the liver graft. As shown in **Figure 2**, LSECs can take up alloantigens through PPRs, notably the mannose receptor (MR), process and transfer them to MHC-I for the priming of naïve CD8 T cells, which is called cross-presentation as MHC-I normally exhibit endogenous antigens rather than exogenous peptides (alloantigen as foreign antigen in transplant setting) (55, 56). However, the priming of naïve CD8 T cells by LSECs upregulates the expression of the co-inhibitory molecule B7-H1(PDL1) on LSECs whereas the expression of co-stimulatory molecule CD80/CD86 is not changed, thus the binding of B7-H1 on LSECs and PD-1 on naïve CD8 T cells leads to the apoptosis of the alloreactive CD8 T cells, creating therefore the tolerogenic environment within the liver graft. Interestingly, the LSECs-induced tolerance is highly correlated with antigen load and the strength of TCR stimulation in mice. Tolerance only occurs in low-dose antigen stimulation while high-dose antigen load results in the differentiation of effector memory T cell phenotype; this process is determined partly by IL-2 secretion of naïve CD8 T cells upon early antigen priming. Furthermore, exogenous IL-2 overrides B7-H1 mediated tolerance by LSECs and induces cytotoxic T lymphocytes (CTL) differentiation (57–59). Nevertheless, LSEC lectin (LSECtin), a member of the dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) family, specifically recognizes activated T cells and negatively regulates the intrahepatic immune responses (60, 61).

Similar with CD8 T cells priming, LSECs can prime naïve CD4 T cells with expression of MHC-II, especially under inflammatory conditions, but fail to stimulate the proliferation of these cells due to the low expression of co-stimulatory molecules. Importantly, LSECs also regulate the fate of naïve CD4 T cell differentiation within the liver graft. Neumann et al. found that LSECs could suppress the differentiation of pro-inflammatory Th1 cells and promote the secretion of immune suppressive cytokines such as IL10 via the Notch pathway (62). As we addressed before, Tregs are another fundamental mediator for keeping allograft tolerance. There are several different Tregs including natural Tregs (nTregs), induced Tregs (iTregs), IL10 producing Type 1 regulatory T cells (Tr1 cells), and TGF-β producing Th3 cells. nTregs are mainly developed from the thymus while iTregs are induced from naïve T cells with the presence of a low amount of antigen and TGF-β. iTregs play an essential role in keeping immune homeostasis at mucosal interfaces with expression of probably a distinct TCR repertoire as nTregs (63). Under the condition of vast antigens in the liver and TGF-β secreted by DCs, hepatic iTregs are the major source of peripheral iTregs and lead to transplant tolerance together with nTregs in both humans and mice (17, 64–68). LSECs also promote cytokine secretion of the immune suppressive Th2 cells in addition to iTregs induction in animal models (69). Furthermore, *in vitro* stimulation of Th1 and Th17 by LSECs actively inhibits their capacity to secrete IFNγ and IL17, which is tightly correlated with the dominate inhibitory (B7-H1) over co-stimulatory (CD80/CD86) signals on LSECs and IL10 production by other tolerogenic cells such as DCs (70). As Th1 and Th17 cells are important mediators of transplant rejection post LTx (71, 72), the enrichment of Tregs contributes a lot to the tolerance induction as transient accumulation of total Tregs in peripheral blood of transplant recipients, especially non-rejection recipients at 1 or 2 weeks post LTx, was observed. Similar enrichment of Tregs was also proved in tolerogenic kidney transplant recipients, suggesting the priming of T cell response by the graft antigens (17, 67, 73).

Notably, the crosstalk between LSECs and T cells largely depends on cell-cell contact by different expression of adhesion molecules and chemokine receptors. Recruitment and accumulation of CD8 T cells within the liver depend primarily on TCR activated intercellular adhesion molecule 1 (ICAM1) expressed by LSECs and slightly on vascular cell adhesion molecule 1 (VCAM1), which does not need the recognition of intrahepatic antigens, thereby passively sequestering activated CD8 T cells (74). On the other hand, liver-resident T cells express lymphocyte function-associated antigen-1 (LFA-1) (CD11a or αLβ2 integrin) rather than CD103, an integrin that is required to retrain tissue-resident T cells in many epithelial tissues, to interact with ICAM1 on LSECs (75, 76). Chemokine receptor CXCL16 with its ligand CXCR6 is also involved in intrahepatic T cell and NKT cell recruitment, whereas Tregs bind to different chemokines due to their expression of CCR5 or CCR4; they are also reported to use distinct combination of adhesion receptors such as stabilin 1 to migrate cross LSECs (77).

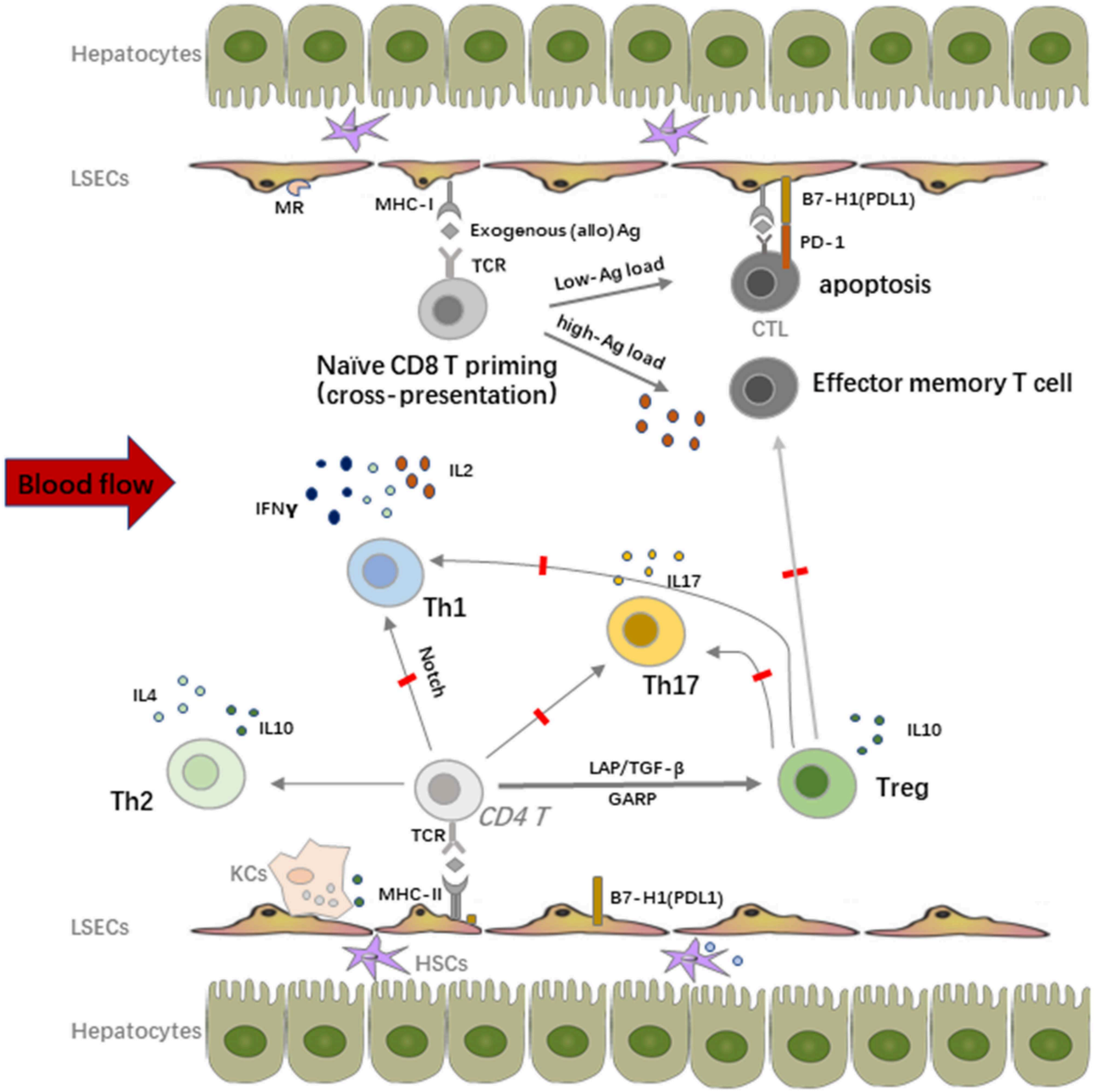

FIGURE 2 | Crosstalk between alloreactive T cells and LSECs. LSECs constitute a unique vascular bed with fenestrae organized in sieve plates without basal membrane in the liver. They are the most powerful scavenger system by expression of pattern recognition receptors (PRRs), notably the mannose receptor (MR). On one hand, LSECs process and transfer the MHC-I to the naïve CD8 T cells, which is called "cross-dressing." This priming process upregulates expression of the co-inhibitory molecule B7-H1(PDL1) on LSECs, whereas the expression of co-stimulatory molecule CD80/CD86 is not changed, thus the binding leads to the apoptosis of the alloreactive CD8 T cells. The LSECs induced tolerance is also highly correlated with antigen load and the strength of TCR stimulation. On the other hand, LSECs also prime naïve CD4 T cells with expression of MHC-II, especially under the inflammatory conditions, but fail to stimulate the proliferation of these cells due to the low expression of co-stimulatory molecules. LSECs also regulate the fate of naïve CD4 T cell differentiation within the liver graft. They suppress the differentiation of Th1 and Th17 cells but favor the enrichment of immune suppressive Th2 and Tregs, which promote the allograft tolerance.

INTERACTIONS OF HEPATOCYTES AND ALLOREACTIVE T CELLS

Through interaction of immune cells with LSECs and adhesion cascade in the hepatic sinusoids, the survived lymphocytes from the LSECs immune surveillance can transmigrate across the LSECs line with help from the orchestra of chemokines and adhesion molecules through several different routes paracellularly, transcellularly, or intracellularly, to finally get a chance to crosstalk with hepatocytes (52). The paracrine factors that were secreted by hepatocytes also accelerate the recruitment of lymphocytes. The interaction of hepatocytes and immune cells plays an important role in inducing liver transplant tolerance. In general, hepatocytes mainly serve as non-professional APCs with expression of MHC-I to interact with CD8 T cells under physiological conditions while expression of MHC-II is also inducible under inflammatory conditions, especially in the presence of IFNγ. However, low expression of co-stimulatory molecules on hepatocytes leads to apoptosis of the alloreactive T cells (10). Paul-Heng et al. have found that direct recognition of hepatocyte expressed MHC-I alloantigen (cross presentation) is required for tolerance induction, whereas the indirect recognition of the processed and presented allogeneic peptide on MHC-II by CD4 T cells is not sufficient for tolerance induction although it can prolong the graft survival and generate Tregs to promote transplant tolerance (78, 79). Additionally, processing of the soluble antigens into peptide presented by MHC-I is impaired in hepatocytes lacking collectrin, which is an intracellular chaperone protein within the endoplasmic reticulum-Golgi intermediate compartment and positively regulated (80). Different from other liver cells, hepatocytes can produce exosomes to control the active T cells response and clear the activated T cells through the non-apoptotic way of suicidal emperipolesis (SE), which is a process leading to cell-in-cell structures and promotes cell death through degradation within endosomal/lysosomal compartments (**Figure 3**) (81, 82). Recently, Beringer et al. have found that the interaction of hepatoma HepaRG and human peripheral blood mononuclear cells (PBMCs) in the inflammatory response can be divided into two phases. At the early phase, PBMC-HepaRG interaction can modulate the T cell polarization into Th1 cells and suppress the differentiation into Th17 cells through direct cell-cell contact with increased secretion of IL6, IL8, CCL20, and MCP-1 (**Figure 3**), whereas the PBMC-hepatocyte crosstalk at the late phase may down-regulate the immune response with decreased expression of HLA-DR on hepatocytes to induce the immune tolerance in the liver (83). Moreover, it is not clear yet whether the similar kinetic interaction of alloreactive T cells and hepatocytes also exist in the LTx setting.

INTERACTIONS OF HSCS, CHOLANGIOCYTES, AND IMMUNE CELLS

Hepatic stellate cells (HSCs), also known as perisinusoidal cells or fat-storing cells, are crucial in liver inflammation and fibrosis by producing inflammatory and fibrotic mediators. In the context of LTx, migrating host immune cells also interact with graft liver resident cells. Both cell-cell contact and soluble cytokines or factors contribute to the graft function and transplant outcome. Inflammatory cell derived IL17A induced HSC to express collagen I directly and TGF-β from activated KCs induced expression of collagen I on HSCs indirectly, promoting the graft fibrosis progression (84). Activated HSCs produce inflammatory cytokines and chemotactic factors to accelerate the migration and deposition of immune cells, which could be further enhanced by paracrine signals from damaged hepatocytes (85–87). However, due to the low amount of MHC-I expression and co-inhibitory molecule B7-H1 on HSCs, interaction of HSCs with activated alloreactive T cells mainly leads to apoptosis of these T cell. In addition, mature HSCs can stimulate allogeneic Treg proliferation with the manner of cell-cell contact and enhance the suppressive capacity of Tregs regarding inhibiting of Teff proliferation *in vitro*. Adoptive transfer of HSC-stimulated Tregs significantly reduced liver injury in mice with autoimmune hepatitis by modulating the balance between Tregs and Th17 cell responses (88).

Cholangiocytes express MHC-I under physiological conditions and a low amount of MHC-II only in the context of inflammation (89). It was reported that through expression of MHC-I like molecule CD1d, murine cholangiocytes could present both exogenous (cross-presentation) and endogenous lipid antigens to NKT cells and activate them to mediate inflammation in the bile ducts. The human cholangiocytes also present exogenous antigens in a CD1d-restricted way to invariant NKT cells. However, CD1d expression was down-regulated in the biliary epithelium of patients with late primary sclerosing cholangitis and primary biliary cirrhosis compared to healthy controls, suggesting their potential role in the pathology of these diseases (90, 91). On biliary epithelial cells (BECs) in biliary atresia patients, increased ICAM-1 expression was also observed in association with MHC-I, but not MHC-II. The major lymphocytes within the portal tracts are CD4 T cells expressing LFA-1, indicating the potential crosstalk between them (92). MHC-I expression level on cholangiocytes might correlate with cholangitis post LTx. Interestingly, BECs express a relatively higher amount of MHC-I compared with other liver cells (12).

THERAPEUTIC TARGETS FOR LIVER TRANSPLANT TOLERANCE INDUCTION

Operational tolerance, characterized with stable graft function in the absence of IS for at least 1 year, is the final goal of all allogenic solid organ transplantation (SOT). To achieve this, several approaches for immune modulation, including adoptive cell therapy, have been conducted in the clinical trials. We and others have showed that both recipient and donor Tregs play an essential role in maintaining the graft tolerance in SOT (17, 18, 93–95). Adoptive Treg-based therapy is a very promising approach to support allograft acceptance with minimizing or potentially eliminating IS treatment. A phase II international multicenter proof-of-concept clinical trial of Treg therapy for

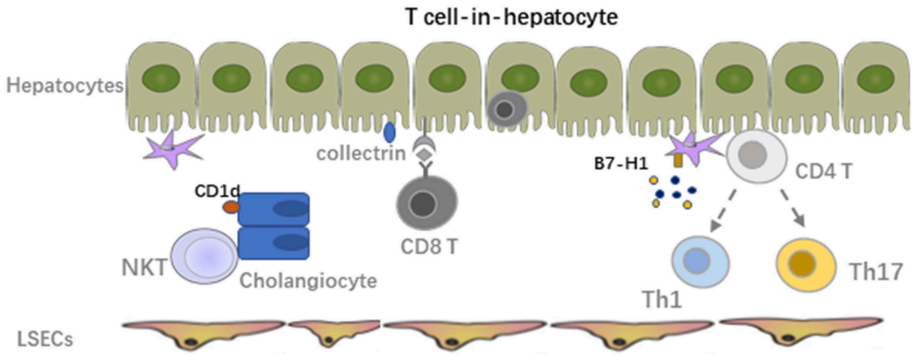

FIGURE 3 | Crosstalk between alloreactive T cells and hepatocyte, HSCs, cholangiocytes. The survived lymphocytes from LSECs immune surveillance can transmigrate across the LSECs line to crosstalk with hepatocytes. The paracrine factors secreted by hepatocytes also accelerate the recruitment of lymphocytes. Hepatocytes mainly serve as non-professional APCs with expression of MHC-I to interact with CD8 T cells under physiological conditions while expression of MHC-II is also inducible under inflammatory condition especially in the presence of IFNγ. Interaction of HSCs with activated alloreactive T cells mainly lead to apoptosis of these T cells due to the low MHC-I expression. Expression of MHC-I like molecule CD1d on cholangiocytes can activate NKT cells and mediate inflammation in the bile ducts.

SOT patients has been conducted in the European Union (The ONE Study). Our group have shown that nTregs from even end-stage renal disease patients could be expanded *ex-vivo* for adoptive cell therapy, whereas alloantigen specific Tregs exhibit superior immune suppressive capacity for tolerance induction (96, 97). Moreover, adoptive Treg transfer in the inflammatory phase of viral-induced myocarditis protects the heart against inflammatory damage and fibrosis via modulation of monocyte differentiation in favor of the anti-inflammatory $Ly6C^{low}CCR2^{low}Cx3Cr1^{high}$ subset (98). In the liver transplant setting, Todo et al. have published a very exciting pilot study that 7 of the 10 liver transplant recipients receiving a single dose of donor antigen specific Tregs and splenectomy become operationally tolerant (99). Several clinical trials for adoptive cell therapy employing either *ex vivo* expanded polyclonal Tregs or alloantigen specific Tregs are also being conducted worldwide. The ThRIL trial at King's College Hospital, UK [clinical trials.gov NCT02166177] utilizes polyclonal Tregs in their therapeutic setting. The DeLTA and ARTEMIS trials at University of California, San Francisco, USA, use donor antigen reactive Tregs for tolerance induction in both deceased donor LTx [NCT02188719] and living donor LTx [NCT02474199]. These clinical trials will not only show the efficiency and safety of Treg therapy but also indicate the survival and homing of these adoptively transferred cells as they are labeled with deuterium (100). Another clinical trial at Nanjing Medical University, China, utilizes donor antigen specific Tregs for chronic rejections in LTx patients at early and late time points, with multiple Treg injections and IS withdrawal [NCT01624077] (101).

In addition to *ex vivo* expansion of Tregs for adoptive cell therapy, other strategies regarding *in vivo* expansion of Tregs are also very appealing. For instance, low-dose IL-2 administration could expand Tregs *in vivo* up to 8 times without a significant increase in Teff cells because Tregs express a higher amount of IL-2 receptor α-chain (CD25) and thus respond to a very low amount of IL-2 while Teff could not. This brings the possibility to expand Tregs pool *in vivo* without requirement of very expensive and large-scale GMP facilities for clinical grade Treg products (102). Low dose IL-2 also restores Treg homeostasis or dysfunction in chronic GvHD patients (103, 104). A corresponding phase IV clinical trial, LITE Trial (NCT02949492), is in progress at King's College London. Scientists there are using low dose IL-2 to promote the selective expansion of endogenous Tregs in liver transplant recipients at the time of immunosuppression (101). Recently, Ratnasothy et al. even showed that IL-2 treatment in mice preferentially enhances the proliferation of the adoptively transferred allospecific Tregs in an antigen-dependent manner and increases the expression of regulatory-related markers, such as CTLA4 and inducible co-stimulator (ICOS). Based on this, combination therapy of both low-dose IL-2 and adoptively transferred alloantigen specific Tregs could provide an appropriate condition to enhance the immunoregulation toward alloimmune response in clinical transplantation (105). Low-dose IL-2 enriched Treg therapy is also investigated intensively in autoimmune diseases and GvHD after hematopoietic stem cell transplantation (106–109).

Notably, as antigen specific Tregs are superior to polyclonal Tregs in controlling Teff responses, improving approximately 100-fold of the efficacy, and theoretically safer due to avoiding bystander compromised immunity (110, 111), it is more appealing to use this Treg population for adoptive Tregs therapy. However, expansion of these Tregs *in vitro* is a big obstacle for clinical translation. Therefore, engineering human T cells to express a chimeric antigen receptor (CAR) is a new approach to create antigen specific T cells. For instance, autoantigen-based chimeric immunoreceptors can direct T cells to kill autoreactive B lymphocytes through the specificity of the B cell receptor (BCR) (112). Meanwhile, CAR Tregs can also be generated with CAR technology to develop alloantigen specific Tregs, which have showed potent and markedly enhanced therapeutic potential for the protection of allografts (113–115). Co-administration of antigen with tolerogenic nanoparticles (tNPs), which comprised

of biodegradable polymers with encapsulated rapamycin, could inhibit ag-specific transgenic Teff proliferation and induce ag-specific Tregs. This suggests another potential strategy to expand ag-specific Tregs *in vivo* and suppress T cell-mediated autoimmunity or graft rejection (116–118). On the contrary, Treg plasticity refers to their capacity to produce inflammatory cytokines and lose FoxP3 expression (119, 120). In this case, they could transform into pathogenic Teff cells, thus contributing to disease pathogenesis, which might represent a risk for adoptive Tregs therapy. Based on these concerns, genetic "editing" through CRISPR-associated protein 9 (Cas9) system could generate optimal Tregs while ensuring stability (121, 122). However, our group have recently published that gene-editing with CRISPR-Cas9 system might cause significant safety issues because of the pre-existing ubiquitous effector T cell response directed toward the Streptococcus pyogenes (SpCas9) within healthy humans. Therefore, modification of Tregs with the CRISPR-Cas9 system still needs further careful evaluation (123).

Similar to Tregs, regulatory B cells (Bregs) function as a form of active immune regulation, which was first reported experimentally through anti-CD45RB treatment of mice receiving a cardiac allograft (124). Moreover, the field of Breg-mediated tolerance is relatively immature and their function is somehow also related with Tregs (125, 126). Regulatory DCs (DCregs) with capacity to suppress allograft rejection and promote transplant tolerance in pre-clinical models can readily be generated from bone marrow precursors or circulating blood monocytes. Donor-derived DCregs are short-lived but can induce robust donor-specific T cell hyporesponsiveness. Infusion of donor-derived DCregs could achieve IS withdrawal in patients 18 months post LTx (38, 127). Furthermore, down-regulation of HLA-1 expression level on hepatocytes can reduce the strength of allogeneic immune responses and improve the graft survival. Alternatively, gene transfer of alloantigen to hepatocytes induces the expansion of CD8 Tregs, which further prevent the allograft rejection in mice pancreatic islets transplantation. These gene-modified hepatocytes may also provide some possible tolerance induction strategy in the future (128, 129).

SUMMARY AND OUTLOOK

Based on the alloimmune responses mediated transplant rejection, interactions of alloreactive T cells with both innate immune cells and liver cells including hepatocytes, LSECs, HSCs, and cholangiocytes contribute dramatically to the transplant outcome. The capacity of alloantigen presenting and inflammatory mediator secretion by liver cells dominates the fate of alloreactive T cell differentiation and transplant outcome. As Tregs play an essential role in inducing and maintaining the allograft tolerance, Treg based therapy either with adoptively transferred *ex vivo* expanded Tregs or low-dose IL-2 *in vivo* enriched Tregs pool is very promising and appealing for clinical translation. However, more efficient Treg expansion protocols have to be developed and evaluated to improve the efficiency of the therapy and reduce the cost for the clinical cell products. In addition, combination of several tolerance induction strategies might provide synergistic results, but more clinical studies from multiple centers still need to be conducted for successful translation from bench to bedside.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Targeting Metabolism as a Platform for Inducing Allograft Tolerance in the Absence of Long-Term Immunosuppression

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Transplant tolerance in the absence of long-term immunosuppression has been an elusive goal for solid organ transplantation. Recently, it has become clear that metabolic reprogramming plays a critical role in promoting T cell activation, differentiation, and function. Targeting metabolism can preferentially inhibit T cell effector generation while simultaneously promoting the generation of T regulatory cells. We hypothesized that costimulatory blockade with CTLA4Ig in combination with targeting T cell metabolism might provide a novel platform to promote the induction of transplant tolerance.

Keywords: mouse, model, transplantation, immunology, metabolism, costimulation blockade, rejection

INTRODUCTION

Transplantation is now recognized as the most effective therapy for patients with end stage organ failure. Despite outstanding short-term graft and patient survival, organ transplantation continues to face several major challenges including poor long-term graft survival resulting from chronic rejection (1–4) and major side effects from the need for long-term immunosuppressive therapy (5, 6). Along these lines, a long elusive goal of human organ transplantation has been the development of therapeutic prophylaxis to prevent graft rejection that ultimately induce transplant tolerance in the absence of long-term immunosuppression.

To this end, maintenance of immunosuppression and treatments for graft rejection rely heavily on the use of calcineurin inhibitors (CNIs) (7–10). These regimens consist of truly potent immunosuppressive agents in that not only do they inhibit immune activation but they also inhibit the induction of tolerance (11, 12). Indeed T regulatory cell generation, activation induced cell death, and T cell anergy are all inhibited by calcineurin inhibitors (13, 14). More recently, the use of costimulatory blockade has been employed in experimental and clinical transplant protocols. T cell activation require signals elicited by the T cell receptor (TCR), costimulatory receptors and the immune microenvironment (15, 16). CD28 is expressed on the surface of the majority of naïve CD4+ and CD8 T+ cells and is the major costimulatory molecule in initial T cell activation (17).

Targeting CD28/B7 T-cell co-stimulation pathways with CTLA4Ig to reduce pathological T-cell responses has met with therapeutic success in transplantation, but challenges remain (18). Data from phase III clinical trials have shown promising results with significant improvement in risk of death, graft loss, donor-specific antibodies and better graft function with CTLA4Ig compared to CNIs (19, 20). However, regimens with CTLA4Ig have also shown higher incidence and severity of acute rejection, especially during the early phase post-transplantation (21, 22). Furthermore, while costimulatory blockade has been shown to promote tolerance in animal models of transplantation, such has not been achieved with these agents in human trials (23, 24).

It is becoming increasingly clear that immune cell activation, differentiation, and function is intimately linked to cellular metabolic reprogramming (25, 26). Similar to cancer cells, activated T cells markedly upregulate glycolysis even in the presence of oxygen. Simultaneously, metabolites generated through the tricarboxylic acid cycle (TCA cycle) can be employed to generate the substrates (amino acids, lipids and nucleic acids) for the prodigious anabolic demands of activation. To this end, glutamine plays a critical role in promoting this process through its conversion to glutamate and subsequently alpha ketoglutarate (27–30). While these metabolic processes are critical for T cell effector function, naïve T cells and regulatory T cells rely on more conventional metabolic programs such as oxidative phosphorylation and fatty acid oxidation. With this in mind, we recently demonstrated the ability of anti-metabolic therapy to prevent graft rejection in mouse models of transplantation (31). Using this approach we were able to maximally inhibit the expansion and function of antigen specific effector cells while promoting the generation of antigen specific regulatory T cells. Nonetheless, long term allograft survival in heart transplants required continued drug treatment. That is, while our metabolic therapy inhibited effector function and promoted regulatory T cells, this was not enough to promote allograft tolerance in the absence of treatment.

In this study, we investigated the effect of combining CTLA4Ig abatacept with our previously defined metabolic inhibitor (MI) therapy. This regimen consists of the glucose analog 2DG which blocks glycolysis, the glutamine analog 6-Diazo-5-oxo-L-norleucine (DON) and the diabetes drug metformin (which blocks complex I of the mitochondria). Our data demonstrate that the addition of CTLA4Ig to continuous metabolic therapy not only results in enhanced skin allograft survival but also promotes long-term cardiac allograft acceptance in the absence of maintenance treatment.

METHODS

Mice

C57BL/6 (H-2b), BALB/c (H-2d), FVB/N (H2q), FVB-Tg(CAG-luc,-GFP)L2G85Chco/J (H2q), B6(Cg)-Tyrc-2J/J (H2b) mice were purchased from The Jackson Laboratory. 5C.C7 mice were purchased from Taconic Farms. All animal procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University.

Antibodies and Reagents

Antibodies against the following proteins were purchased from eBioscience: CD44 (IM7, 1:500), IFN gamma (XMG1,2, 1:500), T-bet (4B10, 1:500), Ki-67 (501A15, 1:1000), KLRG1 (2F1, 1:500), Foxp3 (FJK-16S, 1:250), B220 (RA3-6B2, 1:500), IgG and Fc Block (2.4G2, 1:100). Antibodies against CD3 (145-2C11, 1:1000), Bcl6(K112-91, 1:200), CD4 (RM4-5, 1:1000), CD8 (53-6,7, 1:1000), IFN gamma (XM61,2 1:1000), CD25 (PC61, 1:1000) were purchased from BD Biosciences. Annexin V (BD Bioscience) staining was performed to manufacturer's protocol using Annexin V buffer. Antibodies against granzyme B (GB11, 1:500), PD1 (29F1.A12, 1:500) were purchased from Biolegend. The following antibodies were purchased from Cell Signaling: p-S6 (S240/244, D68F8, 1:1000), phospho PLC gamma (Tyr 783, 1:2000 for immunoblotting). OVA-I peptide (SIINFEKL) was purchased from AnaSpec. Stimulatory anti-CD3 (2C11) and anti-CD28 (37.51) were purified from hybridoma supernatants prepared "in-house." CFSE was obtained from Invitrogen. Cell Proliferation Dye-eFluor450 and fixable viability dye eFluor780 were purchased from eBiosciences. PMA, and ionomycin were purchased from Sigma Aldrich. Class I OVA peptide was obtained from AnaSpec. Vaccinia-OVA (1E6 pfu) and listeria-OVA (5E6 cfu) are modified vaccinia and listeria that contain the full-length ovalbumin protein but lack lytic ability and were generated as previously described (32).

OTI CD8+ T Cell Adoptive Transfer

Naïve Thy1.1 OTI CD8+ T cells were labeled with eF-450 cell proliferation dye. 1-2.5 $\times$ 10^6 cells CD8+ T cells were transferred into naïve C57/Bl6J (Thy1.2) hosts already infected with vaccinia-OVA (4 h prior). Mice were subsequently treated with same drug regimen for transplantation but received DON for both days rather than every other day. On Day 2, spleens were harvested and donor T cells (Thy1.1) were analyzed for cell proliferation and cell death.

Cell Culture

Splenocytes or T cells were cultured in RPMI 1640 media supplemented with 10% FBS, penicillin/streptomycin, glutamine and 50 μM BME. For naïve stimulation and proliferation studies, splenocytes from C57BL/6 mice were labeled with 5 μM eFluor 450 cell proliferation dye (eBioscience) and were stimulated with anti-CD3 (1 μg/ml). For preparation of pre-activated CD8+ T cells, splenocytes from C57BL/6 mice were stimulated with anti-CD3 (1 μg/ml) for 48 h, followed by gradual 2–3-fold media expansion with IL-2 (10 ng/ml; Peprotech) for 5 days. Live cells were collected by density gradient separation (Ficoll, GE Healthcare) and then re-stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (2 μg/ml) in the presence of GolgiPlug (BD Biosciences) overnight. For short term stimulation, CD4+ T cells were harvested from 5C.C7 mice and

purified by negative selection with CD4+ MACS cell isolation protocol (Miltenyi Biotec).

Immunoblot Analysis

CD4+ T cells from 5C.C7 CD4+ transgenic mice were isolated with MACS. Samples were flash frozen and lysed in RIPA lysis buffer with protease and phosphatase inhibitor cocktails. Proteins were detected by ECL Plus substrate (GE Healthcare). All images were obtained using UVP Biospectrum500 Imaging System.

Transplantation

Full-thickness BALB/c or FVB-Tg(CAG-luc,-GFP)L2G85Chco/J trunk skin grafts (1 cm^2) were transplanted onto the flank of C57BL/6, FVB/N, or B6(Cg)-Tyrc-2J/J recipient mice, sutured with 6.0 Nylon and secured with dry gauze and a bandage for 7 days as previously described (33). Grafts were clinically observed every day thereafter and considered rejected when ≥90% of the graft tissue became necrotic.

Heterotopic Heart Transplantation

BALB/c mice served as heart donors and C57BL/6 mice serve as allograft recipients.

Either abdominal or cervical heterotopic heart transplantation was performed as previously described (34, 35). Functionality of the transplanted heart was monitored daily by palpation was scored from 0 (no palpable heart beat) to 4 (strong, fast, rhythmic) according as previously described (36). Clinical rejection was defined by cessation of palpable heartbeats and confirmed by autopsy. Loss of graft function within 48 h of transplantation was considered as a technical failure, and animals in which this occurred were omitted from the analysis.

Treatment Protocols

CTLA4Ig (Abatacept; Bristol-Myers) was administered at a dose 0.5 mg on days 0, 2, 4, 6 after transplantation. Triple metabolic therapy consisted of 2-DG, metformin and DON. 2DG 500 mg/kg and metformin 150 mg/kg were administered every day. DON 1.6 mg/kg was administered every other day. 2DG was purchased from Carbosynth. Metformin and DON were purchased from Sigma-Aldrich and Bachem.

For all *in vivo* experiments, CTLA4Ig and individual metabolic inhibitors were dissolved in PBS and administrated intraperitoneally (i.p.).

In vivo Bioluminescence Imaging of Mice

Mice were anesthetized with 2% isoflurane and placed in a light-tight chamber. A photographic (gray-scale) reference image was obtained at 5 min after D-luciferin (Sigma) injection (150 mg/kg i.p.); bioluminescent images were collected immediately thereafter. Bioluminescence of the mice was detected via the IVIS Imaging System 200 Series. The region of interest from displayed images was designated and quantified as total flux (photons/sec) using Living Image 2.50 software (Xenogen).

Donor Specific Antibody Assay

Donor Balb/c splenocytes (1×10^6 cells) were incubated with diluted (1:50) serum from transplanted, sensitized or naïve recipients. After two washes, cells were stained with anti-B220, anti-CD3 and anti-IgG antibodies. Mean fluorescence intensity on the B220-negative cells were measured by flow cytometry.

Flow Cytometry and Intracellular Cytokine Staining

Flow cytometry data were acquired with FACSCelesta (BD Biosciences) and were analyzed with FlowJo7.6 software (TreeStar). For intracellular staining, cells were stimulated at 37°C for 4 h in the presence of monensin (GolgiStop; BD Biosciences), phorbol 12-myristate 13-acetate (PMA; Sigma), and ionomycin (Sigma). Cells were surface stained and underwent fixation/permeabilization with either a Cytofix/Cytoperm kit (BD Biosciences) or a Fixation/Permeabilization kit (eBioscience), followed by staining for intracellular cytokines. Gates were determined appropriately using un-stimulated control cells. Voltages were determined from unstained controls.

Statistical Analysis

Prism software version 7.0 (GraphPad Software) was used for statistical analyses, including one-way ANOVA non-parametric Kruskal–Wallis test, two-way ANOVA and log-rank analysis. A p-value less than 0.05 was considered statistically significant.

RESULTS

CTLA4Ig and Metabolic Inhibitors Differentially Affect T Cell Activation

Initial studies were performed to examine the effects of CD28/B7 costimulation blockade and metabolic inhibitors (MI) on T cell activation. To this end, we stimulated T cells and performed immunoblot analysis and measured activation parameters, proliferation and cytokine production in the presence of CTLA4Ig or 2DG+metformin+DON (triple metabolic inhibitor (MI) therapy). As expected, T cell activation was impaired by CTLA4Ig, as indicated by reduced phosphorylation of PLC gamma upon early T cell signaling and also reduced expression of the activation marker CD44 (5 and 10 min) (**Figures 1A,B**). That is, blocking costimulation with CTLA4Ig inhibited TCR-induced signaling necessary for full T cell activation. Alternatively, blocking metabolism with the triple metabolic therapy only minimally affected proximal TCR-induced signaling and expression of CD44. In contrast, CD25 expression, which is induced by both TCR signaling and IL-2 signaling was equally inhibited by MI and costimulatory blockade (**Figure 1C**). In light of the important role of mTORC1 signaling in promoting T cell activation, differentiation and function (15), we also examined the effects of costimulatory blockade and MI on mTOR activation. As seen in **Figure 1D**, that while early mTORC1 signaling was not affected, both costimulatory blockade and MI led to a marked decrease in mTOR signaling by 24 h.

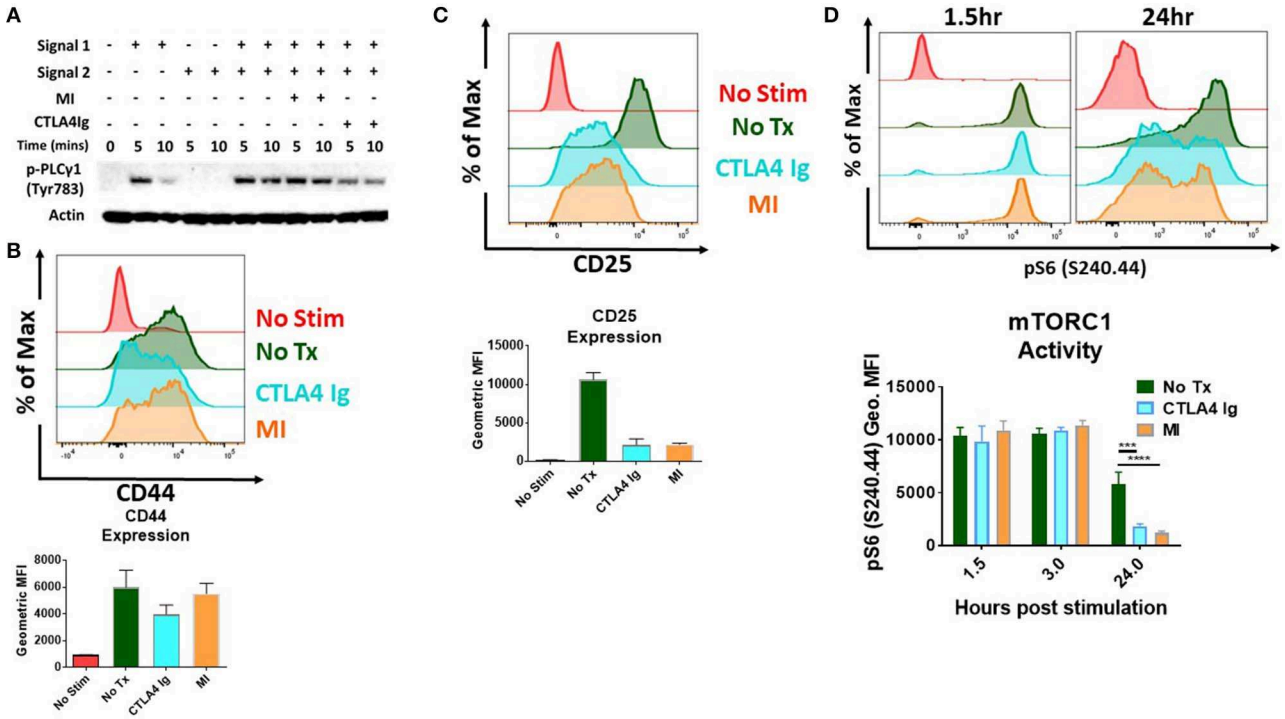

FIGURE 1 | Effect of metabolic inhibitors (MI) and CTLA4Ig on T cell activation and mTORC1 activity **(A)** Immunoblot analysis of phospho-PLC gamma (Tyr 783) activity in naive CD4+ T cells stimulated with anti-CD3 (Signal 1) and/or anti-CD28 (Signal 2) in the presence of CTLA4Ig (50 μg/ml) or MI (2DG 0.6 mM, metformin 1 mM, DON 5 μM) for 5 and 10 min. **(B–D)** Splenocytes from naïve WT C57BL/6 mice were stimulated with anti-CD3 (1 μg/ml) and cultured in the presence of CTLA4Ig or MI as in **(A)**. **(B)** Expression of the activation marker CD44 on viable CD8+ T cells at 24 h under CTLA4Ig or MI treatment. **(C)** Expression of the activation marker and high affinity IL-2 Receptor CD25 **(D)** T cell activation induced mTORC1 activity determined by the phosphorylation state of the S6 ribosomal protein of viable CD8+ T cells at 1.5, 3, and 24 h. ***$p < 0.001$ ****$p < 0.0001$ (one-way ANOVA nonparametric Kruskal–Wallis test) Data are representative of at least three experiments **(A–D)**, (**B–D**, $n = 4$ biological replicates).

CTLA4Ig and Metabolic Inhibitors Differentially Affect T Cell Proliferation, Activation Induced Cell Death and Function

Next, we sought to determine the potential differential effects of costimulatory blockade and MI on T cell proliferation and activation induced cell death. As seen in **Figure 2A**, unlike CTLA4Ig, MI strongly inhibited proliferation based on cell proliferation dilution (CPD) as cells were not able to fully enter cell cycle based on the expression of the proliferation marker, Ki-67. Having demonstrated that MI more robustly inhibited proliferation compared to costimulatory blockade, we next examined the effect of these regimens on activation induced cell death. As seen in **Figure 2B**, MI treatment resulted in markedly enhanced apoptosis of undivided T cells as determined by Annexin V positive staining. Thus, when compared to costimulatory blockade, MI inhibits clonal expansion by both blocking proliferation and promoting activation induced cell death. Next, we examined the effect of costimulatory blockade and MI on T cell function. We also observed both IFN-g and Granzyme B (GzB) production were markedly inhibited by both CLTLA4Ig and MI (**Figure 2C**). Finally, to test the ability of CTLA4Ig costimulation blockade and MI in suppressing alloimmune response, we performed BALB/c to C57BL/6 full thickness skin transplants and treated the recipients from the day of surgery with CTLA4Ig or 2DG+metformin+DON. As previously shown (31), blocking glycolysis, OXPHOS and glutamine metabolism significantly increased skin graft median survival time (MST) 38 days) compared to those that received CTLA4Ig (MST 15 days) or no treatment (MST 14 days) (**Figure 2D**). These findings suggest that CTLA4Ig and metabolic therapy have distinct immunosuppressive effects and that the ability of MI to limit clonal expansion by both preventing proliferation and enhancing activation induced cell death is associated with a more robust ability to prevent skin allograft rejection.

Metabolic Inhibitors Have Increased Efficacy During Acute Rejection

Clinical trials with CTLA4Ig costimulation blockade have shown higher incidence of acute rejection during the early post-transplant period (21, 22). In light of the differences we observed comparing CTLA4Ig and MI in terms of inhibition of proliferation and activation induced cell death, we wanted to compare these two modalities in terms of their ability to inhibit acute rejection. First, we compared the effects of CTLA4Ig or MI on pre-activated T cells. We activated splenocytes with anti-CD3 for 48 h, expanded in media containing IL-2 for 5 days and

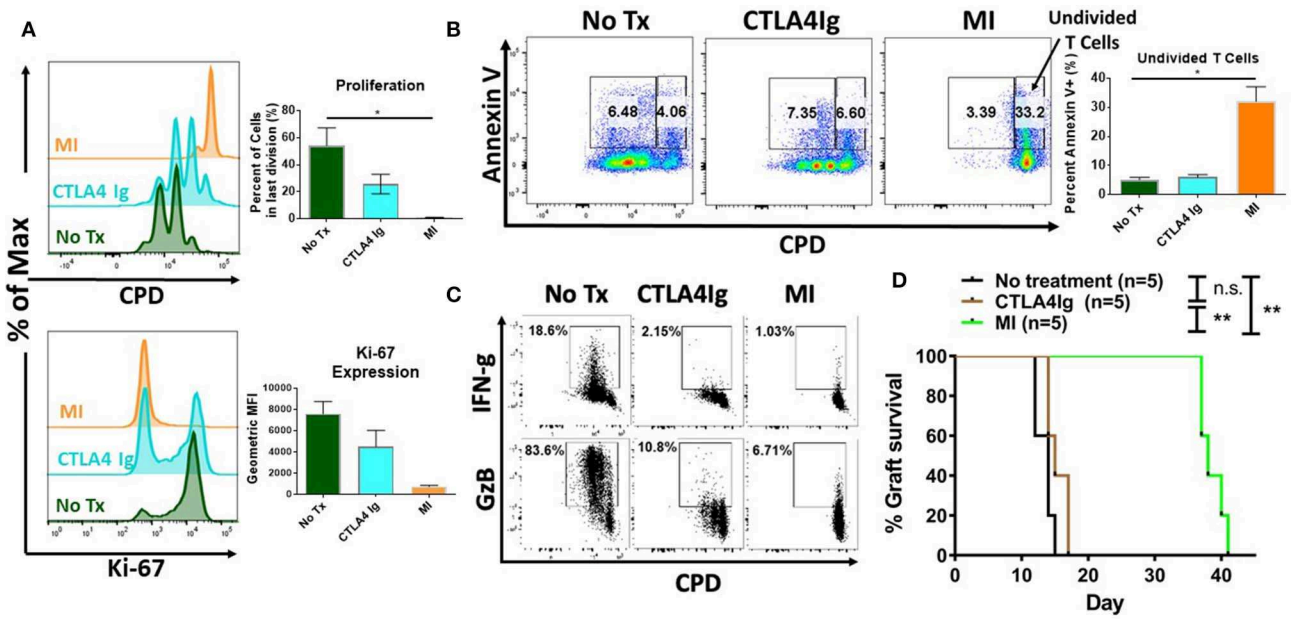

FIGURE 2 | Effect of metabolic inhibitors (MI) and CTLA4Ig on T cell activation and BALB/c (H2d) to C57BL/6 (H2b) complete MHC-mismatched full thickness skin transplantation. Cell proliferation dye eFluor 450 (CPD) labeled splenocytes from naïve WT C57BL/6 mice were stimulated with anti-CD3 (1 μg/ml) and cultured in the presence of CTLA4Ig (50 μg/ml) or MI (2DG 0.6 mM, metformin 1 mM, DON 5 μM). **(A)** Proliferation and Ki-67 expression of viable CD8+ T cells at 48 h. **(B)** Percentage of Annexin V+ staining vs. proliferation in undivided cells (right gate) vs. proliferative cells (left gate) shown. **(C)** At 36 h after activation, brefeldin A was added to each culture for 12 h and IFN-gamma (IFN-g) and Granzyme B (GzB) production was compared to CPD dilution in CD8+ T cells. **(D)** Balb/c to C57BL/6 complete MHC-mismatched full thickness skin transplantation. Therapy was started from the day of transplantation (day 0) until complete graft rejection. No treatment MST = 14 days; CTLA4Ig MST = 17 days; MI MST = 38 days. n.s., not significant, $*p < 0.05$, $**p < 0.01$(log-rank analysis) (one-way ANOVA non-parametric Kruskal–Wallis test) Data are representative of at least three experiments **(A–C)**, (**A,B**, $n = 4$ biological replicates) and two experiments **(D)**.

then re-stimulated with plate bound anti-CD3 and soluble anti-CD28 for additional 48 h in the presence of CTLA4Ig or the metabolic inhibitors. Notably, CTLA4Ig had minimal effect on either proliferation or cytokine production, whereas metabolic therapy still had a partial effect on inhibiting previously activated T cell responses (**Figure 3A**). Next, we sought to compare the effects of CTLA4Ig/MI in a transplant model of acute rejection. To this end, we transplanted skin grafts from luciferase+ mice, FVB-Tg(CAG-luc,-GFP)L2G85Chco/J (H2q), to B6(Cg)-Tyrc-2J/J (H2b) recipients and monitored graft viability by tracking the intensity of their bioluminescent signal. Luciferase+ mice have been widely used as donors for monitoring engraftment of transplanted heart, ovary, hepatocyte and islet (37–40). In skin transplants, allogenic grafts lose viability and decrease bioluminescent signal early after day 6 post-surgery. By Day 12, they are rejected completely. In contrast, syngenic grafts maintain the bioluminescent signal over time (**Supplementary Figure 1**). Given that MI has been shown to be more effective than other conventional immunosuppressive regimens such as cyclosporine or rapamycin in prolonging allograft survival (33), for this acute rejection model, we first treated all the recipients with MI for 10 days to minimize the initial inflammation and stabilize the graft. On Day 10, we paused treatment to induce an alloimmune response and acute rejection, and we resumed treatment after 3 days either with CTLA4Ig or MI (**Figure 3B**). Under CTLA4Ig treatment, total graft loss, as evidenced by bioluminescence and histology, occurred on day 18 (**Figures 3C–F**), which is equivalent to the normal rejection time of non-treated allogeneic grafts (**Supplementary Figures 1B,C**). With MI, however, graft viability persisted at least for another 7 days. Thus, consistent with previous reports which suggested that memory cells may be resistant to CD28/B7 costimulation blockade (41, 42) MI appeared to be superior to CTLA4Ig in terms of treating acute rejection.

CTLA4Ig and MI Have Synergistic Effects on Inhibiting Acute and Memory T Cell Responses

Having demonstrated distinct differences between the ability of MI and CTLA4Ig to inhibit T cell function, we next sought to test the ability of *combination* therapy to inhibit antigen–specific T cell responses in a robust infection model. To this end, C57BL/6 mice were infected with vaccinia-OVA and treated with CTLA4Ig, MI or CTLA4Ig+MI for 6 days. After 30 days of the primary infection, a secondary infection with listeria-OVA was performed to induce antigen-specific memory recall. This model enables us to specifically track and examine the effect of MI + CTLA4Ig on robustly activated antigen specific T cells *in vivo*. To this end, the antigen-specific CD8+ T cell response was interrogated by Class I OVA+ tetramer staining. The acute response was analyzed in peripheral blood on Day 7

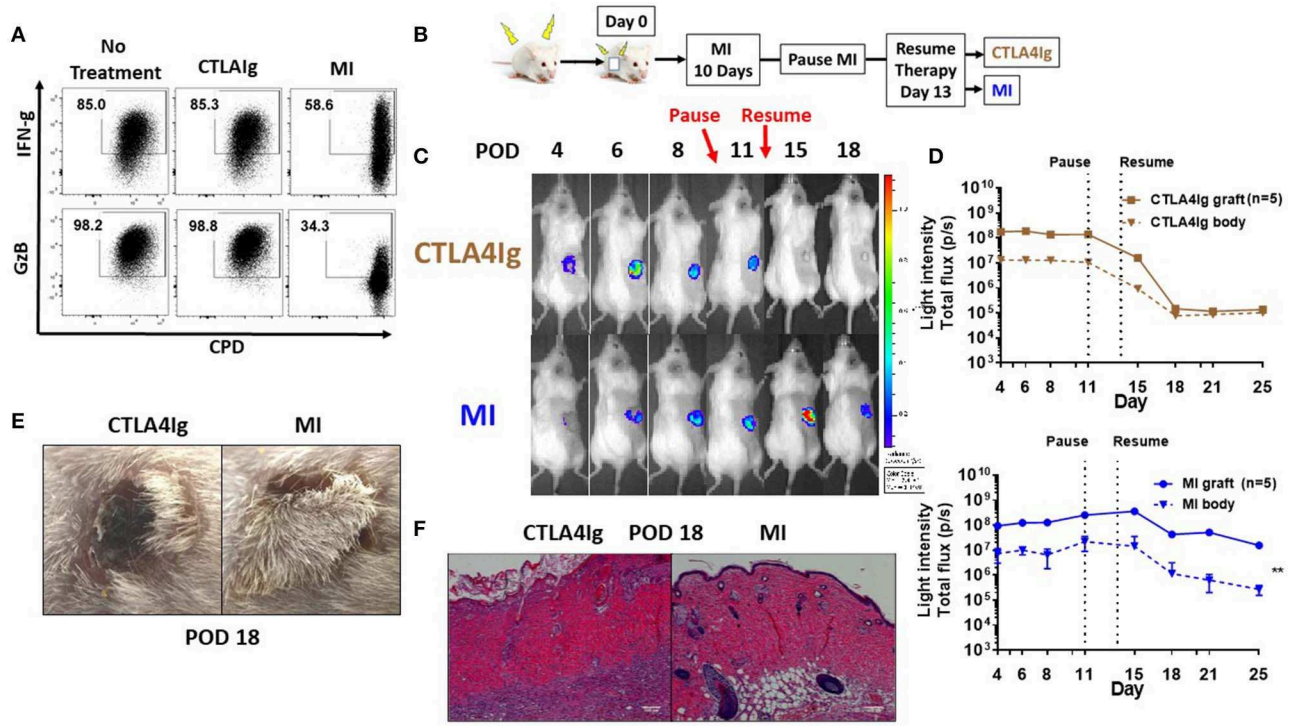

FIGURE 3 | Effect of metabolic inhibitors and CTLA4Ig on pre-activated CD8+ T cells and a model of acute skin allograft rejection**. (A)** Previously activated and resting *in vitro* T cells were re-stimulated for 48 h with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (2 μg/ml) in the presence of CTLA4Ig and MI as in **Figure 1C**. At 36 h, brefeldin A was added to each culture and IFN-gamma (FN-g) and Granzyme B (GzB) production was compared to eFluor 450 dilution in CD8+ T cells. **(B)** Schematic of transplant and treatment model. FVB-Tg(CAG-luc, -GFP)L2G85Chco/J (H2q) to B6(Cg)-Tyrc-2J/J (H2b) complete MHC-mismatched full thickness skin transplantation. MI therapy was started from the day of transplantation (day 0) until POD 10 and stopped for 3 days to induce acute rejection. On POD 14, treatment was resumed with either CTLA4Ig or MI. MI: metabolic inhibitors: 2DG+ metformin + DON. 2DG, 500 mg/kg once daily; metformin 150 mg/kg once daily; DON 1.6 mg/kg once every other day; CTLA4Ig 0.5 mg every other day starting on day 14. **(C)** Representative images from a single mouse for each of the indicated time points. **(D)** Skin graft viability measured by luminescent light intensity (photons/sec). **(E)** Skin graft appearance on POD 18. **(F)** Hematoxylin and eosin stain (X200) of skin grafts on POD 18. $^{**}p < 0.01$ (2-way ANOVA). Data are representative of three experiments **(A)** and two experiments **(B–D)**.

and memory re-call was examined in splenocytes on Day 35, 5 Days after the secondary infection (**Figure 4A**). Consistent with the ability of MI to robustly inhibit clonal expansion, treatment with MI inhibited the expansion of tetramer+ CD8+ T cells more significantly than CTLA4Ig (**Figure 4B**). However, combination with CTLA4Ig resulted in a more profound inhibitory effect and furthermore, a stronger decrease in T-bet expression of those T cells (**Figure 4B**). In addition, we monitored cell proliferation of adoptively transferred CD8+ OTI T cells with acute drug therapy in response to vaccinia-OVA infection. Similar to our *in vitro* findings (**Figure 2**), MI or CTLA4Ig+MI therapy significantly decreased proliferation of transferred CD8+ T cells (**Supplementary Figure 2A**). Also, we observed increased cell death in the undivided T cell population with MI or CTLA4Ig+MI therapy unlike proliferative T cells that typically die during the course of infection seen in the no treatment group (**Supplementary Figure 2B**). Following secondary infection (re-challenge), CTLA4Ig combined with MI not only markedly suppressed memory recall (**Figure 4C**), but also led to a stronger inhibition of IFN-g or Granzyme B secreting CD8+ T cells (**Figure 4D**). Together, these results show that combination therapy of CTLA4Ig with MI had the most robust effect on CD8+ T cell expansion, both during the initial antigen encounter and during memory recall.

Combined CTLA4Ig and MI Prevent Allograft Rejection in Skin Transplantation and Promote Graft Acceptance Upon Stopping Therapy in Heart Transplantation

Having demonstrated the effect of CTLA4Ig and MI in inhibiting acute and memory responses in virus infection, we next examined the ability of the combination therapy in improving Balb/c to C57BL/6 skin and heart allograft survival. Not surprisingly, CTLA4Ig+MI significantly prolonged skin graft survival (**Figure 5A**). Levels of peripherally circulating T cells were analyzed periodically and showed decreased frequency of activated (CD44+) and terminally differentiated (KLRG1+) CD8+T cells during early time points (POD 10 and 20) in the combination group. Regulatory T cells were decreased initially, but then recovered over time (POD 40) (**Figure 5B**). Additionally, CTLA4Ig+MI strongly inhibited IFN gamma production of CD8+ T cells upon re-challenge

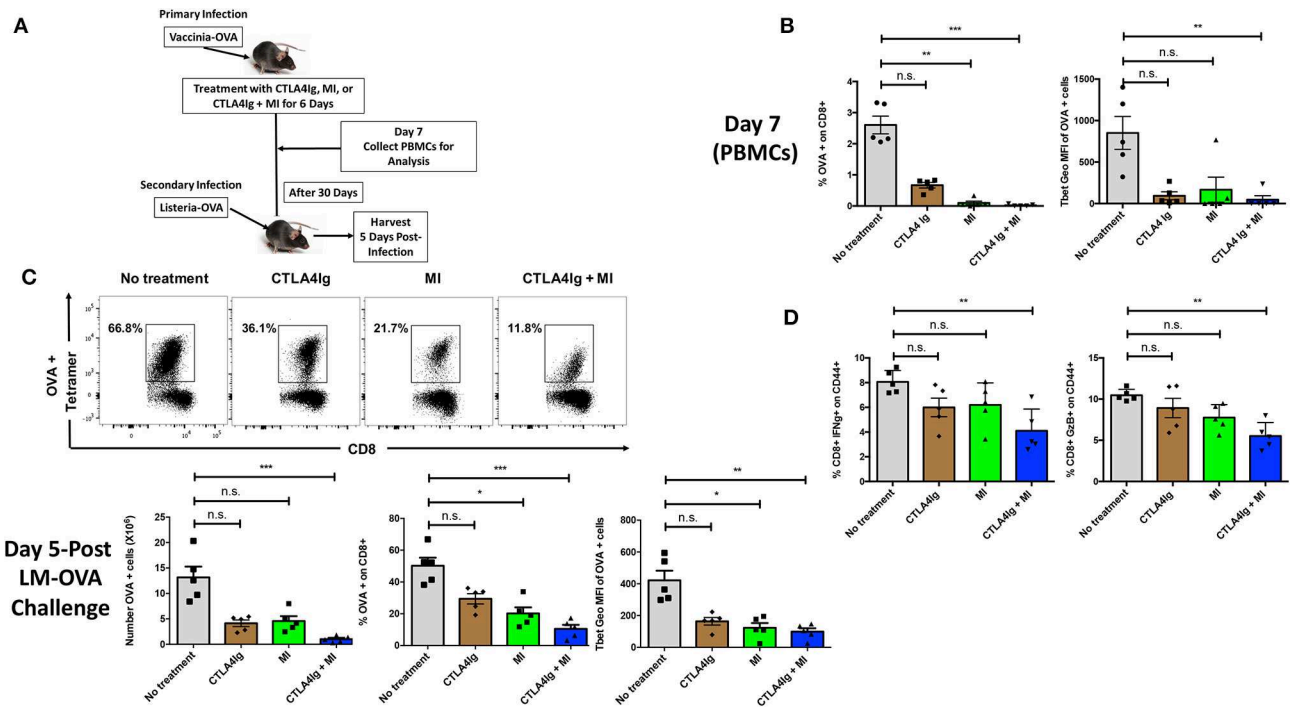

FIGURE 4 | Effect of combining MI and CTLA4Ig on a robust model of T cell effector response. **(A)** Schematic of infection model with treatment. WT C57BL/6 mice were infected with OVA-expressing Vaccinia virus and treated with MI (2DG + metformin + DON; 2DG, 500 mg/kg once daily; metformin 150 mg/kg once daily; DON 1.6 mg/kg once every other day) or MI + CTLA4Ig (CTLA4Ig 0.5 mg once every other day) for 6 days. Peripheral blood was collected on Day 7 to analyze antigen-specific CD8+ OVA tetramer+ T cells. 30 days after the primary infection, the same mice were infected with OVA-expressing Listeria monocytogenes for memory T cell recall response. Five days following the secondary infection, splenocytes were isolated to analyze memory response. **(B)** Frequency and T-bet expression (geometric MFI) of tetramer+ CD8+ T cells on Day 7. **(C)** Total number, frequency and T-bet expression of OVA+ CD8+ T cells after re-challenge. **(D)** Percentage of IFN-gamma (IFN-g) + and Granzyme B (GzB) + CD8+ CD44+ cells after 5 h *ex vivo* re-stimulation with OVA peptide. n.s., not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (one-way ANOVA non-parametric Kruskal–Wallis test). Data are representative of two experiments.

ex vivo (**Figure 5C**) and donor specific antibodies (DSA), were steadily low over time (**Figure 5D**). This correlated with decreased levels of follicular T helper cells (**Figure 5E**), which is in line with data showing that CTLA4Ig interferes with T cell- B cell help and decrease antibody-mediated rejection (43, 44). Thus, in the robust model of skin allograft rejection, combining MI therapy with CTLA4Ig led to prolonged graft survival.

Finally, we tested the CTLA4Ig+MI regimen in heart transplants. Previously, we have shown that continuous therapy with MI alone could achieve more than 100-day graft survival (31). However, stopping treatment resulted in rejection of the hearts 80 days later (data not shown). Therefore, we investigated whether costimulatory blockade could be able to improve the efficacy of short-term anti-metabolic therapy. Consistent with previous preliminary studies, treatment with only 30 days of MI resulted in rejection of the grafts ∼60 days later (**Figure 6A**). In contrast, adding CTLA4Ig the first week after transplant could achieve 100% 100-day graft survival and near 80% 130-day graft survival, which represents 100 days without treatment (**Figure 6A**). Nevertheless, clinical assessment of the beating scores showed a decline in the beating quality of the transplanted hearts, although to a lesser extent than the MI alone group (**Figure 6B**). The addition of CTLA4Ig also resulted in an initial decrease in DSA production that then increased after stopping therapy (**Figure 6C**). Interestingly, histologic examination of the hearts on POD 100 revealed more viable heart tissue with less necrosis and fibrosis in the setting of equivalent lymphocytic infiltration between the groups (**Figure 6D**).

DISCUSSION

In a previous study (31), we demonstrated that metabolic inhibition therapy (MI) in the form of 2-DG (a glucose analog), DON (a glutamine antagonist) and metformin (a diabetes drug) could prevent allograft rejection. However, discontinuation of treatment led to graft rejection. Thus, the overall goal of this study was determine if the addition of CTLA4Ig might promote long-term graft acceptance in the absence of long-term immunosuppressive therapy. To this end, in this current study we have (**i**) Demonstrated distinct immunologic effects between MI and CTLA4Ig therapy (**ii**) The ability of MI therapy to treat acute graft rejection (**iii**) The synergistic ability of MI + CTLA4Ig to prevent skin allograft rejection (**iv**) The ability of short term MI + CTLA4Ig therapy to promote long-term heart allograft acceptance. While our studies specifically focused on the combination of MI + CTLA4Ig, we

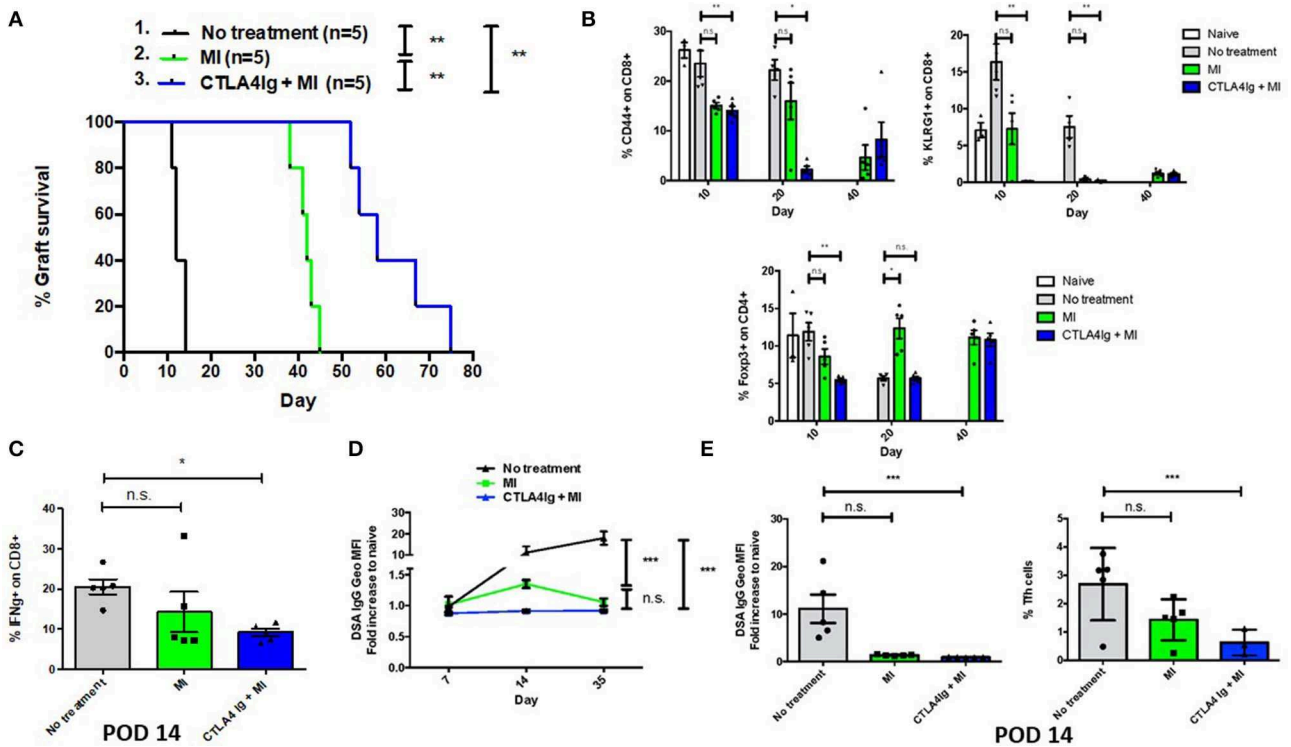

FIGURE 5 | Effect of combining metabolic inhibitors and CTLA4Ig on promoting allograft tolerance in BALB/c (H2d) to C57BL/6 (H2b) complete MHC-mismatched full thickness skin transplantation. The dosage of all drugs was the same as described in **Figure 2D**. Therapy was started from the day of transplantation (day 0) until complete graft rejection. **(A)** Skin allograft survival comparing MI alone with MI + CTLA4Ig. No treatment MST = 12 days; MI MST = 42 days; CTLA4Ig + MI MST = 58 days. **(B)** Dynamics of levels of CD44+ and KLRG1+ activated effector CD8+ T cells and Foxp3+ CD4+ regulatory T cells in peripheral blood over time. **(C)** Percentage of IFN-gamma (IFN-g) + CD8+ cells after 4 h *ex vivo* re-stimulation with PMA and ionomycin **(D)** Levels of donor-specific IgG antibodies (DSA, presented as fold increased relative to naive mice) during early and late post-tranplant period. **(E)** Levels of DSA and frequency of follicular helper T (Tfh) cells (CD4+ ICOS+ PD1+ Bcl6+) in draining LNs on POD 14. n.s., not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (log-rank analysis and one-way ANOVA non-parametric Kruskal–Wallis test). Data are representative of two experiments.

believe that these results provide the rationale for employing metabolic therapy as a platform for a potential wide variety of tolerance-inducing regimens.

CTLA4Ig costimulatory blockade is currently widely used in clinical organ transplantation (45–48). Recent long-term clinical trials in kidney transplant patients have shown improved graft function, better cardiovascular/metabolic risk profile and similar patient and graft survival when compared to CNIs. However, these trials also noticed higher rates of acute rejection within the first months post-transplantation (21, 22). The acute rejection episodes were not typically associated with DSA (49). T cell mediated acute cellular rejection may be alternatively involved and our study demonstrated that anti-metabolic therapy had a more profound effect on suppressing effector T cell function during acute rejection or acute viral infection. Indeed, similar to other immunosuppressive regimens the profound ability of metabolic therapy to block anti-allograft clonal expansion would also suppress clonal expansion in response to concomitant infections. Nonetheless, metabolic therapy has a number of advantages over treatment regimens that include CNIs. First, a major problem of conventional immunosuppression with CNI's is Post-transplant lymphoproliferative disorder (PTLD) as well as the reactivation of herpes viruses such as CMV. Metabolic therapy on the other hand directly inhibits herpes virus activation as well as inhibits cancer cell growth (50, 51). Second, in as much as CNI's inhibit tolerance induction, transplant patients require life-long immunosuppression. Metabolic therapy promotes tolerance thus potentially mitigating the need for prolonged immunosuppression and hence susceptibility to infection. Finally, from a clinical perspective CNIs (and steroids) are associated with multiple adverse events including hyperglycemia, accelerated atherosclerosis, gastro intestinal bleeding, neurotoxicity, and nephrotoxicity all of which are avoided and in some cases (for example metformin and hyperglycemia) improved with metabolic therapy.

Further, while CTLA4Ig was associated with decreased TCR-induced activation, MI was associated with a marked decrease in clonal expansion. This decrease was mechanistically secondary to both a decrease in proliferation and an increased in activation induced cell death. Along these lines the initial antigen-specific T-cell precursor frequency has been shown to be an important factor in determining the effectiveness of CTLA-4Ig in a murine model of transplantation suggesting that patients with an initially high precursor frequency of alloreactive T cells (poor major histocompatibility complex donor and recipient matching) might also be particularly refractory to treatment with CTLA4Ig

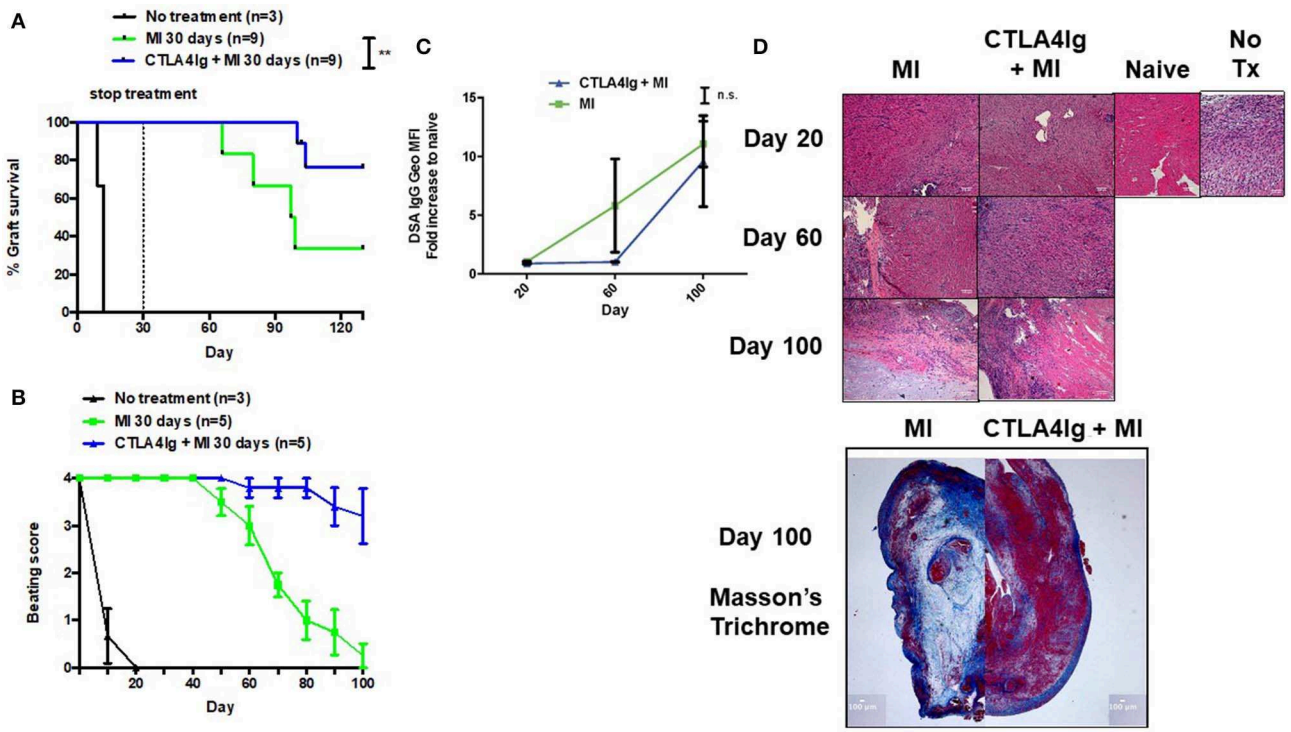

FIGURE 6 | Effect of combining short-term metabolic therapy and CTLA4Ig on BALB/c (H2d) to C57BL/6 (H2b) complete MHC-mismatched heterotropic heart transplantation. The dosage of all drugs was the same as described in **Figure 2D**. Metabolic therapy was started from the day of transplantation (day 0) until POD 30. **(A)** Heart allograft survival. **(B)** Clinical assessment of the heart grafts by cervical or abdominal palpation (grade 0–4). **(C)** Levels of DSA over time. **(D)** Hematoxylin and eosin stain (X200) of heart grafts at POD 20, 60, and 100. Masson's trichrome stain (X20) of heart grafts at POD 100. n.s., not significant, $^{**}p < 0.01$ (log-rank analysis and one-way ANOVA non-parametric Kruskal–Wallis test). Data pooled from two experiments.

costimulatory blockade (52). Our data suggest that the addition of MI therapy can help overcome this hurdle. Furthermore, we observed that combination therapy led to further decreased DSA formation by decreasing follicular T helper cells and abrogating T cell- B cell help. Likewise, MI therapy led to a decrease in mTORC1 activation that could further contribute to the ability of MI + CTLA4Ig to promote long-term graft acceptance.

In as much as we observed graft acceptance in the absence of continued immunosuppression, our results suggest that MI + CTLA4Ig can induce a state of "functional" tolerance. That said, it should be noted that upon stopping therapy we observed an increase in DSA and that in spite of having less fibrosis and more functional heart tissue in the MI + CTLA4Ig treated hearts when compared to the MI treated hearts, we did observe equivalent lymphocytic infiltration POD 100. Also, secondary skin transplants to those recipients in the combination group that survived more than 150 days showed normal rejection of the skin grafts (data not shown), indicating that robust tolerance was not induced. However, neither the heart graft of those recipients rejected after the secondary re-challenge nor the beating quality further decreased. To this end, while our work supports the concept of MI therapy as a platform for inducing tolerance, the details of robustly achieving this goal remain to be determined.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All animal procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University.

AUTHOR CONTRIBUTIONS

GB and JP designed research. C-HC, C-FL, BO, GF, and CP performed research. C-HC analyzed data. C-HC and JP wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00572/full#supplementary-material

Supplemental Figure 1 | *In vivo* bioluminescence to assess changes of skin allograft viability during the course of alloimmune rejection. FVB-Tg(CAG-luc,-GFP)L2G85Chco/J (H2q) to FVB/N (H2b) and B6(Cg)-Tyrc-2J/J (H2b) syngenic and allogenic full thickness skin transplantation. **(A)** Representative images from a single mouse for each of the indicated time points. **(B)** Skin graft viability measured by luminescent light intensity (photons/sec). **(C)** Skin graft appearance on POD 12. Data are representative of two experiments.

Supplemental Figure 2 | Effect of metabolic inhibitors (MI) and CTLA4Ig on T proliferation and cell viability in response to viral infection. WT C57BL/6 mice were infected with OVA-expressing Vaccinia virus prior to receiving different congenic marked ef450-labeled CD8+ OTI T cells. Mice were then treated with MI (2DG + metformin + DON; 2DG, 500 mg/kg once daily; metformin 150 mg/kg once daily; DON 1.6 mg/kg once daily) or MI + CTLA4Ig (CTLA4Ig 0.5 mg once) for 2 days. On Day 2, spleens were harvested to analyze CPD dilution and cell viability of donor CD8+ T cells. **(A)** Percent proliferation based on CPD dilution of donor CD8+ T cells. **(B)** Percent death seen in undivided cells vs. divided cells $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ $n = 5$–8 per group (one-way ANOVA non-parametric Kruskal–Wallis test). Data are representative of three independent experiments.

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REVIEW
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Targeting Signal 3 Extracellularly and Intracellularly in Graft-Versus-Host Disease

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Allogeneic hematopoietic stem cell transplantation (allo-HCT) holds curative potential for many hematological disorders. However, the pathophysiology of the desired graft-versus-tumor effect is linked to life-threatening complications of acute graft-versus-host disease (GVHD). Allogeneic donor T lymphocytes are essential for causing GVHD, and their activation relies on the coordination of TCR engagement and co-stimulation, also known as Signal 1 and Signal 2. In addition to these signals, a network of secreted cytokines by immune cells provides a third signal, Signal 3, that is critical for the initiation and maintenance of GVHD. Strategies to target Signal 3 in human diseases have shown therapeutic benefit for inflammatory disorders such as Rheumatoid Arthritis and Inflammatory Bowel Disease. However, despite our growing understanding of their role in GVHD, the success of targeting individual cytokines has been modest with some notable exceptions. This review aims to describe current approaches toward targeting Signal 3 in clinical GVHD, and to highlight emerging studies in immune cell biology that may be harnessed for better clinical translation.

Keywords: bone marrow transplantation, graft-versus-host disease, cytokines, alloimmunity, intracellular trafficking

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INTRODUCTION

Acute Graft-versus-Host Disease (GVHD) is a major cause of non-relapse morbidity and mortality in patients receiving allo-HCT. While the development of GVHD is dependent on numerous factors, the HLA major and minor antigen-induced activation of donor-derived T cells is the key determinant for induction and severity of GVHD (1). T cell activation occurs as a result of the engagement of three signals (2, 3). Signal 1 is provided by the T cell receptor with cognate peptide:HLA, and Signal 2 follows the engagement of T cell co-stimulatory receptors by cognate ligands on antigen presenting cells (2). Interaction with both antigen and co-stimulatory ligands is critical for initiating the intracellular signaling cascade that promotes T cell proliferation, survival, and effector functions, and therefore a number of approaches aim to target these steps in the prevention and treatment of GVHD (4–6). In addition to these two signals, Signal 3 which is provided by surrounding cytokines controls the differentiation of T helper (Th) subsets, influences the polarization of specific T effector responses, and shapes the balance between immune activation and tolerance (3, 7, 8).

The cytokine milieu in patients following bone marrow transplantation is complex, and includes both immune cell and target tissue sources. It is released following conditioning treatments and

amplified by tissue destruction from T cell-mediated lysis (9). In both experimental models of allo-HCT and patients affected by GVHD, the cytokines that provide Signal 3 significantly impact the alloreactive T cell response (10). Selectively targeting the cytokines that promote alloreactive T cells is therefore an attractive therapeutic strategy.

Many existing therapies for GVHD are aimed at targeting donor T cells or inflammatory byproducts of immune cells which contribute to symptoms and pathology. However, donor T cells are also critical for the graft-versus-tumor (GVT) effect, and as such, balancing therapies has been a challenge to preserve sufficient GVT activity while minimizing GVHD-related tissue damage. One strategy has been to target the cytokines that promote alloreactive T cell toxicity and also cause direct inflammation-related organ damage. While the success of these approaches has been modest thus far, a growing basic science understanding of relevant cytokines, the regulation of cytokine secretion, and the specific impact each has on immune and target cells will inform future strategies for the prevention and treatment of GVHD.

Several outstanding recent articles have reviewed the biology and the important role of cytokines in both acute and chronic GVHD (10–13). In this review, we will only highlight cytokines that serve as Signal 3 to T cells (**Figure 1**) and have been targeted in clinical acute GVHD. Specifically, we will first briefly review approaches that directly target Signal 3 secreted cytokines, the majority of which have been targeted upon their release extracellularly (i.e., post-synthesis and release, in the extracellular space). We will then focus on therapies that target the induction of cytokine synthesis intracellularly in immune cells, focusing on specific cell signaling pathways that lead to cytokine synthesis (i.e., pre-synthesis by targeting intracellular signaling cascades). Finally, we will review an as yet understudied area to target the cytokines following their synthesis intracellularly, including post-translational pathways, and the intracellular trafficking pathways that regulate their release. We will discuss why understanding the pathways by which these cytokines are transported intracellularly may represent an effective approach toward the rational design of GVHD therapies.

DIRECT T CELL INTRINSIC CYTOKINES AND PROLIFERATIVE RESPONSES

Strategies to control donor T cell activity begin with broadly acting anti-inflammatory prophylactic agents. The most widely used approaches today include methotrexate in combination with cyclosporine or tacrolimus. Methotrexate, a folate antagonist, can target rapidly proliferating allogeneic T cells and be cytotoxic to their growth. Cyclosporine and Tacrolimus inhibit the calcineurin-dependent activation of NFAT transcription factors and their translocation from the cytoplasm to the nucleus, reducing the transcription of inflammatory cytokines such as IL-2 and IFNγ by T cells. Targeting the immune response at this level inhibits key T effector functions as well as their proliferation.

While instrumental in reducing GVHD risk, however, standard prophylaxis measures are not completely effective in preventing the onset of GVHD. Furthermore, they confer non-specific anti-inflammatory functions that can increase the risk of tumor relapse and infection. Systemic glucocorticoids which remain the mainstay of first-line treatment of acute GVHD is also broadly immunosuppressive. However, T cells are responsive to the influence of select cytokine signals which promote their growth, proliferation, cytotoxicity, and secretion of effector molecules. Important signals include cytokines that promote inflammation and which also tend to be increased following conditioning and allo-HCT such as IL-12, IL-4, IL-1, TNFα, and IL-6 (14, 15). Therefore, agents that attenuate the cytokine signals that promote the overactivity of T cells could be beneficial in GVHD treatment. While all of these cytokines have been shown to be critical sources of Signal 3, agents that block TNF, IL-6, and IL-1, as well as the T cell-derived growth factor IL-2 have been studied as potential modes of treatment in acute GVHD. There remains active interest in the use of specific anti-inflammatory cytokine blockade including agents that directly target activating cytokine signals to T cells extracellularly.

EXTRACELLULAR SIGNAL 3 BLOCKADE

Anti-TNF

Tumor Necrosis Factor (TNF) is a cytokine that acts on multiple immune cell types, and promotes the production of other Signal 3 cytokines including IL-1 and IL-6 (16). Overexpression of TNF is implicated in the development of multiple autoimmune and inflammatory disorders, and its blockade has improved disease management and quality of life for patients with rheumatoid arthritis, inflammatory bowel disease, and others (17). In addition to innate immune cell-derived TNFα, T cell-derived TNFα is implicated in the development of GVHD in experimental murine models, and in humans, it has been observed that early increased serum levels of TNFα are associated with major transplant-related complications (18, 19). TNFα levels are initially increased following conditioning treatments as measured by levels of TNF receptor-1 which correlate with those of plasma TNFα (20, 21). TNFα is appreciated to play direct roles in acute GVHD pathogenesis by both effecting direct tissue damage and by promoting allogeneic T cell cytotoxicity (22). Therefore, the use of neutralizing anti-TNFα agents has been studied for efficacy in GVHD prevention and treatment. One such agent is infliximab, an anti-TNFα monoclonal antibody that has been studied in prophylaxis, and treatment of steroid-refractory acute GVHD (SR-aGVHD) (23, 24). However, in patients with SR-aGVHD, the addition of infliximab did not improve survival and instead increased post-transplant risk of infection when compared to treatment with corticosteroids alone (24, 25). Clinical trials have also been performed to test the efficacy of etanercept for the blockade of soluble TNF. However, early results were not borne out by a multi-center randomized study that demonstrated no impact of etanercept on GVHD or on overall survival, infection, and relapse of the primary malignancy (26–30). While TNFα is best known for its inflammatory properties in promoting T cell effector responses, its dual role as a suppressive cytokine is increasingly being appreciated with the characterization of

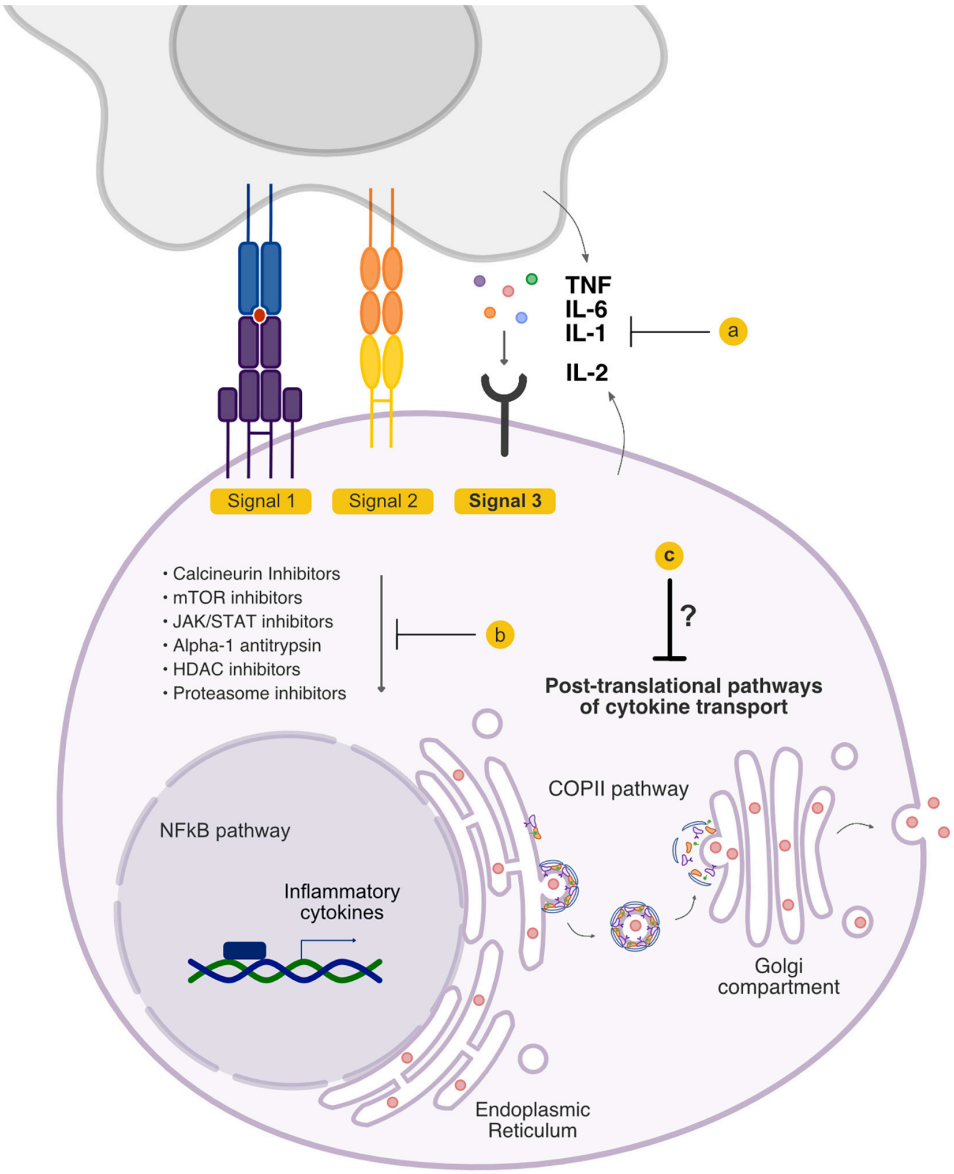

FIGURE 1 | Signal 3 pathways that are targeted in graft-versus-host disease (GVHD). Signal 3 critically shapes the allo-response, and existing treatment options have the potential to modulate the cytokine milieu that accompanies allogeneic T cell activation. Current treatment strategies include **(a)** blockade of cytokines extracellularly and blockade of cytokine receptors, and **(b)** inhibition of the downstream signaling cascades that culminate in the production of inflammatory cytokines. **(c)** The pathways that regulate cytokine secretion following their synthesis but preceding their release have not been therapeutically targeted.

functionally disparate TNF receptors and their actions on different cell types such as T regulatory cells (Tregs) (31). While the vast majority of current therapies target TNF directly, future studies of specific inhibitors of TNF production, signaling, and oligomerization, may clarify their potential in the treatment of GVHD (17).

Anti-IL-6

IL-6 is a member of a family of cytokines that shares the receptor complex gp130, a widely expressed signaling complex that leads to the activation of associated Janus kinase (JAK) and signal transducer and activation of transcription (STAT) pathways. Levels of circulating IL-6 can increase dramatically in settings of inflammation, and consequently, IL-6 is associated with the acute phase inflammatory response (32). The biological consequences of IL-6 are wide-ranging and include the pathologic stimulation of proinflammatory responses, such as by promoting Th17 cell development and inhibition of regulatory T cell differentiation (33). In murine models of GVHD, donor T cell-derived IL-6 critically contributes to disease severity, and donor T cell-specific deficiency of IL-6 decreases GVHD-related mortality (34). Blockade of the IL-6 signaling in experimental models also improved GVHD survival and led to an increase in regulatory T cells, and decreased Th1 and Th17 cells in target organs while

preserving GVT effects (34, 35). A clinical study examining the effect of tocilizumab, a humanized monoclonal antibody against the IL-6 receptor, showed favorable outcomes in a small number of patients with either acute or chronic GVHD (36). A phase I/II clinical trial further studied the effect tocilizumab administered 1 day prior to allogeneic peripheral blood stem cell transplantation in patients that received cyclosporine and methotrexate as GVHD prophylaxis (37). A phase II trial in which patients received busulfan-based conditioning prior to receiving tocilizumab with tacrolimus and methotrexate showed a low incidence of gastrointestinal GVHD (38). Recently, a phase III randomized and double-blinded trial observed a trend toward a reduced overall incidence of grade II-IV GVHD in patients receiving tocilizumab, but no difference in long term survival compared to controls (39).

Anti-IL-1

In addition to TNF and IL-6, IL-1 is an inflammatory cytokine that is increased following conditioning and is critical for immune homeostasis, but when dysregulated, potentiates GVHD pathology (10). Although small early studies showed that targeting soluble IL-1 with a recombinant human IL-1 receptor or administration of a recombinant IL-1 receptor antagonist could ameliorated SR-aGVHD, these results were not confirmed in a randomized controlled trial (40–42). Given its known potency in inflammatory disorders and association with GVHD in both experimental models and humans, strategies that target IL-1 may be effective depending on the phase of acute and/or chronic GVHD (43, 44). Key upstream regulators of IL-1 include intracellular immune sensors NLRP3 and NLRP6, which assemble into inflammasomes in settings of cellular damage and stress such as those induced by pre-transplant conditioning therapies. Following allogeneic transplant in experimental models, NLRP3 inflammasomes induced the secretion of pathogenic levels of IL-1β by multiple intestinal cellular sources, and also controlled the IL-1β-dependent skewing of Th17 differentiation critical to the development of GVHD (44). By contrast, donor myeloid derived suppressor cells, which have immunoregulatory functions in GVHD, can lose suppressive capacity following activation of the inflammasome (45). Thus, the cellular source is an important determinant of the impact of inflammasome dependent effects on GVHD. In host non-hematopoietic target tissues, NLRP3 inflammasomes serve a protective role in promoting intestinal epithelial cell integrity and repair by increasing IL-18 secretion (46). NLRP6, which has protective roles in intestinal colitis, plays a role in aggravating gastrointestinal GVHD when expressed in host-non-hematopoietic tissue, and its absence in host intestinal epithelial cells helps maintain gut homeostasis following allogeneic BMT in experimental models (47). It is likely that the effects mediated by NLRP6 may be IL-1 independent or dependent depending on the type of immunopathology.

Anti-IL-2

IL-2 expression is increased upon activation, is released by T cells, and serves as a growth factor for T effector cells and Tregs. IL-2 is one of the earliest cytokines to be studied as a target for immunosuppression therapeutically (48–50). Intracellular targeting of the production and secretion of IL-2 with calcineurin inhibitors remains the first line prophylaxis strategy in the prevention of GVHD. Studies have also explored the use of monoclonal antibodies against IL-2 receptor including daclizumab, basiliximab, and inolimomab, to target the activity of secreted IL-2. One randomized trial found that the addition of daclizumab to corticosteroids as an initial therapy for acute GVHD resulted in increased GVHD-related mortality (51). A phase II study found that while treatment of SR-aGVHD with daclizumab led to an increased complete response rate, it was associated with higher rates of long-term complications of chronic GVHD (52). Although basiliximab appears to be better tolerated by patients and not associated to the same degree of adverse events in initial studies, future studies are needed to determine its safety and efficacy (53–55). Targeting IL-2 is nuanced by its dual roles, as in addition to promoting the T cell-mediated toxicity in GVHD, it is essential for the development and maintenance of Tregs which are important regulators of immune tolerance, and may in turn be employed in the prevention of GVHD (56–60). Therefore, efforts to target IL-2 must balance its inflammatory and immunoregulatory effects that minimize GVHD but still prevent relapse of the primary disease. The administration of low-dose IL-2 is of interest in the treatment of chronic GVHD, and has been associated with expansion of Tregs, suppression of conventional T cell proliferation, and long-term reduction of chronic GVHD symptoms (61–63).

TARGETING THE INTRACELLULAR AFFERENT ARM OF SIGNAL 3 CYTOKINE RELEASE

Following their activation, T cells engage distinct signaling pathways that lead to the increased synthesis of important effector molecules including cytokines and cytotoxic factors. These culminate in a pro-inflammatory milieu that shapes the allogeneic T cell response and also causes direct tissue damage. While targeting cytokines following their release by immune cells is gaining increasing interest for their promising outcomes in both experimental models and clinical trials, there has been renewed interest in targeting earlier steps following T cell activation such as the intracellular signaling pathways that increase cytokine production. Therapeutic strategies have included targeting the intracellular signaling pathways that lead to proinflammatory cytokine transcription and translation with agents such as calcineurin inhibitors as described above, mTOR inhibitors, JAK inhibitors, Alpha-1 Antitrypsin, histone deacetylase inhibitors, and proteasome inhibitors.

mTOR Inhibition

The mammalian target of rapamycin (mTOR) pathway is a major regulator of cellular growth and metabolism that is also critical for T cell activation, differentiation, and function (64). Sirolimus, an inhibitor of mTOR, has been demonstrated to exhibit anti-inflammatory effects through multiple mechanisms including inhibition of both conventional T cell and dendritic cell activity,

and promotion of Treg development (65–67). Early studies showed that sirolimus can be well tolerated in patients and may be associated with a lower risk of GVHD (68, 69). A prospective randomized trial found that in combination with tacrolimus, sirolimus is a safe alternative to cyclosporine and methotrexate for GVHD prophylaxis (70). A recent phase III trial reported that the addition of sirolimus to cyclosporine and mycophenolate mofetil for prophylaxis showed efficacy in lowering the incidence of GVHD (71). However, its efficacy as a therapy for SR-aGVHD in combination with other agents may be limited depending on the stage of GVHD, and warrants further studies (72).

JAK1/2 Inhibition

T cells are responsive to inflammatory cytokines including IL-6 and interferons via their propagation of JAK/STAT pathways. Activation of the JAK family of proteins leads to the phosphorylation of STATs, which translocate to the nucleus and are critical regulators of T cell alloreactivity (73). Pre-clinical models demonstrated that targeting JAK1/2 targets GVHD but preserves GVL, with the contribution of decreased serum levels of proinflammatory cytokines including IL-6 (74–76). This led to testing the effects of JAK inhibitors such as ruxolitinib, baricitinib, and itacitinib. Ruxolitinib, a selective inhibitor of JAK1/2, in patients with SR-aGVHD. In an early study, six patients experienced reduced GVHD in correlation with a decrease in proinflammatory cytokines in the serum (75). Additional clinical trials are underway to examine the effects of ruxolitinib in patients with SR-aGVHD (77). Itacitinib, a selective JAK1 inhibitor, has also demonstrated safety in a phase I trial and studies of its efficacy in the treatment of SR-aGVHD are ongoing (78).

Alpha-1 Antitrypsin

Alpha-1 Antitrypsin (AAT) is an endogenously circulating serine protease inhibitor that, when deficient or mutated, has been described in the pathogenesis of disorders including COPD, cirrhosis, and multiple neurodegenerative diseases (79). In addition, AAT has a suppressive role in inflammatory settings with an appreciable inhibitory effect on TNF levels (80, 81). When AAT is administered in models of murine allo-HCT, it has been shown to reduce GVHD-induced mortality while preserving the allogeneic T cell GVL effect (82–84). The therapeutic benefit in these models has been linked to a decrease in alloreactive effector T cells and inflammatory cytokines, and an increase in Tregs and immunoregulatory cytokines such as IL-10 (84). AAT is an effective modulator of the profile of circulating cytokines following allo-HCT leading to significantly reduced disease murine models, underscoring the therapeutic potential of AAT and strengthening the rationale for studying the effect of AAT therapy in humans. Recent studies showed complete recovery in 4 of 12 patients and improvement in the other 8 patients with SR-aGVHD (85). A prospective multi-center study that followed tested AAT as a first line therapy for SR-aGVHD led to an overall response rate of 65% and complete response rate of 35% by day 28 (86). Ratios of T effector cells and Tregs were consistent with those observed in experimental models (86). Both studies found that AAT is well tolerated by patients, is not associated with an excessive risk of infection, and are now being studied in a randomized manner in a phase III study.

Histone Deacetylase Inhibition

Histone deacetylase (HDAC) inhibitors represent a diverse class of drugs that cause reversible inhibition of HDAC enzymes, remodel chromatin structure, and differentially modify gene expression depending on the specific HDAC, cell type, and context. A clinically significant consideration of HDAC inhibitors is that in addition to acting on histones, they can have non-specific effects on other protein deacetylases that broadly regulate cell growth and signaling (87). However, at non-cytotoxic doses, HDAC inhibitors have recently been appreciated to be well tolerated and exhibit immunoregulatory properties, lending to growing interest in their potential to treat inflammatory diseases (88). Among their diverse effects, HDAC inhibitors have shown immunomodulatory effects on dendritic cell and macrophage antigen presentation, TLR pathways, and IFN signaling (88). As a consequence, they can reduce the expression of cytokines involved in Th1 and Th17 differentiation such as IL-6 and IL-12 (89, 90). In experimental models of GVHD, HDAC inhibition has been observed to lead to reduced secretion of proinflammatory cytokines including IL-12, IL-6, and TNFα by dendritic cells through enhancing the expression of indoleamine 2,3 dioxygenase (91–93). Two phase II clinical trials have examined oral HDAC inhibitor vorinostat in the prevention of GVHD. One study investigated the addition of vorinostat to tacrolimus and mycophenolate in patients that received reduced intensity conditioning prior to related donor hematopoietic stem cell transplantation (94). Another study tested the effect of vorinostat when combined with tacrolimus and methotrexate following myeloablative conditioning prior to unrelated donor allo-HCT (95). Both studies showed that vorinostat is well tolerated and associated with a lower incidence of acute GVHD (94, 95). A third study is ongoing to evaluate vorinostat as preventive therapy in adolescents and young adults receiving allogeneic BMT when combined with standard preventive therapy (NCT03842696). Future studies will elucidate the clinical benefit of HDAC inhibitors including vorinostat, as well as other agents such as panobinostat that are more recently being evaluated as primary therapy for acute GVHD (96).

Proteasome Inhibition

The ubiquitin proteasome pathway is central to the selective of maintenance of proteins, and regulates a diverse set of intracellular processes including quality control for misfolded proteins, regulation of the cell cycle, and peptide processing for antigen presentation (97). In immune cells, the proteasome is also involved in cell signaling, notably by regulating the expression of NF-κB, a transcription factor that promotes cell survival and the expression of numerous inflammatory cytokines (98). Proteasome inhibitors have thus emerged as a drug class that is associated with a number of immunomodulatory effects, and is currently approved for the treatment of a number of hematologic disorders (99). Proteasome inhibitors have been shown suppress NF-κB activation, in part due to

the reduction of proteasome-dependent degradation of IκB (100, 101). The inhibition of NF-κB is associated with reduced proliferation, survival, and toxicity of allogeneic T cells, and has also been shown to abrogate T cell cytokine production (102, 103). In addition to its effect on T cells, proteasome inhibitors such as bortezomib have suppressive effect on dendritic cell maturation and inflammatory cytokine production, while increasing dendritic cell apoptosis, highlighting their influence on multiple processes and cell types (104). In murine models of acute GVHD, treatment of recipients with bortezomib led to increased survival and protection from GVHD while maintaining GVT activity (105, 106). However, the timing of bortezomib administration may be critical in determining its efficacy as well as its overall safety, as delayed administration (i.e. 5 or more days after BMT) compared to 0 to 3 days following BMT results in increased gastrointestinal toxicity. This mechanistically correlates in other studies with amplified IL-1β production by dendritic cells (107, 108). While an early phase I/II study to test a prophylaxis regimen of bortezomib combined with tacrolimus and methotrexate showed that this combination was well tolerated and associated with a lower incidence of GVHD, a randomized controlled trial failed to show an improvement in grade II-IV acute GVHD incidence with the addition of bortezomib, compared to methotrexate and tacrolimus alone (109, 110). Another proteasome inhibitor ixazomib improves acute GVHD upon early administration, impairs dendritic cell development, cytokine production, and expression of co-stimulatory molecules consistent with reduced proliferation of T cells, and clinical trials are underway to determine its efficacy in post-transplant patients (111).

TARGETING THE INTRACELLULAR EFFERENT ARM OF SIGNAL 3 CYTOKINE RELEASE

The majority of pre-clinical studies have provided the foundation for the development of therapies that target cytokines or cytokine receptors directly, or the signaling pathways that govern their transcription and translation. A gap in knowledge remains, however, in the post-translational intracellular pathways that coordinate the transport mechanisms that regulate cytokine release by immune cells. Multiple transport steps coordinate the membrane biogenesis, transport, and fusion events that carry cytokines between intracellular compartments and toward the cell surface for secretion. Better understanding of these intracellular secretory pathways utilized by cytokines in immune cells may provide important insights into novel therapeutic targets.

The post-Golgi Apparatus Transport of Cytokines

In the classical secretory pathway, proteins are co-translationally inserted into the endoplasmic reticulum (ER), and transported to the Golgi compartment where they undergo further processing and are delivered to other intracellular compartments, or the extracellular space. Activated T cells undergo morphologic changes that affect intracellular cytokine transport by first establishing polarity and forming immune synapses with antigen presenting cells. A dynamic cytoskeleton enables T cells to both adhere to the APC, and transport secretory vesicles containing cytokine cargoes (112). $CD8^+$ T cells engaged with cognate APCs reorient their microtubule organizing center toward the immunological synapse, and transport secretory granules along microtubules toward the point of cell-cell contact for targeted lysis of the APC (113). $CD4^+$ T cells remain less well characterized in their regulated secretory pathways than $CD8^+$ T cells. However, distinct post-Golgi pathways have been elucidated, including a directional pathway that directs cytokines toward the immunological synapse and minimizes non-specific cytokine release, and a multi-directional pathway to promote more generalized inflammation (114). Studies to elucidate the molecular mediators of regulated T cell secretion may enable novel approaches toward controlling targeted cytokine release in disease states. These studies and others underscore that in addition to cytokine expression, regulation of the membrane-bound organelles that transport them significantly impact the consequences of T cell activation. However, to date, studies have been limited to understanding the secretory pathway of cytokines through events that occur after their egress from the Golgi apparatus.

Targeting Early Intracellular Phases of the Efferent Arm of Signal 3 Release

About one-third of encoded proteins are estimated to be targeted to the ER and destined for the secretory pathway (115, 116). Coat Protein Complex II (COPII), a complex of five highly conserved proteins (Sec23/Sec24, Sec13/Sec31, and Sar1), assembles at the ER membrane and forms vesicles that incorporates proteins for transport to the Golgi compartment, including many secreted proteins (**Figure 1**). The molecular components of COPII were first described in *Saccharomyces cerevisiae* (117, 118), and COPII-mediated ER-Golgi transport is conserved in all eukaryotes including humans (119). As our understanding of the COPII-dependent secretory pathway increases, the characterization of cell- and context-specific activities and regulation of protein secretion will be critical. Fundamental gaps remain in our knowledge about the role of the early secretory pathway in specific cytokine secretion, and the relevant molecular regulators of this process by immune and other cells. Recently, we have begun to decipher the role of the COPII pathway in the release of cytokines by T cells. We observed that disrupting COPII coat formation by targeting SEC23 results in greatly reduced pathogenicity of donor T cells in experimental models of GVHD (120). Future studies on how the COPII pathway regulates secretion of critical Signal 3 cytokines may further shed light on immune cell secretory pathways and provide insight into potential novel therapeutic targets.

Targeting the Timing of Signal 3 for Mitigating GVHD

Cytokine secretion and its downstream effects are dynamic and context dependent. Signal 3 cytokines are typically studied and understood as discussed above in the context of APC activation and induction of T cell response. The role of signal 3 in the perpetuation of an ongoing T cell response is unclear. Based on the known data the timing of targeting signal 3, it may be critical for mitigating GVHD. Specifically, given its role in induction of allogeneic T cell response, it may be more effective to target signal 3 in prevention strategies for either incidence of GVHD or in preventing steroid-refractoriness following onset of severe GVHD. However, because cytokine cascades and inflammatory responses may wax and wane, the exact timing will need to be carefully determined experimentally and in clinical studies.

CONCLUDING REMARKS

The relevance of cytokines that serve as Signal 3 for robust T cell responses is increasingly well established in their role in promoting GVHD, and as promising therapeutic targets. However, current approaches have yielded modest success and additional strategies are warranted. Moving forward, identifying shared intracellular trafficking pathways that control cytokine release may be of value in developing newer approaches to target Signal 3. Basic science research on the fundamental and critical determinants of intracellular trafficking pathways that coordinate their release remain to be understood. With a better mechanistic understanding of these pathways, the identification of key molecular mediators in the allogeneic setting will be essential. Exploring these questions will both enhance our fundamental understanding of immune regulation, and may pave the way for controlling T cell immunity in inflammatory disorders.

AUTHOR CONTRIBUTIONS

SK and PR wrote and edited the manuscript.

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REVIEW
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Innate Functions of Dendritic Cell Subsets in Cardiac Allograft Tolerance

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Survival rates after heart transplant have significantly improved over the last decade. Nevertheless, long-term allograft viability after 10 years remains poor and the sequelae of transplant-associated immunosuppression increases morbidity. Although several studies have implicated roles for lymphocyte-mediated rejection, less is understood with respect to non-major histocompatibility, and innate immune reactivity, which influence graft viability. As immature and mature dendritic cells (DCs) engage in both Major Histocompatibility Complex (MHC)-dependent and MHC-independent immune responses, these cells are at the crossroads of therapeutic strategies that seek to achieve both allograft tolerance and suppression of innate immunity to the allograft. Here we review emerging roles of DC subsets and their molecular protagonists during allograft tolerance and allograft rejection, with a focus on cardiac transplant. New insight into emerging DC subsets in transplant will inform novel strategies for operational tolerance and amelioration of cardiac vasculopathy.

Keywords: dendritic cell, transplant, tolerance, innate, cardiac

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CLINICAL RELEVANCE

The volume of heart transplants performed worldwide has continued to rise as surgical transplantation remains a standard of care for patients with advanced heart failure (1). According to a recent report, 3,273 heart transplants were performed in the United States in 2017 in addition to a continued increase in new listings for transplantation (2). While cutting-edge surgical tools and advances in immunosuppression have improved acute posttransplant mortality, significant morbidity is still experienced by heart transplant recipients.

Chronic allograft vasculopathy (CAV), an accelerated version of atherosclerosis characterized by diffuse thickening of arterial walls, remains a noteworthy cause of long-term graft attrition with 29.3% of transplant recipients experiencing CAV 5 years post-transplant and an astounding 47.4% of patients within 10 years (3). While the specific pathogenesis of CAV has yet to be fully elucidated, a significant factor recognized to contribute to CAV is a maladaptive immune response (4, 5). Thus, the need for improved strategies to suppress chronic immune reactivity to the allograft remains.

Numerous studies have attributed chronic rejection to lymphoid and antibody-mediated mechanisms where graft reactive antibody constitute a persistent inflammatory state. Experimental and clinical data indicate that graft-reactive antibody triggers inflammatory signaling by graft endothelium and interstitial cells, thereby activating donor-reactive, and non-specific innate and adaptive immune mechanisms (6). Such persistent inflammatory insults subvert natural wound healing pathways and may lead to a state of non-resolving inflammation often associated with

chronic graft interstitial fibrogenesis and vascular injury. This inflammatory response is difficult to resolve and therefore a key obstacle in preventing progression of chronic graft injury.

Relative to studies on T lymphocytes, less is appreciated regarding the role of innate immune cells, including monocytes, dendritic cells (DCs), and macrophages, following solid organ transplantation and their involvement in this chronic inflammatory state. While these cells are classically thought of as first responders, emerging evidence encourages us to reevaluate this "surface level" thinking and consider a deeper investigation as events occurring early after transplant have the potential to contribute to or even perpetuate long term damage. While much could be said about each of the innate immune cell types, our discussion will focus specifically on the role of DCs in heart transplantation.

DC SUBSETS

DCs are canonically recognized as professional antigen presenting cells (APCs) that serve as a key linking cell between the non-specific innate immune response and the memory producing, antigen specific adaptive immune response. As such, DCs are a remarkably heterogenous population of cells, existing in a variety of subtypes that differ in surface phenotype, function, and location in the body (7). Attempts to accurately classify DCs into their appropriate subtypes has been fraught with challenges as cell surface markers commonly used in classification schemes are often not unique to a particular cell subtype and vary based upon activation state of a cell or location in the body. A nomenclature for DC subsets based primarily on ontogeny and secondarily by location, function, and phenotype was proposed by Guilliams et al. (8) which we will apply in an attempt to maintain clarity in this discussion.

DCs develop from a common progenitor cell in the bone marrow into classical DCs (cDCs) with potent antigen presenting abilities, or plasmacytoid DCs (pDCs) implicated in the production of type I interferons (IFNs) and subsequent innate immunity against viral infection (7) (**Figure 1**). Recent evidence has emerged suggesting very early separation of these lineages during development, where pDC precursor cells differentiate from a lymphoid progenitor cell that is independent of the myeloid cDC lineage (9). However, it is important to note a fair amount of disagreement exists as to the identity of a true DC progenitor that exclusively gives rise to the DC lineage and ensuing subsets. Nevertheless, our appreciation of DC subset heterogeneity and lineage is undergoing continuous evolution driven by current studies that leverage transcriptomic and single cell sequencing, coupled with genetic lineage tracing.

Additionally, there is some controversy concerning a final category of monocyte-derived DCs (moDC) which are routinely generated *in vitro* (10) but are defined uniquely by researchers as either macrophage-like or DC-like based upon expression of CD11c; further scenarios complicated by inflammation alters this phenotypic profile (8). However, moDC have remained of interest to researchers due to their use in DC vaccination immunotherapy for cancer treatment (11). Below we focus our discussion on cDC and pDC subsets.

Classical Dendritic Cells

Following migration of a committed precursor cell (pre-cDC) from the bone marrow to peripheral lymphoid and non-lymphoid tissues (12), cDCs will complete their development into cDC1 and cDC2 subsets dependent upon a unique set of transcription factors where BATF3 and IRF8 have been recognized as crucial for regulation of cDC1 development (13, 14) and IRF4 for cDC2s (15, 16). These subsets can be differentiated by surface markers across multiple tissues as $XCR1^+$ $Cadm1^+$ $CD172a^-$ cDC1s and $XCR1^-$ $Cadm1^-$ $CD172a^+$ cDC2s (17), or with additional tissue specific markers such as splenic $CD8\alpha^+$ cDC1 and $CD4^+$ cDC2 or lung $CD103^+$ cDC1 and $CD11b^+$ cDC2.

The predominate function of cDCs is recognized to be antigen presentation, where $XCR1^+$ $CD172^-$ cDC1s present to and subsequently stimulate a $CD8^+$ T cell response (18) while $XCR1^-$ $CD172^+$ cDC2s are more adept at stimulating $CD4^+$ helper T cells and humoral immunity (19). Importantly, DC subsets exhibit remarkable plasticity dependent upon their microenvironment (20), allowing for $XCR1^-$ $CD172^+$ cDC2s and pDCs to retain the ability to cross-present antigens to $CD8^+$ T cells when appropriately stimulated (21, 22).

Plasmacytoid Dendritic Cells

The development of pDCs requires the transcription factor E2-2 (23) and is regulated by cytokine FLT3-ligand in both mice and humans (24, 25). Unlike cDCs, development of pDCs is completed in the bone marrow prior to their migration to secondary lymphoid organs and peripheral tissues. The complex biology of pDC has been reviewed extensively by others (26), however a brief overview of their unique phenotype and functionality is warranted.

Identification of pDCs requires the use of multiple surface markers in order to accurately delineate a pure pDC population. Murine pDCs are known to express CD11c (though at lower levels than cDCs), CD45R (B220), Sca-1, Siglec-H, Bst2, and CCR9 in addition to markers that are thought to be related to maturation state such as Ly6C, CD4, and CD8 (27).

Functionally, activated pDCs are able to perform the canonically associated antigen presenting role of a DC, however they do so much less efficiently than cDCs (28, 29). pDCs exhibit a lower expression of MHC class II and costimulatory molecules compared to their cDC counterparts, but mature pDCs are still able to generate an effective, and immunogenic T cell response (30). This response has been revealed to be variable, polarizing to direct Th1 or Th2 differentiation dependent upon factors including antigen dose, stimulation type, and cell maturation state (31).

With these somewhat "weak" antigen presenting capabilities and ability to prime T cells, pDCs are more recognized for their role in production of type I Interferon in response to viral stimulation (32). This subset specific high level production of type I interferon is known to activate NK cells yielding induction

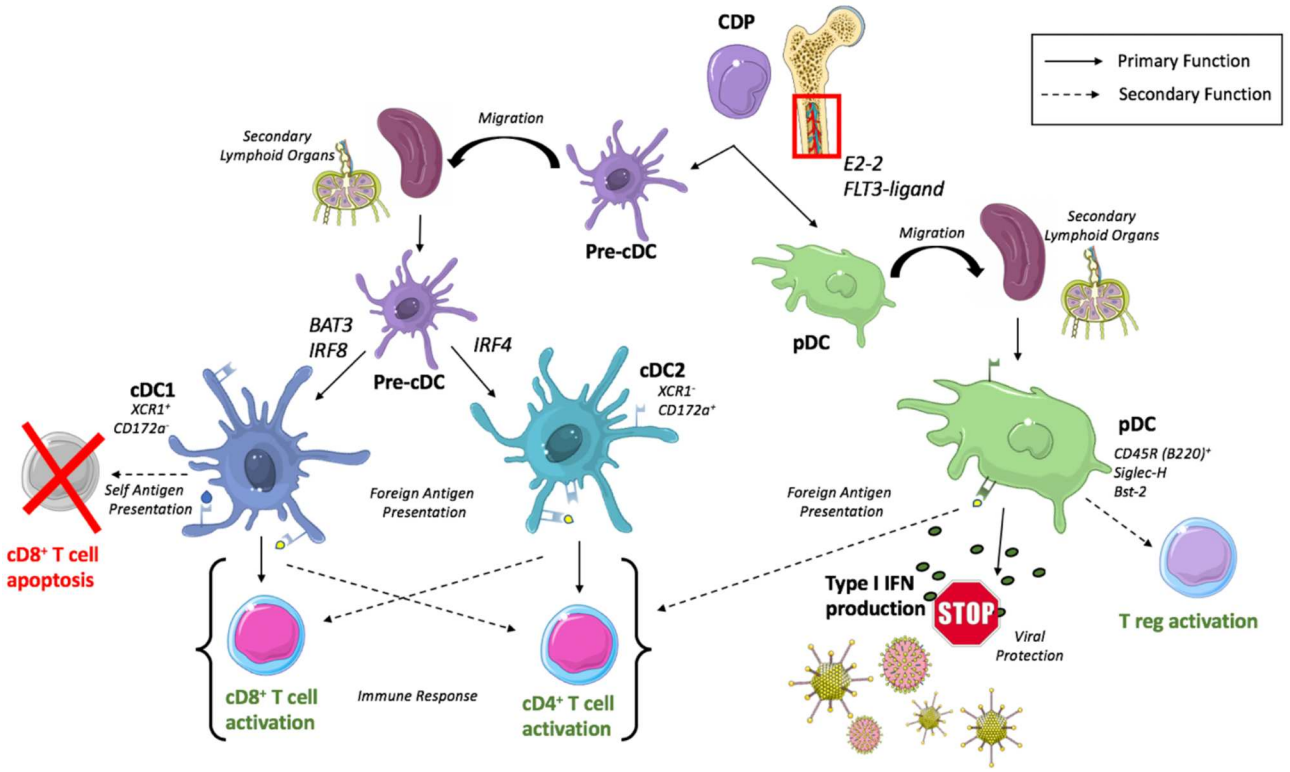

FIGURE 1 | Development of DC subsets and conically associated functions. cDCs arise from a common dendritic cell precursor (CDP) originating in the bone marrow. CDPs mature into pre-cDCs and then migrate to secondary lymphoid organs where they differentiate into cDC1s or cDC2s dependent upon transcription factors, BAT3/IRF8 and IRF4, respectively. cDC1s are recognized to predominantly activate $CD8^+$ T cells but secondarily can induce $CD8^+$ T cell apoptosis with the presentation of self-antigen while cDC2s predominantly activate $CD4^+$ T cells. pDCs complete their maturation within the bone marrow before migrating to secondary lymphoid organs where they participate in type I IFN production for viral protection and participate (to a lesser extent) in antigen presentation.

of cytotoxicity and IFN-γ production (33), helping to orchestrate the TLR9 mediated control of viral infection (34).

Beyond this predominant function of cytokine production, it has been suggested that given appropriate stimuli, pDC are able to induce the development of $CD4^+CD25^+$ regulatory T cells (Tregs) as demonstrated following co-culture of $CD4^+CD25^-$ naïve T cells with pDCs enriched from human peripheral blood mononuclear cells (PBMCs) (35). Relatedly, pDCs have been shown to activate resting $CD4^+CD25^+Foxp3^+$ Tregs *in vivo* isolated from murine tumor draining lymph nodes in an indoleamine 2,3-dioxygenase $(IDO)^+$ pDC dependent manner (36).

INNATE RESPONSE OF DCS IN CARDIAC TRANSPLANT

As we begin to assess the innate response of the aforementioned DC subsets in cardiac transplant, it is important to consider the environment these cells currently or will soon occupy. Organ transplantation induces rapid activation of the innate immune system as damaged vascular and parenchymal tissue from organ procurement, organ storage, and engraftment yield numerous inflammatory stimuli derived from dead or dying graft cells. These released damage-associated molecular patterns (DAMPs) are then recognized by toll-like receptors (TLRs), a type of pattern-recognition receptor (PRR), which initiate a signaling cascade that results in production of multiple cytokines and cellular responses to further enhance this inflammatory milieu (37).

The onslaught of immune cell infiltration has long been assumed to be damaging to the graft with cell-specific depletion studies further confirming. Depletion of macrophages in a mouse model of heart transplantation revealed markedly reduced development of CAV lesions (4) and a related attenuation of experimental transplant vasculopathy has been documented in association with reduced numbers of graft-associated macrophage and dendritic cells (38). Complimentarily, the presence of intravascular macrophages within the first year after human heart transplant was found to be predictive of donor specific antibody and potential development of antibody mediated rejection (39). However, a subset of macrophages have also been shown to play a role in angiogenesis and may aid in microvascular repair of the injured graft (40). Thus, we must ask, how is it that DCs contribute to the innate response following cardiac transplantation?

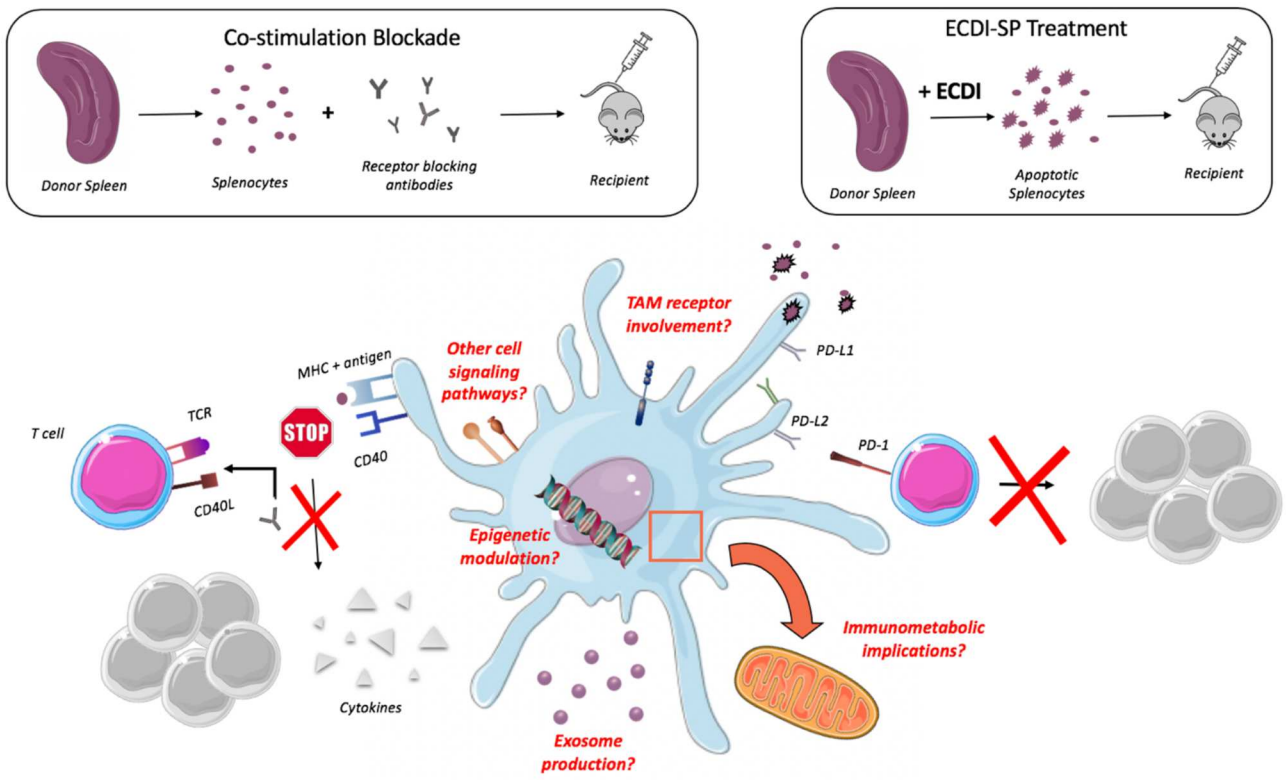

FIGURE 2 | Impact of tolerogenic therapy on DCs. Administration of donor antigen with CD40L blocking antibodies results in decreased production of cytokines by DCs while administration of ECDI-induced apoptotic cells results in upregulation of negative costimulatory molecules (PD-L1, PD-L2) yielding an inhibition of T cell proliferation. Questions remain as to the role of TAM receptors, epigenetic modulation, exosome production, and Immunometabolic implications.

The innate immune response is composed of physical, cellular, and chemical components that function as a first line defense to protect the body from invading pathogens and/or foreign antigens. As such, the innate response can be thought to consist of elements that respond directly to this invader and elements responsible for signaling to members of the adaptive response, allowing for formation of immunological memory (41). DCs have revealed that they participate in both of these elements, in many ways serving as a cellular director who orchestrates the recruitment of necessary cell populations dependent upon the pathology occurring.

Immature DCs specialize in the capture and processing of antigens, after which they mature and lose much of their endocytic capacity while upregulating the expression of MHCII and other costimulatory molecules such as CD40, CD80, and CD86 (42). Engagement with either MHCII or CD40 on DCs has long been known to result in their production of IL-12 (43) which plays an important role in the activation of NK cells and their subsequent production of IFN-γ (44) known to regulate Th1 cell development (45). This interplay between DCs and NK cells within the innate immune system has garnered attention of recent as increasing numbers of molecular and cell-to-cell interactions between these two cells are uncovered. A cellular conversation occurring reciprocally between DCs and NK cells has been found to result in a diversity of outcomes including NK cell activation and proliferation as well as either DC maturation or elimination [reviewed by Degli-Esposti and Smyth (46)]. The importance of NK cell activation in transplant has been argued by data showing adoptive transfer of NK cells 1 day prior to heart transplant in T/B/NK-deficient mice resulted in the development of CAV, which was not seen in mice who remained NK cell deficient (47). While it was not an objective of this study to assess the involvement of DCs in this NK-cell required pathology, it is important to consider the importance and presence of upstream cellular signals that may allow for the end result. Additionally, when we consider the conversation that occurs between NK cells and DCs in the context of transplantation, we must add an additional layer as both recipient and donor "passenger" immune cells of both cell types could be involved. However, It has been demonstrated that recipient NK cells will quickly eliminate donor allogeneic DCs found within the transplanted organ (48), indicating it is likely recipient DCs that play the predominant role in the innate response.

Continuing with the concept of DCs as cellular director of the innate response, elegant experiments from the lab of Florent Ginhoux have identified skin cDC1s (distinct from epidermis associated antigen presenting Langerhans cells) as producers of the cytokine VEGF-α, recognized to be important in the recruitment of neutrophils (49). Depletion of this DC subset via diphtheria toxin injection yielded a significant decrease in

neutrophils to the cutaneous injury site which could be recovered following cDC1 adoptive transfer. Interestingly, neutrophils isolated from cDC1 depleted mice revealed a downregulation of genes associated with priming, mobility, and neutrophil recruitment compared to their cDC1 sufficient counterparts. Additionally, neutrophils from a cDC1 deficient environment exhibited decreased functional capacity and survival. The recency of this study have not allowed for evaluation of this finding in other pathologies, such as the setting of transplant. However, given that a low lymphocyte to neutrophil ratio was shown to be a potential biomarker to predict acute rejection after heart transplant (50), understanding the way in which cDC1s participate in neutrophil recruitment in this setting may be of immense value.

Having discussed the direct responses of DCs to foreign antigen, the second category of elements in the innate response to consider is methods of signaling to members of the adaptive immune system to initiate a primary immune response. While other innate immune cells are recognized for their phagocytic properties (namely macrophages), the responsibility of uptake and subsequent presentation of foreign bodies to lymphocytes to trigger an adaptive response fall on DCs. The efficiency by which different DC subsets present antigen to T cells has already been mentioned, however it is important to recognize the complexity which underlies antigen presentation and allorecognition in the setting of donor and recipient immune cells.

A series of potential allorecognition pathways amongst DC and T cells exist including direct, indirect, and semi-direct allorecognition. Direct allorecognition refers to the recognition of MHC-peptide complexes on donor APCs directly by recipient T cells (51) which likely only play a role during acute graft rejection, as donor-derived APCs will eventually die or be destroyed, prohibiting them from participating in chronic forms of rejection (52). Meanwhile, indirect allorecognition occurs when recipient DCs process graft derived peptides and present these molecules on self-MHC to lymphocytes (53) which has been shown to play a role in both acute and chronic rejection in multiple models (54–57). Finally, the semi-direct pathway involves transfer of intact donor MHC molecules to recipient DCs in a process also referred to as "MHC cross-dressing" by mechanisms still being defined such as cellular "nibbling," also known as trogocytosis, or DC secreted exosomes (58–61). These related but disparate pathways by which recipient or donor DCs uptake or receive graft antigen to utilize in signaling to the adaptive immune system speak of the versatility of this cell within the innate response, A final topic within the innate response of DCs following transplantation worthy of mention is the recognition of allogenic self vs. non-self. Classically, the innate immune system relies upon recognition of conserved microbial molecular patterns also known as pathogen-associated molecular patterns (PAMPs) by PRRs for identification of non-self to subsequently trigger an immune response (62). But what is the innate role of self vs. non-self recognition within the setting of sterile inflammation where PAMPs do not play a role, as occurs in transplant? It has been found that mice devoid of T, B, and natural killer cells are still able to mount an immune response via innate recognition of allogenic non-self (63). This recognition of non-self is shown to be the result of allelic polymorphisms in donor SIRPα membrane protein on donor tissue binding to CD47 (or IAP for integrin-associated protein) on recipient infiltrating DCs (64). Continuing to assess and improve our understanding of the magnitude and effect of this innate non-self recognition response, mediated by DCs, in the setting of solid organ transplant may have important future clinical implications for organ allocation.

DCS IN TRANSPLANT TOLERANCE

A worthy goal in the realm of organ transplantation is the induction of operational tolerance in which there is long-term survival of an allograft without need for immunosuppressive therapies (65). This is an attractive objective, as current pharmacological agents commonly used for maintenance immunosuppression in heart transplant are recognized to result in severe side effects including (but not limited to) nephrotoxicity, dyslipidemia, pancytopenia, and pericardial and pleural effusions (66). Thus, addressing the ability of DCs to induce a tolerogenic state following solid organ transplantation is of great value. Here we will seek to evaluate how DCs and their subsets may play a role in this induction of operational tolerance.

It has also been noted that the relative composition of DC subsets found in the peripheral blood following heart transplantation is mutable, dependent upon factors such as choice of pharmacologic immunosuppressant and length of time since transplant (67). In a study of human heart transplant recipients, an association between lower levels of pDCs and increased rejection grades was observed (68). However, it is important to note this study evaluated two groups of patients treated with different immunosuppressive therapies, tacrolimus (TAC), and cyclosporine A (CsA). The authors report patients treated with TAC have significantly higher values of pDCs than CsA treated patients in addition to decreased rejection. It is very possible there are multiple pharmacologic mechanisms contributing to the rejection phenotype observed, but the DC subset specific differences between these populations is intriguing.

A number of studies have begun to further probe this interesting observation by adoptively transferring pDCs into rodent models of heart or lung transplant and observing graft outcome [reviewed in Rogers et al. (69)]. Remarkably, a consistent story of prolonged graft survival with pDC adoptive transfer begins to emerge. Relatedly, depletion of donor pDCs from bone marrow grafts resulted in accelerated graft-vs.-host disease (GVHD) mortality (70). A similar result was observed in murine heart transplant in which treatment with tolerizing protocol followed by pDC depleting antibody prevented tolerance induction (71). This study further describes the localization of pDCs to high endothelial venules in the lymph nodes with a related distribution of Treg cells. Additional experiments by the authors reveal pDCs to promote Treg development, a cell recognized to play a role in both induction and maintenance of tolerance (reviewed in Tang and Vincenti (72)].

More recently, the cDC1 subset has also begun to reveal its own unique role in the context of tolerance. In a mouse model of peripheral tolerance assessing the renal lymph node, a site where self and foreign antigen are continuously filtered, cDC1 cells were shown to induce apoptosis of $CD8^+$ cytotoxic T cells through programmed death 1 ligand (PD-L1) signaling (73). While this study evaluates cDC1 driven apoptosis as a mechanism to prevent auto-immunity, the potential implications in transplant are readily apparent as a means to deplete graft reactive T cells, although this, to the authors' knowledge, has yet to be formally assessed.

cDC1s have also been found to play a vital role in central tolerance by presentation of cell-surface antigens from apoptotic medullary thymic epithelial cells yielding development of a diverse repertoire of regulatory T cell receptors (74). Interestingly, this CD36 dependent antigen transfer to $CD8\alpha^+$ DCs (equivalent to cDC1s) was shown to be required for thymic allo-tolerance in a murine model of GVHD following BMT. Implicating this pathway further, a blinded analysis of peripheral blood of patients following BMT revealed a correlation between decreased CD36 expression and $CD141^+$ DCs (the human equivalent of $CD8\alpha^+$ cDC1s) with increased frequency of GVHD development, despite no significant differences in prevalence of other cell types, demographic, or clinical characteristics. While the thymic environment and processes associated with central tolerance are admittedly removed from tolerance induction following solid organ transplant, evaluating the mechanisms by which tolerance in various settings is successfully achieved in parallel with the involved cellular players may help to facilitate the generation of new tolerogenic therapies.

DCS IN TOLERANCE INDUCING THERAPIES

A variety of protocols have been developed and refined to commandeer and direct the interactions of immune cells in a manner that would promote a tolerogenic environment following organ transplantation leading to graft acceptance. Of special interest to the authors is the use of donor cells, with or without additional combinatorial therapy, as a means to harness the body's ability to clear naturally occurring apoptotic cells via phagocytosis without damaging healthy neighboring cells or initiating an inflammatory milieu (75). We will discuss the currently known role of DCs in this process, but a broader discussion of the mechanisms that underly the use of apoptotic cell-based therapies in the promotion of tolerance can be found in the review by Morelli and Larregina (76).

Treatment of donor splenocytes with the chemical crosslinker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI-SP) induces apoptosis, allowing for the processing and presentation of donor antigens in a non-immunogenic manner (77). Donor ECDI-SP is intravenously infused into the recipient 7 days before and 1 day after transplantation, resulting in indefinite survival of full MHC-mismatch allogenic pancreatic islet grafts (78) and prolonged survival of heart allografts in the absence of immunosuppression. Interestingly, when ECDI-SP treatment was combined with a short course of rapamycin (from day −1 to day +8), long term cardiac graft survival (>150 days) was achieved in all recipients (79). The differential success of this treatment strategy in two unique solid organ transplants could point toward an organ specific immune response, variations in mechanism for tolerance induction reliant upon organ resident or infiltrating immune cells, or other such hypotheses that must be addressed. Still, this tolerizing treatment strategy exhibits potential, revealing both safety and tolerability in a phase I trial of patients with MS (80) and in a phase I/IIa trial as prophylaxis for GVHD (81).

The role of DCs in this tolerizing protocol has been revealed to be essential, as only depletion of $CD11c^+$ DCs via administration of diphtheria toxin to $CD11c^+$ DTR mice was able to inhibit islet allograft survival following ECDI-SP treatment (82). In the same study, internalization of ECDI-SP was seen to occur with varying proportions across the various DC subsets, however all of these populations were simultaneously depleted in the $CD11c^+$ DTR mouse model when defining the necessity of DCs. Additional research, such as the use of subset specific depletion models, are required to delineate if specific roles and/or differing levels of importance exist within the respective DC subsets in the induction and maintenance of ECDI-SP induced tolerance.

The manner in which DCs are impacted by ECDI-SP treatment deserves consideration as this knowledge may help to elucidate other powerful molecular targets for tolerance induction. Kheradmand et al. (82) reported an upregulation of negative costimulatory molecules, PD-L2, and PD-L1, with no increase in positive costimulatory molecules on $CD11c^+$ DCs of mice treated with ECDI-SP, a balance which was essential for tolerance. The PD-1 pathway and its associated ligands are recognized to be involved in the T cell response (83) where engagement of PD-1 on activated T cells by its known ligands inhibits T cell proliferation (84, 85). Again, it has yet to be determined if the upregulation of these molecules (or others not investigated in this study) following ECDI-SP treatment are differentially attributed to specific DC subsets. This type of result would prompt preferential targeting of the identified subset in order to yield an enhanced response while minimizing off-target effects.

Other techniques to exploit DC interactions have been explored in which donor antigens, usually in the form of splenocytes, are delivered to recipients in conjunction with costimulation blockade (86, 87). Classically, naïve T cells require two signals for their activation and subsequent proliferation: (1) recognition of APC presented antigen by T cell receptor (2) costimulatory signal (88). Thus, the administration of antigen in combination with an antibody that blocks the required costimulatory signal, such as anti-CD154(CD40L), allows for an altered functional DC phenotype similar to that established in the setting of peripheral tolerance (89). This technique has been shown to yield robust tolerance in the setting of murine cardiac allograft transplantation, in which only a triple intervention strategy (depletion of Tregs, PD-L1 antagonism, low dose T cell transfer) was able to break established tolerance (90).

Some concern has been raised of clinical viability of the CD40-CD40L blockade strategy following the discovery of

CD40L on endothelial cells and stimulated platelets (91) and a high incidence of thromboembolic events in primates after receiving monoclonal antibody against CD40L (92). However, a newly generated CD154 blocking antibody utilizing a mutated IgG1 construct lacking Fc activity was able to prolong kidney graft survival in a non-human primate without evidence of thromboembolic complications, helping to demonstrate that obstacles for clinical utility of this strategy can be overcome (93).

When CD40 (expressed on DCs) binds to CD40L (expressed on activated $CD4^+$ T cells), a complex pathway of downstream signaling is initiated that alters DC phenotype and functionality in order to promote an effective T cell response. This includes increasing MHC and costimulatory molecule expression, increasing production of inflammatory cytokines (94), and encouraging DC longevity (95). Although costimulation blockade with CD154 occurs at the level of the T cell, it is important to consider how interrupting this DC to T cell interaction effects DCs due to the absence of the CD40:CD40L signal. Following CD40/CD154 blockade, (96) demonstrated a significant reduction in inflammatory cytokines secreted by DCs and delayed expansion and differentiation of host reactive T cells. However, somewhat surprisingly DCs were shown to express similar levels of positive costimulatory molecules (CD80, CD86) as untreated controls.

While both ECDI-SP and CD40-CD154 costimulation blockade strategies exhibit promise for inducing tolerance in transplant recipients, it's important to note such tolerance appears to be achieved through dissimilar mechanisms (upregulation of inhibitory molecules vs. reduced cytokine secretion) as far as DCs are concerned. Continuing to further delineate the cellular and molecular mechanisms by which tolerance is induced and maintained may allow for use of such therapies in combination and improved pharmacological targeting (**Figure 2**).

DC IMMUNOMETABOLISM AND TRANSPLANTATION

Over the past 5 years there has been a developing interest in the relationship between cellular metabolism and its influence on cell function. Recent studies have shown alterations to metabolic pathways, including glycolysis, the Krebs cycle, and fatty acid metabolism, are able to profoundly influence the function of macrophages and DCs in notably specific ways (97). While metabolic pathways are unmistakably complex, the advent of increasingly sophisticated, and sensitive molecular tools have allowed us to begin to unravel this intricate network of potential therapeutic targets.

The bioenergetic requirements of DCs are highly dependent upon their activation or lack thereof, and yet viewing the concept of metabolism purely from an "energy providing" point of view is a vast oversimplification. Exciting work has revealed metabolites themselves, such as NAD^+ and succinate, are able to provide signals to immune cells that regulate their function (98). For example, it is accepted that toll-like receptor (TLR) agonism is crucial for DC activation from its quiescent state. More interestingly, TLR agonism on DCs was shown to result in a metabolic transition from oxidative phosphorylation (inactive) to aerobic glycolysis (active) which could be inhibited by adenosine monophosphate activated protein kinase (AMPK) (99). This direct inhibition of DC activation by the hand of cellular metabolism should encourage us to postulate how immunometabolism contributes to a tolerogenic or rejecting DC phenotype in the setting of solid organ transplant.

With the help of deep mRNA sequencing and molecular pathway analysis software, it has been shown that tolerogenic DCs from human peripheral blood (induced by modulation with 1,25-dihydroxyvitamin D2 and dexamethasone) do indeed differentially express genes associated with metabolic pathways. Pathways of oxidative phosphorylation, lipid, and sugar metabolism were shown to be two-fold higher in these tolerogenic DCs compared to mature inflammatory DCs (100). Such findings have been seen by others while also observing that tolerogenic DCs express higher levels of proteins involved in mitochondrial fatty acid oxidation (FAO). Interestingly, blocking FAO blunted some of the tolerogenic function of these DCs as measured by increased levels of activated T cells following said blockade (101). It is unknown if these metabolic adaptations function consistently across the identified DC subsets, but this rapid cellular modification to alterations in the metabolic milieu resulting in observable change in the tolerogenic capacity of DCs certainly encourage further investigation.

THERAPEUTIC IMPLICATIONS AND FUTURE RESEARCH

This basic understanding of the powerful role DCs play at the intersection of innate and adaptive immunity in combination with strategies already showing therapeutic potential are an encouragement to the pursuit of achieving operational tolerance. Additional strategies and future research directions should consider complementary or supplemental interrogation of regulatory DC receptors that have yet to be evaluated.

The TAM family of receptor tyrosine kinases—TYRO3, Axl, and Mer—are expressed among cells of the immune system including macrophages, resting and activated DCs, and natural killer cells. These receptors are recognized to play essential roles in innate immunity including inhibition of the inflammatory response, phagocytosis of apoptotic cells, and maturation of natural killer cells (102). However, in the case of Axl and Mer, these roles have been identified as diverging with Axl expression increasing following inflammatory stimuli and Mer expressed on resting macrophages and enhanced following tolerogenic stimuli, such as in culture with immunosuppressive dexamethasone (103). Zagorska et al. (103) identified *in vitro* bone marrow-derived DCs as having greater levels of inflammation associated Axl in comparison to Mer, a finding further supported by similar levels of expression on $CD11c^+$ DCs isolated from murine spleen. Characterization of these receptors within specific DC subsets has not performed and use of gene and cell specific knockout models or receptor specific antibodies could aid in elucidating targetable pathways amongst the TAM receptors for use in transplant.

Currently identified ligands of the TAM receptors include growth-arrest specific six protein (GAS6) and Protein S (104). These proteins serve as so-called "linker molecules" to the TAM receptor as they are simultaneously bound to phosphatidylserine present on apoptotic cell membranes. Interestingly, in a murine model of autoimmune thyroiditis, the prevalence of thyroiditis, and inflammatory infiltrate was shown to be significantly decreased in mice that received recombinant Gas6 (105). These mice also showed distinct differences in the distribution of T cell subsets following treatment with Gas6, however though APCs are required for T cell activation, the impact of Gas6 administration on APCs was not evaluated.

A potential strategy toward harnessing the selective activation of the aforementioned receptors may be through the use of nanobiologics that selectively target DCs, such as dendritic cell-targeted polymersomes (106). This idea of selectively targeting innate immune cells has already begun to be investigated with promising results. Braza et al. (107) utilized a short term high-density lipoprotein nanobiologic to encapsulate the mTOR inhibitor, rapamycin in order to preferentially target myeloid cells, and inhibit trained immunity. Significantly higher uptake of these particles was seen in macrophages compared to other cell types (DCs, neutrophils) with a related decrease in TNFα and IL-6 protein expression by flow sorted macrophages from mice receiving this non-biologic treatment. Perhaps most exciting is treatment with this myeloid targeting nanobiologic yielded significantly increased heart allograft survival, even when compared to oral and intravenously administered rapamycin. Thus, it appears combining cell-specific targeting and nanobiologic treatment could serve as a powerful tool in the promotion of tolerance.

CONCLUDING REMARKS

In this review, we have sought to highlight what is currently known as it relates to DCs in the setting of cardiac transplantation. We highlight this unique cell as a prominent director of the innate immune response with the ability to activate and recruit additional cell types, such as NK cells and neutrophils, and participate in allogenic recognition and signaling to the adaptive system. We describe DC subset classification and what has been found regarding subset specific roles in transplantation tolerance where both pDCs and cDC1s could serve as important cellular mediators of tolerance, although this has yet to be fully elucidated. We assess the impact of tolerization therapies, such as delivery of apoptotic cells or costimulation blockade, acting on DCs in related albeit dissimilar mechanisms through upregulation of inhibitory molecules vs. reduced cytokine secretion, respectively. Finally, we address areas in which research has only just begun including the implications of immunometabolic modulation and the interrogation of regulatory DC receptors such as the TAM family with nanobiologics.

As our ability to probe deeper into specific immune cell populations continues to advance, we find ourselves at a unique time in immunology to be able to ask questions and address pathways and cellular functions in ways like never before. There has been a drastic increase in understanding of the innate immune system over the past few years, yet gaps in knowledge certainly remain within the realm of transplantation and DCs. Future directions of research require careful consideration of subset specific receptors and subsequent responses in order to better delineate DC roles in tolerance and maximize potential therapeutic targets. We must also deepen our awareness of the mechanisms by which DCs mediate both the adaptive and innate response in transplant, evaluating DC signaling utilized for cellular recruitment, innate sensing of self vs. non-self, and receptors necessary for tolerogenic DC programming. With a growing and evolving knowledge of this complex cell and the innate immune system, true transplant tolerance, considered to be the "holy grail" of transplant research, may become within reach.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Innate Allorecognition and Memory in Transplantation

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Over the past few decades, we have witnessed a decline in the rates of acute rejection without significant improvement in chronic rejection. Current treatment strategies principally target the adaptive immune response and not the innate response. Therefore, better understanding of innate immunity in transplantation and how to target it is highly desirable. Here, we review the latest advances in innate immunity in transplantation focusing on the roles and mechanisms of innate allorecognition and memory in myeloid cells. These novel concepts could explain why alloimmune response do not abate over time and shed light on new molecular pathways that can be interrupted to prevent or treat chronic rejection.

Keywords: allorecognition, innate immunity, transplantation, monocyte, dendritic cell

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Activation of the innate immune system is necessary for driving adaptive immune responses (1, 2). In infection, pathogen-associated, non-self, molecules trigger host innate defenses and induce maturation of antigen-presenting cells (APCs) by binding to germline-encoded pattern recognition receptors [e.g., Toll-like receptors (TLRs)]. Mature APCs then initiate and sustain adaptive immunity by presenting antigen and providing co-stimulation to T cells.

How transplanted organs (allografts) induce APC maturation is less clear. Initial, landmark experiments suggested a role for TLRs by demonstrating that deletion of Myd88 downstream of TLRs blocks dendritic cell (DC) maturation and prevents rejection of single minor histocompatibility antigen-mismatched grafts (3). Later studies however showed that the rejection of MHC- or multiple minor antigen-mismatched allografts can still proceed in the absence of TLR signaling (4, 5). Moreover, deletion of additional microbial sensing pathways failed to completely prevent rejection (6–8). Similarly, the alternate hypothesis that "danger" molecules released at time of transplantation due to tissue injury trigger APC maturation could not account for alloimmune responses initiated after injury has subsided (9, 10). For example, allografts parked for a long time in T cell-deficient hosts were promptly rejected when T cells were replenished despite absence of discernible inflammation or injury in the graft at the time of T cell transfer (11–15). These observations raise the possibility that innate receptor systems, other than those involved in sensing microbes and danger, sense allogeneic non-self on transplanted tissues and cause APC activation. Here, we will summarize evidence that monocytes and macrophages distinguish between self and allogeneic non-self and review the mechanisms and functional consequences of this form of innate

allorecognition. We also touch on the allospecific memory in these innate immune cells and discuss the translation of the findings into clinical situations.

EVIDENCE FOR INNATE ALLORECOGNITION

An early study by Zecher et al. demonstrated that *RAG-/-* mice, which lack T and B cells, mount a DTH-like response to allogeneic but not syngeneic *RAG-/-* splenocytes (16). In the same study, it was established that the response is mediated by host monocytes, not NK cells, and is elicited by non-MHC disparities between donor and recipient**.** A subsequent publication by Liu et al. independently reported that macrophages in alloimmunized hosts engage in allorecognition, acquiring with the help of CD4+ T cells the ability to kill allogeneic cells (17). CD4+ T cell help to macrophages was mediated by CD40 such that the same macrophage allocytotoxic response could be elicited in lymphocyte-deficient mice injected with an anti-CD40 agonistic antibody at the time of alloimmunization.

Prompted by these observations, Oberbarnscheidt et al. studied the innate response of *RAG-/-γc-/-* mice (which lack T, B, NK, as well as all other innate lymphoid cells) to heart, kidney, and bone marrow plug grafts (18). They found that allografts elicit an innate response distinct from syngeneic grafts. Allografts were persistently infiltrated with host-derived mature (MHC-IIhiCD80hi), IL-12+ monocyte-derived DCs (mo-DCs), even several weeks after transplantation, while syngeneic grafts harbored five-fold less mo-DCs, which were transient (present only during the 1st week), less mature, and IL-12neg. Similar differences were observed between allogeneic and syngeneic grafts transplanted to wild-type (WT) recipients and analyzed within 1 day after transplantation (18). Consistent with their IL-12 phenotype, mo-DCs from allografts but not those from syngeneic grafts drove a canonical Th1 (IFNγ+) response *in vitro* and *in vivo*. As in the previous study (16), the innate alloresponse was not dependent on MHC disparities between donor and recipient, or on lymphoid cells in either donor or recipient. Instead, a mismatch in the non-MHC was necessary. Chow et al. made similar observations by injecting allogeneic cells intravenously, to avoid inflammatory reactions, into *RAG-/-γc-/-* mice (19). Therefore, monocytes and macrophages are activated by allogeneic stimuli to become mature DCs that drive the Th1 response and to acquire allocytotoxic functions, respectively.

MECHANISM OF INNATE ALLORECOGNITION: RECOGNITION OF NON-MHC ALLODETERMINANTS

A genetic mapping study was undertaken to identify non-MHC allodeterminants that trigger the innate alloresponse (20). The study was based on the observation that allografts from NOD donors elicit a strong monocyte response in B6. *RAG-/-γc-/-* recipients, while grafts from NOR mice, which share ∼88% of their genome (including the MHC) with NOD, do not (20). Using NOD.NOR congenics, Dai et al. mapped the difference to the gene that encodes SIRPα (signal regulatory protein alpha), a polymorphic IgSF (immunoglobulin super family) protein expressed on neurons and myeloid cells but also present or induced on myocytes, epithelial cells, and endothelial cells (21). They showed that SIRPα triggers monocyte activation via CD47 and that amino acid polymorphisms in SIRPα determine the strength of the innate alloresponse by modulating binding to CD47 (20). The greater binding to its ligand CD47 by NOD variant of SIRPα than other mouse strains of SIRPα was also studied by other groups (22, 23). The allorecognition model (**Figure 1**) that emerged is that non-self SIRPα on donor cells causes host monocyte activation by disturbing the balance between activating and inhibitory signals mediated by CD47 and SIRPα, respectively. Under steady-state conditions, or upon transplanting a syngeneic graft, bidirectional interactions between CD47 and self-SIRPα are of equal affinity and thus prevent monocyte activation. In contrast, transplanting an allograft expressing a mismatched (non-self) SIRPα variant upsets the balance and causes monocyte differentiation to DC (20, 24). This model echoes NK cell allorecognition (25). At the same time, it does not exclude the possibility that other polymorphic ligands/receptors could still participate in fine-tuning the innate alloresponse.

ALLOSPECIFIC MEMORY IN INNATE IMMUNE CELLS: RECOGNITION OF MHC-I MOLECULES

Immunological memory—the ability of immune cells to respond rapidly and provide enhanced protection of the host against previously encountered antigen—is a critical driver of transplant rejection and outcomes (26–28). Although originally confined to T & B lymphocytes, the memory concept has been expanded by discoveries that innate lymphoid and myeloid cells (NK cells and macrophages) (29–35), DCs (36), as well as non-immune cells (epithelial stem cells) (37) acquire memory to prior microbial, phagocytosis of apoptotic cells, or allogeneic exposures. As shown in **Table 1**, immunological memory is not a one-size-fits-all phenomenon but falls on a spectrum of varying biological mechanisms, ranging from epigenetic reprogramming in epithelial stem cells, macrophages, and DCs to clonal expansion and differentiation (with or without gene rearrangement) in NK cells and lymphocytes (36–42). Irrespective of mechanism, all memory enhances protection of the host. Epithelial stem cell memory hastens wound healing, macrophage or DC memory protects against pathogens, and lymphoid cell memory accelerates rejection of microbial and allogeneic non-self (31, 33–37, 43, 44). The lasting state of enhanced innate immunity, innate memory, had been termed "trained immunity" and usually confined to unspecific immunological memory in innate immune cells or does not have to be specific (45–49). Recent studies also revealed extensive changes in cellular metabolism during trained macrophage immunity, such as a switch from oxidative phosphorylation toward the preferential use of aerobic glycolysis through an Akt/mTOR/HIF-1α-dependent pathway induced by *C. albicans*

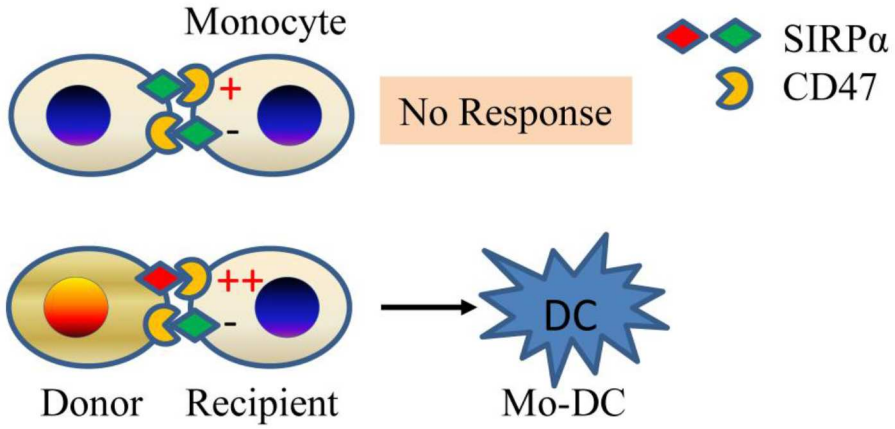


FIGURE 1 | Innate allorecognition model. SIRPα mismatch between donor and recipient (bottom panel) causes imbalance between stimulatory and inhibitory signals in monocytes due to differential affinity of SIRPα to CD47. The mismatch generates mature DCs. If monocytes encounter self (top panel), then no response ensues. Mo-DC, monocyte-derived dendritic cell.

and β-glutan (47). Strategies to regulate trained immunity had shown promise to achieve therapeutic benefits in a range of immune-related diseases (50).

In a series of experiments recently completed by our groups (51), we established that monocytes and macrophages mount an anamnestic memory response to previously encountered allogeneic donor cells but not to third-party cells. This donor specific feature was different from previous concept of "trained immunity," suggesting it is similar to the well-characterized concept of antigen-specific immunological memory in adaptive immune cells (26–28). Memory arose independently of lymphoid cells in either the donor or recipient, underscoring its innate nature. It lasted between 4 and 7 weeks after immunization, which is significantly longer than the average lifespan of a monocyte (∼3 days) (52, 53). Further, we established that memory specificity was to donor MHC-I antigens that were recognized by paired immunoglobulin-like receptor A (PIR-A) molecules expressed on monocytes and macrophages. PIR-A-/- mice or mice treated with PIR-A-blocking agents failed to mount monocyte or macrophage memory. Mouse PIRs are IgSF orthologs of human leukocyte immunoglobulin-like receptors (LILRs) (54). Six linked PIR-A and one PIR-B gene have been identified (55–57). PIR-B contains an ITIM motif and is inhibitory. It binds a wide spectrum of MHC-I molecules (58). PIR-As do not contain ITIM motifs and are stimulatory through association with the Fc receptor common γ (FcRγc) chain, also required for their surface expression (54, 59). PIR-A and PIR-B ectodomains share >92% identity, suggesting that PIR-As also bind MHC-I (58). In fact, PIR-A diversity leads to differential binding of individual PIR-A molecules to distinct MHC-I molecules.

As to the mechanisms by which monocytes acquire allospecific memory, the PIR molecules were preferentially expressed on Ly6Chi monocytes, which significantly expanded after allogeneic antigen exposure. Specific memory independent of lymphoid cells can be transferred to an unimmunized recipient by transferring sorted Ly6Chi monocytes expanded from an immunized recipient, suggesting that clonal expansion of monocytes that express the particular PIR-A molecule that recognizes the particular MHC-I molecule in the immunogen underlies memory (51). This resembles the mechanism established in the case of allospecific NK cell memory (34). We also observed that initial activation of monocytes via the SIRPα-CD47 pathway, which plays an important role in the primary innate allorecognition response (20), is necessary for priming cells toward the memory path (51) (**Figure 2**).

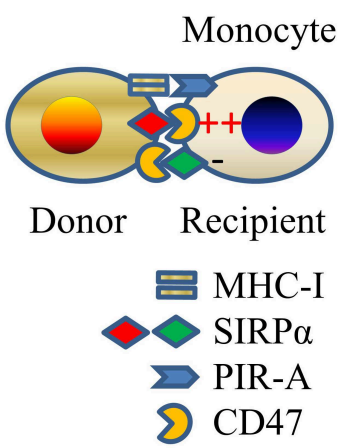
ROLE OF INNATE ALLORECOGNITION IN REJECTION

Evidence that innate allorecognition described above plays an important role in rejection derives from three lines of investigation. In the first (18), OVA-specific OT-II T cells transferred to B6. *RAG*-/- hosts did not reject B6.OVA grafts but rejected (BALB/c x B6)F1.OVA grafts despite similar expression of the antigen, ovalbumin (OVA), recognized by the T cells. Only F1.OVA grafts induced mature mo-DCs and significant proliferation and IFNγ production by OT-II cells, underscoring the importance of monocyte recognition of allogeneic non-self in F1 donors in driving the T cell response. Moreover, short-term mo-DC depletion using the CD11b-DTR transgenic system completely abrogated histological acute rejection at 7 days in lymphocyte-replete mice (18). In contrast, eliminating neutrophils (also CD11b+) with a neutrophil-specific mAb did not affect rejection (18).

In the second study (60), the origin and function of DCs in heart and kidney allografts after transplantation to WT recipients were investigated. It was established that donor-derived DCs were quickly replaced by DCs derived from recipient monocytes and that they closely resembled mo-DCs generated by innate allorecognition. They were mature, IL-12+, and induced

TABLE 1 | Spectrum of immunological memory.

	Longevity	Recall	Specificity	Mechanisms	Enhanced protections
Lymphocyte Memory	++++ (years)	++++	++++	Clonal Expansion Cell Differentiation Gene Rearrangement Epigenetic reprogramming	PATHOGENS: Yes ALLOANTIGENS: Yes
NK Cell Memory	++ (months)	++	++	Clonal expansion Cell differentiation Epigenetic reprogramming	PATHOGENS: Yes ALLOANTIGENS: Yes
Monocyte memory	+ (weeks)	+	++	Clonal expansion Cell differentiation Epigenetic reprogramming	PATHOGENS: Yes ALLOANTIGENS: Yes
Macrophage memory	+ (weeks)	+	+/-	Epigenetic reprogramming	PATHOGENS: Yes ALLOANTIGENS: Yes
DC memory	+ (weeks)	+	+	Epigenetic reprogramming	PATHOGENS: Yes ALLOANTIGENS: Yes
Epithelial stem cell memory	++ (months)	+	-	Epigenetic reprogramming	ENHANCED WOUND HEALING



MHC I mismatch	SIRPα mismatch	PIR-A	CD47	MEMORY
YES	YES	+	+	YES
YES	YES	-	+	NO
YES	YES	+	-	NO
YES	NO	+	+	NO
NO	YES	+	+	NO

FIGURE 2 | Allospecific innate memory mechanism. Mismatches of both MHC I and SIRPα between donor and recipient and expressions of both PIR-A and CD47 molecules on recipient monocytes are required for establishing monocyte allospecific memory.

Th1 differentiation. In the graft, they made stable, cognate interactions with effector T cells and increased T cell proliferation and survival. DC depletion starting on day 5 delayed heart allograft rejection by >30 days in WT recipients (60) and completely prevented rejection in mice that lacked 2° lymphoid organs (splenectomized LTβR-/- mice) after transfer of effector T cells. Therefore, host mo-DCs that persistently infiltrate allografts sustain T cell-mediated rejection locally.

In the third set of experiments (51, 61), innate allorecognition and memory molecular pathways were interrupted. We observed that mouse renal allografts transplanted to recipients that lack either CD47 or PIR-A develop significantly less manifestations of chronic rejection. Similarly, blocking the PIR-A pathway led to long-term heart allograft survival with minimal pathology in recipients simultaneously treated with co-stimulation blockade (CTL4Ig). Acute rejection, however, was either not delayed or only modestly improved if either CD47 or PIR-A was absent. Therefore, the major influence of the SIRPα-CD47 and MHC-I-PIR-A pathways is on chronic allograft rejection and on preventing allograft acceptance. In contrast, rejection was accelerated in the absence of PIR-B signaling in the recipient.

CLINICAL TRANSLATION IN TRANSPLANTATION

In humans, interactions through similar signaling pathways mediated by SIRPα and PIRs' homolog LILRs engaging with CD47 and MHC-I molecules, respectively, also exist (62, 63). By x-ray crystallography, Hatherley D et al. showed that the polymorphism in human SIRPα did not affect binding to its ligand CD47 (64). This suggested the possibility, although

requiring further exploration, that human SIRPα differed in binding features from mouse SIRPα, whose binding affinity to its ligand CD47 was recognized to be dependent on its polymorphic IgV domain (20, 24). Our preliminary data also validated that the amino-terminal ligand binding domain of human SIRPα is highly polymorphic (65). Human LILRs family comprises a set of PIRs (A and B) expressed on myeloid innate immune cells. Similar to PIRs in mice, LILR-Bs contain ITIM motifs and are inhibitory while LILR-As do not contain ITIM motifs but contain ITAM motifs and are stimulatory. Six LILR-As and five LILR-Bs have been identified. Both LILR-As and LILR-Bs bind a wide spectrum of MHC-I molecules (63, 66). Human SIRPα-CD47 interaction has been reported to be implicated in the phagocytosis of red blood cells and leukemia cells by macrophages *in vivo* or *in vitro* (62, 67). There are data suggesting a link between LILR polymorphism and control of HIV infection and autoimmunity in humans (63, 66). However, published human studies on the roles of SIRPα and LILRs in transplantation are not available yet. The similarities in these two pathways (SIRPα-CD47 and MHC-I-PIR-A/LILR-A) between human and mice should trigger investigations into the roles of these pathways in clinical transplantation.

CONCLUDING REMARKS

We have presented evidence that the innate immune cells, namely, monocytes and macrophages, respond to allogeneic non-self independently of T, B, and NK cells. This form of allorecognition initiates or sustains the responses of recipient T cells to allografts by inducing the maturation of APCs. It also provides phagocytic cells with the means to kill allogeneic targets without inflicting damage on self-tissues. One mechanism of innate allorecognition is the differential binding of CD47 on monocytes to polymorphic SIRPα on donor cells. We also summarized data showing that monocytes and macrophages acquire memory specific to allogeneic MHC-I molecules that is dependent on MHC-I sensing by polymorphic PIR-A molecules. Blocking SIRPα-CD47 or MHC-I-PIR-A interaction shows promise in preventing chronic rejection or promoting allograft acceptance. Future studies are expected to establish translation of these findings into clinical transplantation.

AUTHOR CONTRIBUTIONS

DZ and FL wrote manuscript. KA-D and HD contributed data and edited manuscript. MO and XL edited manuscript.

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FTY720 Regulates Mitochondria Biogenesis in Dendritic Cells to Prevent Kidney Ischemic Reperfusion Injury

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Dendritic cells (DCs) are central in regulating immune responses of kidney ischemia-reperfusion injury (IRI), and strategies to alter DC function may provide new therapeutic opportunities. Sphingosine 1-phosphate (S1P) modulates immunity through binding to its receptors (S1P1-5), and protection from kidney IRI occurs in mice treated with S1PR agonist, FTY720 (FTY). We tested if *ex vivo* propagation of DCs with FTY could be used as cellular therapy to limit the off-target effects associated with systemic FTY administration in kidney IRI. DCs have the ability of regulate innate and adaptive responses and we posited that treatment of DC with FTY may underlie improvements in kidney IRI. Herein, it was observed that treatment of bone marrow derived dendritic cells (BMDCs) with FTY induced mitochondrial biogenesis, FTY-treated BMDCs (FTY-DCs) showed significantly higher oxygen consumption rate and ATP production compared to vehicle treated BMDCs (Veh-DCs). Adoptive transfer of FTY-DCs to mice 24 h before or 4 h after IRI significantly protected the kidneys from injury compared to mice treated with Veh-DCs. Additionally, allogeneic adoptive transfer of C57BL/6J FTY-DCs into BALB/c mice equally protected the kidneys from IRI. FTY-DCs propagated from *S1pr1*-deficient DCs derived from *CD11cCreS1pr1*$^{fl/fl}$ mice as well as blunting mitochondrial oxidation in wildtype (WT) FTY-DCs prior to transfer abrogated the protection observed by FTY-DCs. We queried if DC mitochondrial content alters kidney responses after IRI, a novel but little studied phenomenon shown to be integral to regulation of the immune response. Transfer of mitochondria rich FTY-DCs protects kidneys from IRI as transferred FTY-DCs donated their mitochondria to recipient splenocytes (i.e., macrophages) and prior splenectomy abrogated this protection. Adoptive transfer of FTY-DCs either prior to or after ischemic injury protects kidneys from IRI demonstrating a potent role for donor DC-mitochondria in FTY's efficacy. This is the first evidence, to our knowledge, that DCs have the potential to protect against kidney injury by donating mitochondria to splenic macrophages to alter their bioenergetics thus making them anti-inflammatory. In conclusion, the results support

that *ex vivo* FTY720-induction of the regulatory DC phenotype could have therapeutic relevance that can be preventively infused to reduce acute kidney injury.

Keywords: dendritic cell, FTY720, mitochondria, sphingosine-1-phosphate receptor, macrophages, metabolism, acute kidney injury, ischemic reperfusion injury

INTRODUCTION

The pathogenesis of kidney injury following kidney ischemia reperfusion (IR) involves a complex interaction between altered microcirculatory hemodynamics, renal parenchymal cells (endothelial and epithelial) and infiltrating immune cells (1, 2). Dendritic cells (DCs), the major leucocyte subset in the kidney (3–5), contributes to both the innate and adaptive immunity of kidney IR injury (IRI) (6) through aberrant activation of immune cells (7–9). Considerable data supports that the immune system mediates acute kidney injury (AKI) (10), yet many of the underlying mechanisms still remain unclear. In preclinical mouse models, anti-inflammatory pharmacologic treatments have been shown to significantly attenuate tissue injury and loss of function (11–14). However, the side effects of these common anti-inflammatory therapies combined with the lack of clinical data, supporting the involvement of the immune system in AKI pathogenesis, have hindered the development of clinically tenable anti-inflammatory options. Therefore, as of now dialysis remains the only treatment option available to AKI patients, underscoring the need to develop novel approaches to tackle this hurdle to ultimately improve patient quality of life.

Our previously published work using mouse models of AKI (13, 14) and others (15, 16) have demonstrated that modulation of Sphingosine 1 Phosphate receptors (S1PRs) significantly influences AKI development and thus progression to chronic kidney injury. These receptors belong to a family of five G-protein coupled receptors (S1pr1-5) that modulate diverse physiological responses including "cellular growth and proliferation, angiogenesis, apoptosis, and lymphocyte trafficking" (17–20). Similar levels of S1PRs are expressed on both human and mouse leukocytes (21–23). FTY720, a potent immunosuppressant and a synthetic S1P agonist is currently in clinical trials for treatment of autoimmune diseases (24) and is effective in reducing graft rejection in preclinical mouse models (25, 26) because it mediates a potent immunosuppression. Phosphorylated-FTY720 (FTY720-P), the active form of FTY720, is a non-selective S1P analog that binds and activates four (S1PR1, 3–5) of the five known receptors for S1P (24, 27). FTY720-dependent protection or diminished disease severity has been demonstrated in varied acute and chronic disease models, such as diabetes (28–33), multiple sclerosis [MS, review (34)], ischemic injury (35–46), and even clearance of viral infection (47). To date, FTY720 is currently used as FDA-approved treatment (Gilenya) of MS patients (48). In our previously published work, we have shown that this pan-S1PR agonist, FTY720, attenuated kidney IRI by directly activating S1P1 on proximal tubule (PT) cells, independent of its previously known function through binding to S1P1 on B and T cells to induce canonical lymphopenia (14). FTY720 also reduces cisplatin-induced AKI (49). Deletion of S1P1 renderers cultured and kidney PT epithelial cells more susceptible to cisplatin-induced injury (49), whereas overexpression of S1P1 protected PT cells from injury and resistance to cisplatin induced cell death at lower doses (49). One potential mechanism that we previously reported to mediate S1P1 protection in IRI and cisplatin-induced AKI was through possible induction of mitochondrial biogenesis that resulted in higher mitochondria numbers and ultimately preserved kidney function (49). Thus, we previously concluded in these published studies that S1P1 had a central role in stabilizing mitochondrial function and FTY720 administration could represent a novel strategy in the prevention of AKI (14, 49).

However, use of pharmacological agents such as FTY720 has limitations due to off-target (binding to other S1P receptors) and other associated adverse side effects. On the other hand, cell-based therapeutic approaches have advantages; transferred cells are capable of sensing diverse signals, navigating to specific sites in the body, make immunological decisions and executing complex responses. Dendritic cells (DCs) are heterogeneous, professional antigen-presenting cells (APCs) and are distributed throughout the lymphoid and non-lymphoid tissues (50). Our previous studies demonstrated that S1P3 deficient ($S1pr3^{-/-}$) mice are protected from renal IRI through a mechanism that involved BMDCs and their ability to respond as immune modulators to regulate innate and adaptive immune responses (13). Additionally, we had also tested the therapeutic advantage of using $S1pr3^{-/-}$ BMDC in DCs transfer studies in mouse kidney IRI model. Compared to mice treated with wild-type (WT) DCs that had significant rise in plasma creatinine, mice that received $S1pr3^{-/-}$ DCs were significantly protected from kidney IRI (12, 13). $S1pr3^{-/-}$ DCs did not attenuate IRI in splenectomized, $Rag1^{-/-}$, or DC-depleted (*CD11c-DTR*) mice (12) demonstrating that both spleen derived cells, likely macrophages ($CD169^+$ or $F4/80^+$) or DCs ($CD11c^+$ or $CD103^+$) and T cells ($CD4^+$ and Tregs) mediated this protection.

The aim of this study was to determine the potential protective mechanism(s) of FTY720 stimulated BMDCs in a preclinical mouse model of kidney IRI. Treatment of BMDCs *ex vivo* with FTY720 avoids any adverse off-target effects associated with systemic drug injections. Herein, we demonstrate that FTY720 treated BMDCs (FTY-DC) accumulate in the recipient spleen as early as 30 min after adoptive transfer of cells via intravenous injection. FTY-DC mitochondrial content was elevated *in vitro,* and we posit that transfer of FTY-DC mitochondria to splenic macrophages occurs. Indeed, in spleen, FTY-DC interaction with splenic macrophages ($CD169^+$

and F4/80^{+}) was evident. Transplant of mitochondria from FTY-DC reprogrammed macrophage phenotype; macrophages were less immunogenic upon inflammatory stimuli *in vivo* and *in vitro*. Depletion of DC-derived mitochondria through varied approached demonstrated that oxidative capacity of DC was critical to protection from AKI in response to IRI. Splenectomy or pharmacologic ablation of mitochondrial function with combination treatment with rotenone and antimycin A (Rot/AA) of FTY-DC abrogated the protection observed with FTY-DCs. Likewise, inhibiting FYT720 agonism using S1P1 receptor deficient DCs (*CD11cCreS1pr1*$^{fl/fl}$) also reversed FTY-DC therapeutic efficacy. Overall, the interactions between FTY-DC and splenocytes (macrophage) demonstrated that induction of the anti-inflammatory or immunosuppressive phenotype led to reduced injury, an effect that required the recipient spleen. Of note, adoptive transfer of DC worked equally well in allogeneic IRI model (C57BL/6 BMDC → BALB/c mice), suggesting that this cell-based therapy can be efficacious in transplantation. Finally, we provide seminal findings that DCs are mitochondrial donors which illustrate a novel mechanism of how DCs regulate innate immune responses in acute injury.

MATERIALS AND METHODS

Mice

All animals were handled, and procedures were performed in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the University of Tennessee Health Science Center and University of Virginia Institutional Animal Care and Use Committees. *CD11cCre* mice (Jackson Laboratories, Bar Harbor, ME) were purchased and *S1pr1*$^{fl/fl}$ generously provided by Dr. Richard L. Proia, NIH. The lines were crossed and bred as fl/fl with Cre to generate *CD11c*Cre*S1pr1*$^{wt/wt}$ (control) or *CD11c*Cre*S1pr*$^{fl/fl}$ (DC specific S1pr1 knockout) littermates. *Pham*$^{fl/fl}$ mice (51) (Jackson Laboratories, Bar Harbor, ME) were bred with CD11cCre to obtain *CD11c*Cre*Pham*$^{fl/fl}$ mice. For all transfer studies C57BL/6J and BALB/c mice were purchased from the National Cancer Institute, NCI (Frederick, MD). Mice were maintained in standard vivarium housing with a 12 h light/dark cycle on a chow diet and water was freely available.

Renal Ischemia-Reperfusion Injury and Splenectomy (SPLNX)

Mice were anesthetized with an intraperitoneal injection of a ketamine (120 mg/kg) and xylazine (12 mg/kg) mixture and buprenorphine (0.15 mg/kg, subcutaneous injection) was administered as an analgesic and placed on a warm pad to maintain body temperature at 34.5–36°C. Mice were then randomized to sham or IRI operation. Bilateral flank incision was performed and either the renal vessels (vein and artery) on both sides or only on the left side were cross-clamped. Body temperature was checked and maintained throughout the ischemic period using ATC-2000 system (World Precision Instruments, Sarasota, FL). Sham-operated mice underwent the same procedure except for vessel clamping and surgical wounds were closed. Male mice (8–12 wk old, C57BL/6 and BALB/c) were subjected to bilateral IRI (26 min ischemia for C57BL/6 and 28 min for BALB/c mice followed by 20–24 h reperfusion) as previously described (3, 7, 52). Mice that had one kidney with no reperfusion 24 h after ischemia were excluded from all analysis. For experiments that involved splenectomy (Splnx) prior to IRI, mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (12 mg/kg). The spleen was then removed through a small flank incision. Control, sham-operated mice underwent the same procedure except for splenic artery ligation and spleen removal. Sham and splenectomized mice recovered for 7 days prior to BMDC transfer for IRI studies.

Assessment of Kidney Function and Histology

Blood was collected under anesthesia from the retro-orbital sinus, and plasma creatinine (mg/dL) was determined by using an enzymatic method with minor modifications from the manufacturer's protocol (twice the volume of sample; Diazyme Laboratories, Poway, CA) and as previously reported (53). For histology, kidneys were fixed overnight in 0.2% sodium periodate-1.4% DL-lysine-4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (4% PLP) and embedded in paraffin. Kidneys were prepared for Hematoxylin and eosin (H&E) staining as previously described (3) and viewed by light microscopy (Zeiss AxioSkop). Photographs were taken and brightness/contrast adjustment was made with a SPOT RT camera (software version 3.3; Diagnostic Instruments, Sterling Heights, MI). For quantification of tubular injury score, sections were assessed by counting the percentage of tubules that displayed cell necrosis, loss of brush border, cast formation, and tubule dilation as follows: 0 = normal; 1 = <10%; 2 = 10 to 25%; 3 = 26 to 50%; 4 = 51 to 75%; 5 = >75%. Five to 10 fields from each outer medulla were evaluated and scored in a blinded manner. The histological change was expressed as acute tubular necrosis (ATN), scored as previously described (13, 54).

Immunohistochemical Analysis

Kidneys were fixed in 1% PLP (as above except 1% paraformaldehyde) overnight, incubated in 30% sucrose for 24 h at 4° C, and embedded and frozen in Tissue-Tek OCT Compound (Ted Pella Inc., Redding, CA). Frozen sections (5–7 μm) were permeabilized with 0.3% Triton X-100, and non-specific binding was blocked with 10% horse serum and rat anti-mouse CD16/32 (10 μg/ml; clone 2.4G2; BD Pharmingen, San Jose, CA). Sections were labeled by incubation for 1 h with anti-mouse F4/80 (5 μg/ml; clone BM8, Molecular probes, Fredrick, MD), anti-mouse CD169 (7 μg/ml; clone 3D6.112, BioLegend, San Diego, CA), anti-mouse CD169 (7 μg/ml; clone MOMA-1; AbD Serotec/BioRad, Raleigh, NC). All specimens were mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen, Carlsbad, CA) to label cell nuclei. Images were acquired using a Zeiss Axiovert 200 microscopy system with ApoTome imaging and Axiovision 4.8 software (Carl Zeiss Microscopy, Thornwood, NY).

Bone Marrow (BM)-Derived-Dendritic Cell (DC) Culture and Adoptive Transfer

Eight week old C57BL/6J WT, *CD11c*Cre*S1pr*$^{fl/fl}$, *CD11c*Cre*Pham*$^{fl/fl}$ *S1pr3*$^{-/-}$ male mice were used for generating DCs from whole BM precursors (55). GMCSF-rich supernatant was derived from J558L cells stably transfected with mouse GMCSF. The cell line was a generous gift from Dr. Ira Mellman (Dept. of Biology, Yale University). Briefly, freshly isolated BM was cultured with 6 ng/ml recombinant mouse GMCSF (total of 3 treatments) for 8 days in RPMI 1640 (Invitrogen). Eighty to Ninety percentage of resulting cells were CD11c^{+} DCs as determined by flow cytometry with CD11c antibody. The optimal dose of 1 μM FTY720 was decided after testing various doses (0.1–10 μM). Similar to studies done by Zeng et al. (56) FTY720 treated BMDCs were tested for drug induced cellular toxicity (apoptosis) and changes in co-stimulatory molecules (CD40, CD80, CD86, and MHCII) after overnight treatment with LPS. BMDC were treated with 1 μM FTY720 (total of 4 treatments) that was purchased from Cayman Chemicals (Ann Arbor, Michigan). BM-derived DCs were treated with TLR4 agonist lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4; 25 or 100 ng/mL; Sigma-Aldrich); or vehicle (1x PBS) for 24 h in culture medium for syngeneic studies (C57BL/6J BMDCs→ C57BL/6J mice) and left untreated for allogenic studies (C57BL/6J BMDCs→ BALB/c mice). See timeline in **Supplemental Figure 1**. Cells were washed, and 0.5 × 10^{6} cells per mouse were i.v. injected to naive mice 1 day before or in some studies 4 h after kidney IRI. Griess Reagent system (Promega) was used to detect nitrate in media after LPS stimulation. BMDCs were labeled with MitoTracker CMXRos Red or MitoTracker Green (50–100 nM, 30 min @ 37°C, Invitrogen) or Mitosox (5 μM; 10 min @ 37°C; Invitrogen) prior to fixing with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 30 min. Actin was labeled with phalloidin-FITC (2.5 μg/ml; Sigma). Nuclei were visualized using DAPI. All specimens were mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen). Images were acquired using the Zeiss Axiovert 200 microscopy system with ApoTome imaging and Axiovision software (Carl Zeiss Microscopy LLC, Thornwood, NY). RAW264.7 cells were purchased from ATCC (Manassas, VA) and maintained in DMEM (Invitrogen).

Quantitative Real-Time PCR

Total RNA was isolated and reversed transcribed to cDNA, and RT-PCR was performed as previously described (13, 49, 57). Primers span an exon-exon junction and were designed with Primer-BLAST (NCBI). *Pgc1a*, NM_008904.2, 5′GCTCTTCCTT TAACTCTCCGTGTC3′ and 5′CTTGACCTGGAATAT GGTG ATCGG (53). Relative mitochondrial DNA (mtDNA) expression level was measured as previously described (58). Briefly, total genomic DNA was isolated and equal amounts (5 ng) was used for RTPCR using ND1 as surrogate primers for mtDNA and HK2 primers for nuclear DNA (nDNA) for mouse and ND6 for human mtDNA as previously described (58, 59). Total number of mtDNA copies was determined by following formula, delta Ct = nDNA gene (Ct)- mtDNA (Ct); mtDNA copy = $2 \times 2^{\delta}Ct$ (58).

Mitochondria Isolation and Quantification

Mitochondria were isolated from mouse liver or BMDCs as previously described (60). Briefly, 2 pieces of ∼6 mm mouse liver biopsies were homogenized using homogenization buffer (300 mmol/L sucrose, 10 mmol/L HEPES-KOH, 1 mmol/L EGTA-KOH, pH 7.4) in a C tube (Miltenyi Biotec, Cambridge, MA) with GentlyMACS dissociator using the "m-mito tissue" pre-set program. The homogenate was incubated on ice for 10 min with 1 mg Subtilisin A protease from *Bacillus licheniformis* (Sigma-Aldrich, St. Louis, MO). The digested homogenate was serially filtered through 2 × 40 μm Falcon Cell Strainers (Thermo-Fisher, Waltham, MA) and 1 × 10 μm PluriSelect mesh (PluriSelect, San Diego, CA) that was saturated with ice cold homogenization buffer. Mitochondria were collected by centrifuging the filtrate at 3,500 × g at 4°C for 10 min and re-suspended in cold 1x PBS for further use. Protein concentration of isolated mitochondria were determined using Bradford assay according to manufacture recommendations. Isolated mitochondria were kept on ice and used within 1 h after isolation. In some experiments isolated mitochondria were sonicated and kept on ice before injecting, all isolated mitochondria were injected within 1 h of isolation. ATP concentrations of isolated mitochondria were using luminescent CellTiter-Glo reagent (Promega) according to the manufacturer's instructions. Isolated mitochondria were injected (i.v.; 0–100 μg/mouse) 1 day before spleen was harvested for single cells preparation for *in vitro* stimulation with LPS (100 ng/ml) for 6 h. RAW264.7 cells (TIB-71, ATCC, Old Town Manassas, VA) were treated with isolated mitochondria (with and without sonication, 10 μg/ml) from DCs for 24 h before stimulating with LPS (100 ng/ml) or analysis with Seahorse Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Seahorse Flux Bioanalyzer

Seven day old BMDCs were transferred to a Seahorse 24-well tissue culture plates and oxygen consumption rate (OCR) was measured, and parameters were calculated as previously described (49) with the following modification. Prior to the assay, the media was changed to unbuffered DMEM (Gibco #12800-017, pH 7.4, 37°C), and cells were equilibrated for 30 min at 37°C. After measuring basal respiratory rate, Oligomycin (Sigma; 2 μM; uncouples ATP-coupled respiration by inhibiting ATP synthase), FCCP (Sigma; 1.5 μM; carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP), mitochondrial uncoupling agent; uncouples mitochondrial respiration from ATP to determine maximal respiratory rate), and electron transport chain (complex I and III) inhibitors, rotenone (Sigma; 0.5 μM) and antimycin A (Sigma; 0.5 μM; to eliminate all mitochondrial respiration) were injected sequentially during the assay. OCR was measured in 3 min periods of time (over a total period of 2 h). Basal mitochondrial respiration, ATP-linked respiration, proton leak (non-ATP linked oxygen consumption), maximal respiration, non-mitochondrial respiration, reserve respiratory capacity, respiratory control ratio, and coupling efficiency were determined in whole cells according to Brand et al. (61), N = 4–5 wells were used for each experimental group and experiments were repeated a minimum of 3 times.

Flow Cytometric Analysis, Western Blot, ELISA, and 32-Plex Luminex

Flow cytometry was used to analyze kidney leukocyte content. In brief, kidneys were extracted, minced, and digested (1 mg/ml collagenase) as described (7). After blocking nonspecific Fc binding with anti-mouse CD16/32 (2.4G2), fresh kidney suspensions were incubated with fluorophore-tagged anti-mouse CD45 (30-F11) to determine total leukocyte cell numbers. CD45-labeled samples were further used for labeling with different combinations of fluorophore-tagged anti-mouse F4/80 (BM8), GR-1 (Ly6G), CD11b (M1/70), CD11c (integrin alpha X chain-HL3). 7-AAD (BD Biosciences) was added 15 min before analyzing the sample to separate live from dead cells. Appropriate fluorochrome-conjugated, isotype-matched, irrelevant mAbs were used as negative controls. Flow cytometry data acquisition was performed on a FACS Calibur (Becton Dickinson, San Jose, CA) with Cytek 8-color flow cytometry upgrade (Cytek Development, Inc., Fremont, CA). Data were analyzed by FlowJo software 9.0 (Tree Star, Ashland, OR). All antibodies (except as noted) were from eBioscience and were used at a concentration of 5 μg/ml. ELISA: Media was collected from BMDCs or splenocytes treated for 6 or 24 h with wither 25 or 100 ng/ml LPS. TNFα levels were measured by using mouse ELISA kits (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. BMDCs treated with and without LPS for 24 h were used to isolated total protein using RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Vernon Hills, IL). Equal volumes of the lysate supernatants were either boiled for 10 min at 100°C for GAPDH) or left at room temperature for 10 min for rodent OXPHOS cocktail (Abcam, Cambridge, MA) with Laemmli buffer and β-mercaptoethanol. Total of 20 μg of proteins were separated using a 10% SDS-PAGE gel and transferred to PVDF membranes. PVDF membranes were incubated overnight with primary antibody for GAPDH (1:1000, Santa Cruz Biotechnology) and rodent OXPHOS cocktail (1:1000). Blots were then washed and incubated at 1:4000 for 1 h with horseradish peroxidase-conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology). Bands were visualized by chemiluminescence according to the manufacturer's protocol with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and quantified by Image J. The Bio-Plex Pro Mouse Cytokine 23-Plex Immunoassay was used to check serum levels 24 h after bilateral kidney IRI (BioRad, Hercules CA).

Data and Statistical Analysis

GraphPad Prism 8 (GraphPad Inc.), SigmaPlot 11.0 (Systat Software Inc.), and Canvas X (ACD Systems of America Inc.) were used to analyze and present the data. Data were analyzed, after transformation if needed to generate a normal distribution, by 2-tailed *t*-test or 1-way ANOVA with *post-hoc* analysis as appropriate. Two-tailed unpaired *t*-test was used for analysis of two groups. $p < 0.05$ was used to indicate significance.

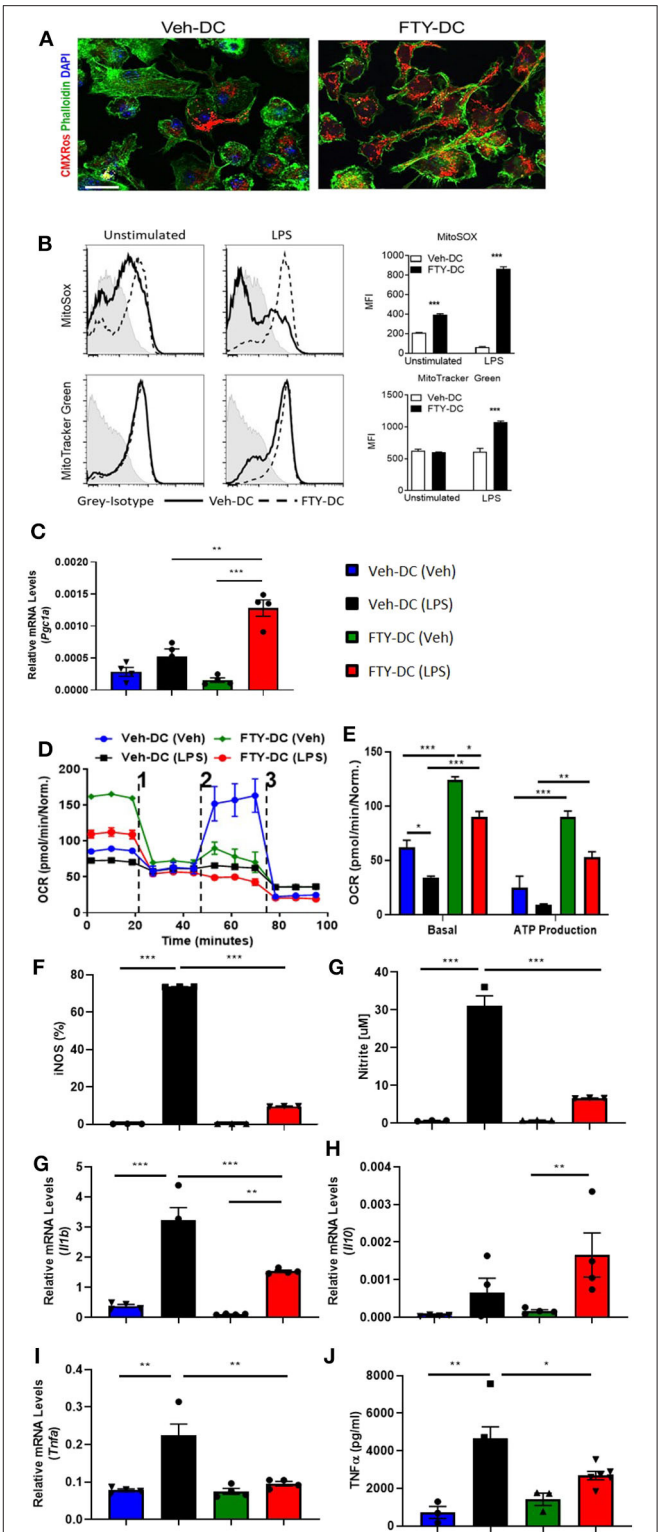

FIGURE 1 | FTY720 treatment induces less immunogenic DCs that have higher mitochondria numbers. **(A)** 8 day old Veh-DC and FTY-DC were labeled with MitoTracker CMXRos (50 nM). Scale bar, 20 μm. **(B)** Veh-DC and FTY-DC treated with 100 ng/ml LPS for 24 h or left unstimulated (unstim). Levels of MitoSox (5 μM) and MitoTracker green (100 nM) were measured after 24 h of

(Continued)

FIGURE 1 | LPS treatment. **(D–E)** DCs were seeded in a Seahorse XF-24e analyzer, stimulated with and without LPS for 24 h, and oxygen consumption rate (OCR) was determined during sequential treatments with Oligomycin (1), FCCP (2) and antimycin A plus rotenone (3). Quantification of basal OCR and ATP production. **(F)** Flow cytometry analysis of iNOS expression was determined with and without LPS stimulation. Quantification of percent of iNOS in Veh-DC and FTY-DC after LPS. **(G)** Nitrite levels were determined in culture supernatants with and without LPS stimulation. mRNA levels of *Pgc1a* **(C)**, *Il1b* **(H)**, *Il10* **(I)**, and *Tnfa* **(J)** in Veh-DC and FTY-DC treated with 100 ng/ml LPS or left unstimulated for 24 h. **(K)** ELISA of TNFα from the Veh-DC and FTY-DC treated with 100 ng/ml LPS for 24 h. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$, one-way ANOVA followed by Tukey's post-test. Data represent means ± SEM of triplicates. One of three experiments is shown.

RESULTS

FTY720 Induces Metabolic Reprogramming in WT BMDCs

WT DCs were isolated and propagated for 8 days in presence of GMCSF and vehicle (1X PBS) or FTY720 (1 μM). Eight day old DCs were labeled with MitoTracker CMXRos Red (50 nM). Compared to vehicle treatment, FTY720 treatment increased mitochondrial content in BMDCs (**Figure 1A**). Similarly, there was significantly higher labeling for MitoSox (5 μM) and MitoTracker Green (100 nM) after over-night LPS stimulation in FTY-DC compared to Veh-DC (**Figure 1B**). FTY-DCs displayed significantly elevated mRNA levels for peroxisome proliferator-activated receptor gamma co-activator 1-alpha (*Pgc1a*) in response to LPS, but this LPS-induction was absent in Veh controls (**Figure 1C**). To determine if the changes in mitochondrial content also altered mitochondrial function, bioenergetic analysis was undertaken. LPS blunted oxygen consumption compared to Veh controls in Veh-DCs as expected (**Figure 1D**, blue to black line) (62). Interestingly, FTY-DCs have higher basal OCR (**Figure 1D**, green to blue line at time zero). Upon treatment with uncoupler FCCP, FTY-DC demonstrate a failure to increase maximal respiratory capacity in unstimulated cells that is even more reduced with LPS stimulation demonstrating that FTY ablates spare respiratory capacity (likely because already at maximal OCR in basal state). When ATP production was quantified, LPS reduced ATP production as measured by OCR (blue to black). FTY-DC demonstrated significantly greater ATP production compared to Veh-DC in both unstimulated and LPS-stimulated DCs (**Figure 1E**). These data indicate that propagation of BMDCs in presence of FTY720 increased mitochondrial content, basal OCR, and ATP production. This suggests the potential for an anti-inflammatory phenotype in DCs.

FTY720 Induces Immune Reprogramming in WT BMDCs

LPS stimulation of BMDCs increased expression of enzymes (iNOS) and cytokines typical of pro-inflammatory DCs. FTY-DC dramatically blunted LPS-induced iNOS expression and nitrate in media compared to Veh-DC (**Figures 1F,G**). Likewise, FTY significantly blunted expression of LPS-induced *Il1b* and *Tnfa* and protein concentrations of TNFα compared Veh-DC treated with LPS (**Figures 1H–K**). *Il12p40* gene expression was not regulated by FTY (data not shown). In contrast, *Il10*, a cytokine often associated with anti-inflammatory immune cells was significantly increased by LPS but only in FTY treated cells (**Figure 1I**). Interestingly, after LPS treatment, FTY-DC have significantly lower expression levels of co-stimulatory antigen presentation molecules (CD80, CD86, and CD40) and MHCII compared to Veh-DC (**Supplemental Figure 1**). Interestingly, FTY-DCs had lower expression level for PDL1 compared to Veh-DCs and maintained the PDL1/CD86 ratio after LPS stimulation compared to LPS treated Veh-DCs (**Supplemental Figure 1**). No significant changes in CD11c expression was observed between Veh- and FTY-DCs, although FTY-DC had lower side scatter signal indicating smaller size of cells after LPS stimulation (data not shown). FTY-DCs had higher relative mtDNA levels compared to Veh-DCs (106 ± 13.03 vs. 165.9 ± 20.6). Additionally, we measured total protein changes in mitochondrial OXPHOS complexes (I-V) in Veh- and FTY-DCs treated overnight with 100 ng/ml LPS. FTY-DCs have higher protein levels of different mitochondrial complexes compared to Veh-DCs with and without LPS treatment (**Supplemental Figures 1H–J**) especially in levels of complex IV (MTCO1) a protein that is encoded by mitochondrial DNA (mtDNA). Mitochondria complex IV was significantly higher in vehicle treated FTY-DC compared to vehicle treated Veh-DC (0.10 ± 0.01 vs. 0.49 ± 0.01, $p < 0.01$) and after LPS treatment (0.04 ± 0.003 vs. 0.16 ± 0.01, $p < 0.01$) along with higher complex III after LPS treatment (**Supplemental Figure 1J**).

Transfer of FTY-DC Protects Kidneys From Ischemic Injury

All DCs were activated with 100 ng/ml LPS prior to transfer in all syngeneic studies (B6 BMDC to B6 mice). Half a million DCs were injected 1 day before bilateral kidney IRI. As control, mice were injected with 1x PBS as no cell (NC) controls. Compared to NC and Veh-DC treated mice, FTY-DC treated mice significantly protected the kidneys from injury (**Figure 2A**). Morphological changes (**Figure 2B**) paralleled functional studies. FTY-DC treatment resulted in less infiltration of immune cells (CD45 labeled) compared to Veh-DC or NC treated mice (**Figure 2C**). Quantitative analysis with flow cytometry further demonstrates that FTY-DC treated mice have few neutrophil infiltrations compared to NC or Veh-DC treated mice (**Figures 2D–F**). To determine if kidney injury genes along with *S1pr1* were regulated we measured by qRT-PCR relative kidney levels in DC treated mice. Mice treated with FTY-DC have significantly lower kidney mRNA levels for *S1pr1, Ngal, Kim1,* and lower levels for *Il6* (**Figure 2G**). These data indicate that FTY-DC treated mice have significantly less inflammation (cytokine levels) that results in less infiltration of innate immune cells (PMNs) after kidney IRI. The expression levels of S1pr1 increase after IRI compared to sham operated mice in a time dependent manner (54), possibility indicating initiation of compensatory mechanism due ischemic injury. Plasma samples from Veh- and FTY-DC treated mice were check 24 h after bilateral ischemia using 23-plex Luminex.

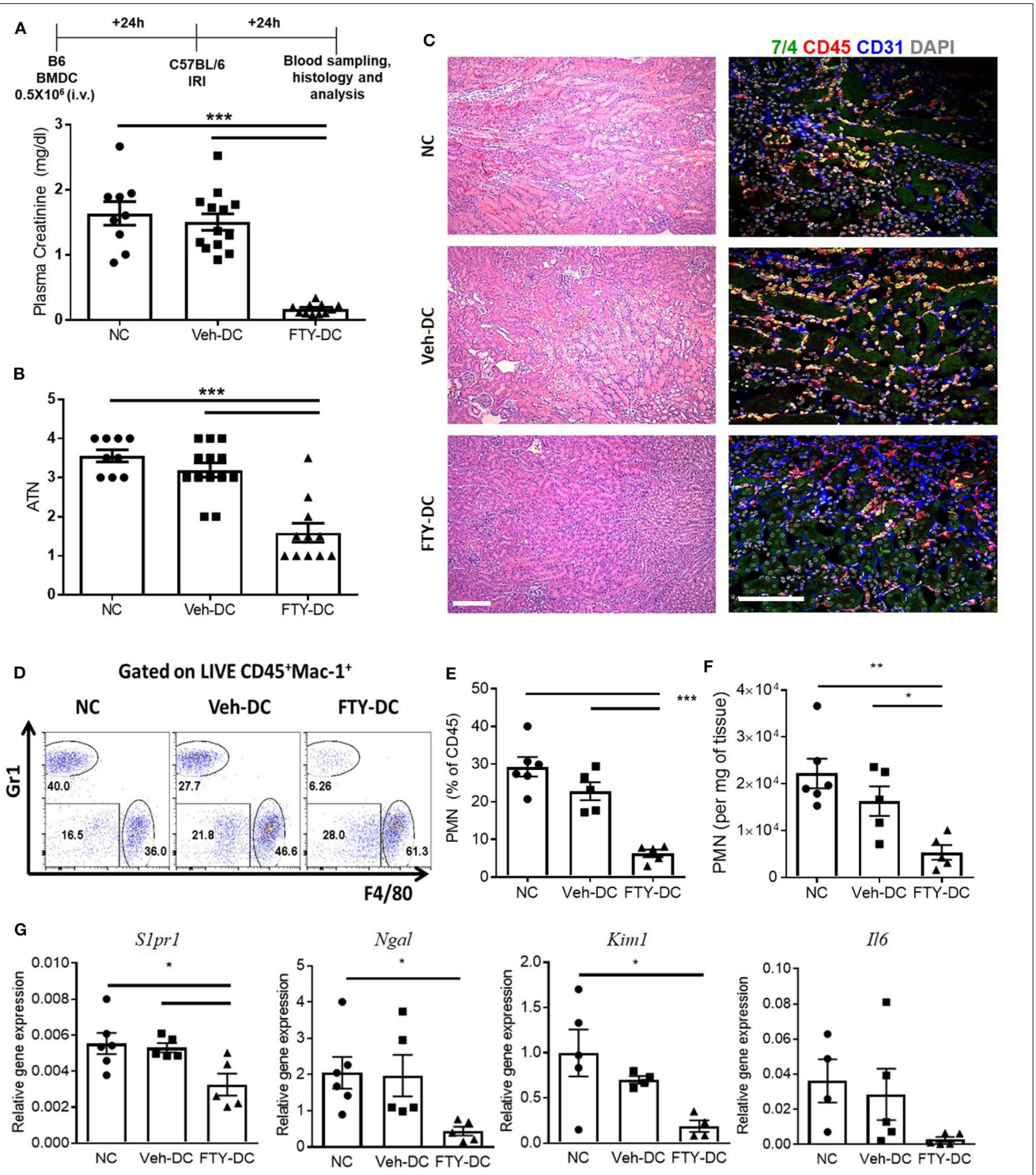

FIGURE 2 | Pretreatment with FTY-DCs protects kidneys from ischemia reperfusion injury. Mice were i.v. injected with 0.5 × 10^6 DCs (Veh-DC or FTY-DC) and as control no cells (NC) 1 day before bilateral kidney IRI. **(A)** Protocol for experimental setup and plasma creatinine. **(B,C)** Quantification of acute tubular injury (ATN). Renal histology (H&E). Scale bar, 100 μm. **(D–F)** Flow cytometry of kidney tissue gated on neutrophils ($CD45^+Mac\text{-}1^+$ $Gr1^+$; PMNs) from mice either treated with NC, Veh-DC or FTY-DC. **(G)** Gene expression of kidney *S1pr1, Ngal, Kim1,* and *Il6*. Data represent means ± SEM, $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$ one-way ANOVA followed by Tukey's post-test.

Only 8 out of 23 cytokines showed low levels of signal. There were no significant changes in circulating levels of MCP-1, IL-9, LIX, or Eotaxin, although trends were toward lower levels in FTY-DC treated mice. Only 3 cytokines were significantly lower (pg/ml; KC [382.06 ± 99.7 vs. 87.22 ± 19.4, $p < 0.05$], IL-1a [508.2 ± 103.1 vs. 245.5 ± 103.4, $p < 0.05$] and G-CSF [910 ± 15.1 vs. 542 ± 135.1, $p < 0.05$]) and significantly higher levels of circulating GM-CSF [46.3 ± 3.6 vs. 112.1 ± 9.8, $p < 0.05$] in FTY-DC treated mice compared to Veh-DC treated mice.

Injected DCs Transfer Mitochondria to Splenic Macrophages

Next to evaluate if DCs transfer mitochondria to recipient cells we harvested BMDCs from *CD11cCrePham*$^{fl/fl}$ mice that contain a fluorescent tag in their mitochondria (**Figure 3A**). Half a million Veh-DC or FTY-DC that were propagated from *CD11cCrePham*$^{fl/fl}$ mice were i.v. injected and signal in spleen was evaluated 30 min or 24 h after injection. The spleen was labeled with anti-CD169 to identify marginal zone (MZ) and anti-F4/80 for red pulp (RP) macrophages and no antibody labeled area are labeled as white pulp (WP) (**Figures 3B,C**). Some green fluorescence signal indicative of mitochondria exchange from DCs to macrophages in CD169^{+} cells at the 30 min after injection time point was evident (data not shown). Strong signal in proximity and inside the various splenic macrophages at 24 h from injected DCs is demonstrated (**Figures 3B,C**). In addition to possibly more mitochondria transfers from FTY-DC, it appears there is disruption in the MZ macrophages (CD169) with FTY-DC treatment along with more mitochondria signal in red pulp compared to Veh-DC (**Figure 3C**). This disruption in MZ macrophages in mice treated with FTY-DC could possibly be due to similar mechanism that we have previously demonstrated using *S1pr3*$^{-/-}$ DCs; that ultimately result in higher CD4^{+}FoxP3^{+} Tregs in white pulp (12).

Splenectomy (Splnx) Abrogates FTY-DC Dependent Protection

Since abundant signal from injected DCs (*CD11cCrePham*$^{fl/fl}$) was found in spleen, to determine if spleen was important in FTY-DC-dependent protection after kidney IRI, mice underwent either sham or Splnx surgeries and were allowed to recover for 7 days. On day 8, half a million LPS treated either Veh-DC or FTY-DCs were injected 1 day before bilateral kidney IRI, as above. In absence of spleen, FTY-DC dependent protection was completely abrogated (**Figure 4A**). Histological evaluation also showed dramatic FTY-DC-dependent protection of kidney architecture (**Figures 4B,C**). Quantitative analysis with flow cytometry further demonstrates that FTY-DC treated Sham mice have few neutrophil infiltrations compared to Veh-DC treated mice (**Figures 4D,E**), no changes in neutrophil percentage or numbers was observed in Splnx-FTY-DC treated mice. In addition to involvement of innate immune cells (macrophages) as possible mitochondria recipients from injected DCs, it is plausible that DCs could donate mitochondria to other adaptive

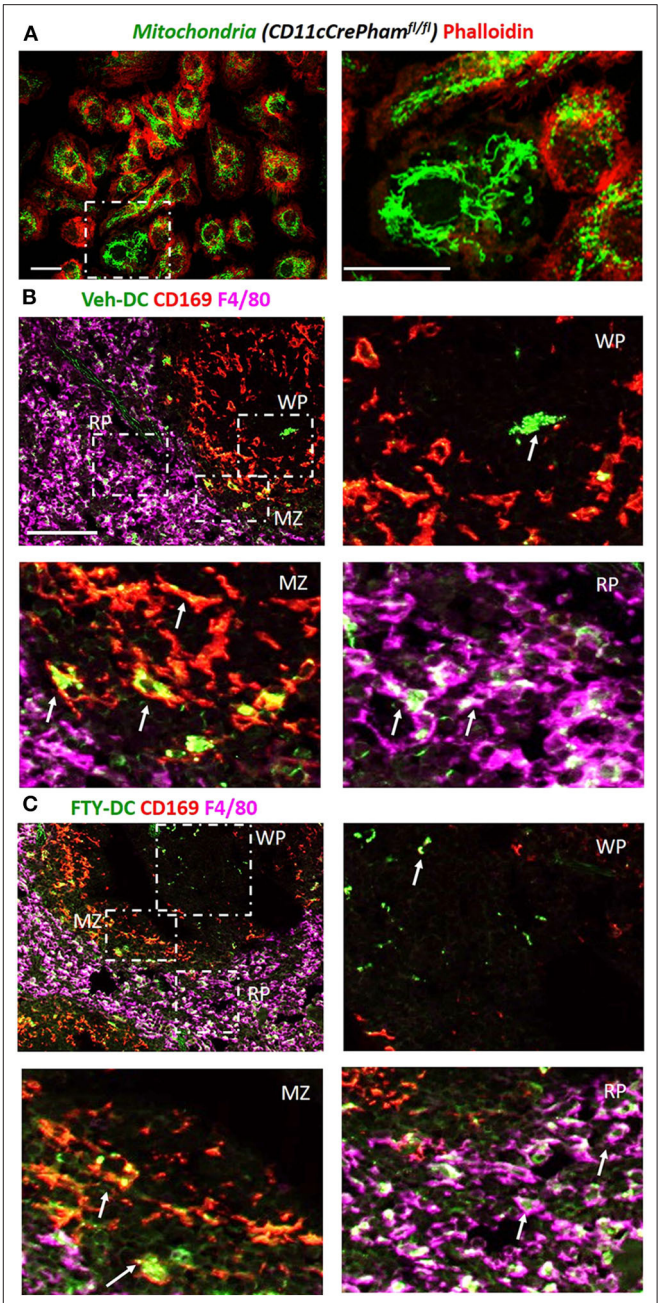

FIGURE 3 | Injected DCs are detected in recipient spleen after adoptive transfer and donate more mitochondria to splenic macrophages. **(A)** BMDCs were propagated from *CD11cCrePham*$^{fl/fl}$ mice. 8 day old BMDCs were grown on coverslip and labeled with phalloidin (red, actin) and endogenous mitochondria (green), Scale bar, 20 μm; inset on right, Scale bar, 20 μm. **(B)** *CD11cCrePham*$^{fl/fl}$ Veh-DC and **(C)** FTY-DC labeled with CD169 (red, clone MOMA-1, marginal zone macrophages) and F4/80 (magenta, red pulp macrophages). The appearance of yellow is colocalization of injected mitochondria (green) with CD169 (red) and white is colocalization of injected mitochondria (green) with F4/80 (magenta). RP, red pulp; WP, white pulp; and MZ, marginal zone. Scale bar, 100 μm (top **B,C**). Scale bar, 20 μm (bottom **B,C**). White arrows point to the taken-up mitochondria from *CD11cPham*$^{fl/fl}$ BMDC.

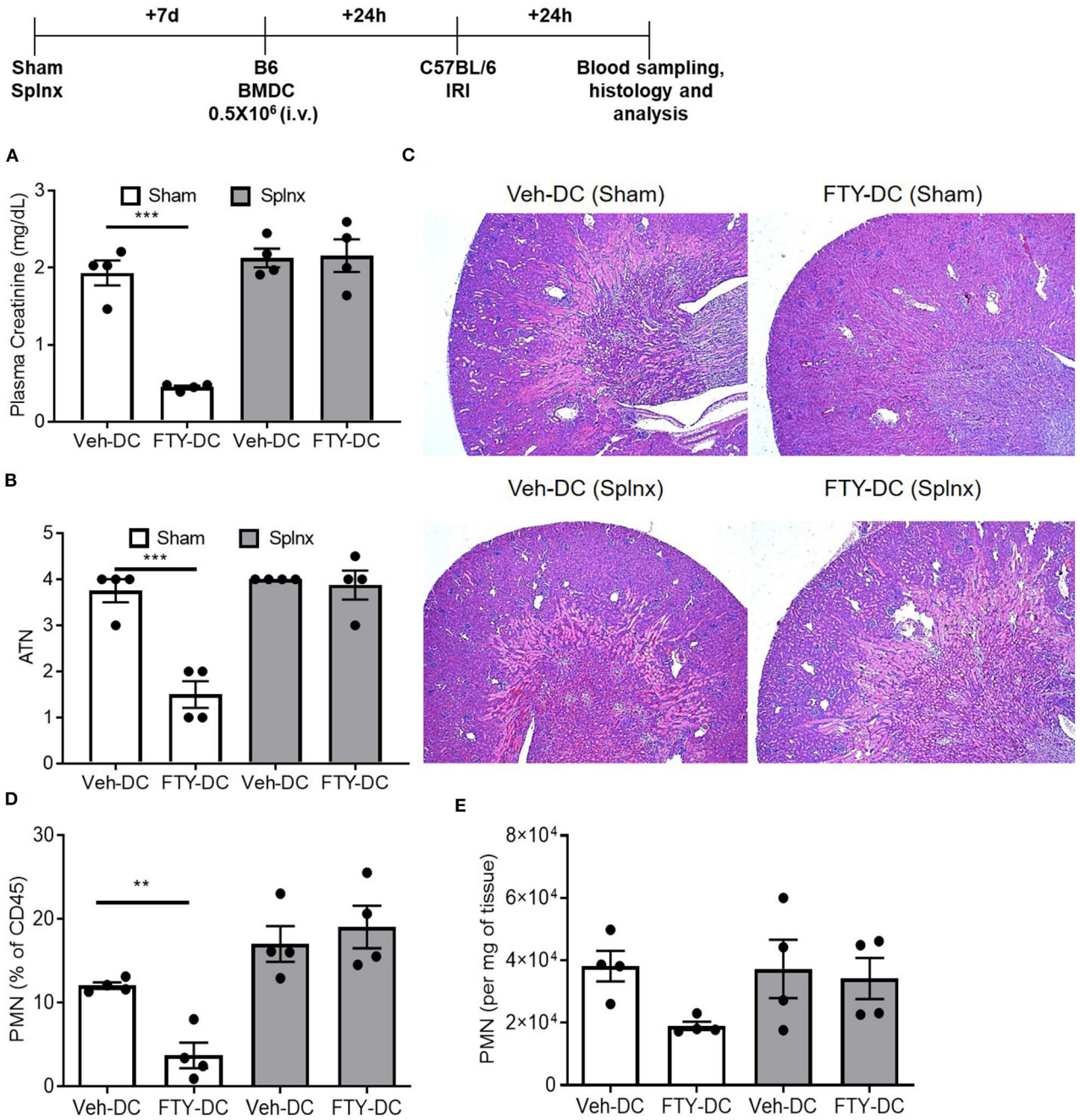

FIGURE 4 | FTY-DC require recipient spleen to protects kidneys from IRI. Protocol for experimental setup. **(A)** Plasma Creatinine (PCr). Mice were i.v. injected with 0.5 × 10^6 DCs (Veh-DC or FTY-DC) 7 days after splenectomy (Splnx) and 1 day before bilateral kidney IRI. **(B,C)** Quantification of tubular injury. Renal histology (H&E). **(D,E)** Flow cytometry of kidney tissue gated on neutrophils from Sham (no Splnx) mice either treated with Veh-DC or FTY-DC. Data represent means ± SEM, $^{***}p \leq 0.001$, $^{**}p \leq 0.01$; one-way ANOVA followed by Tukey's post-test.

immune cells (T and/or B cells). Therefore, we evaluated spleens of sham (no Splnx) mice after kidney IRI that were treated with either Veh-DCs or FTY-DCs. FTY-DC treated mice had higher total number of splenic Tregs ($CD4^+Foxp3^+$) compared to Veh-DC treated mice (44,593 ± 7,136 vs. 71,173 ± 10,436, $p = 0.09$).

Dendritic Cell S1P1 Are Required for FTY-DC Dependent Protection

FTY720 dependent protection is mainly due to its binding to S1P1 at low doses and potentially followed by S1P3 at higher doses. It is unclear which receptor FTY720 may be signaling through to induce such protection from IRI. To

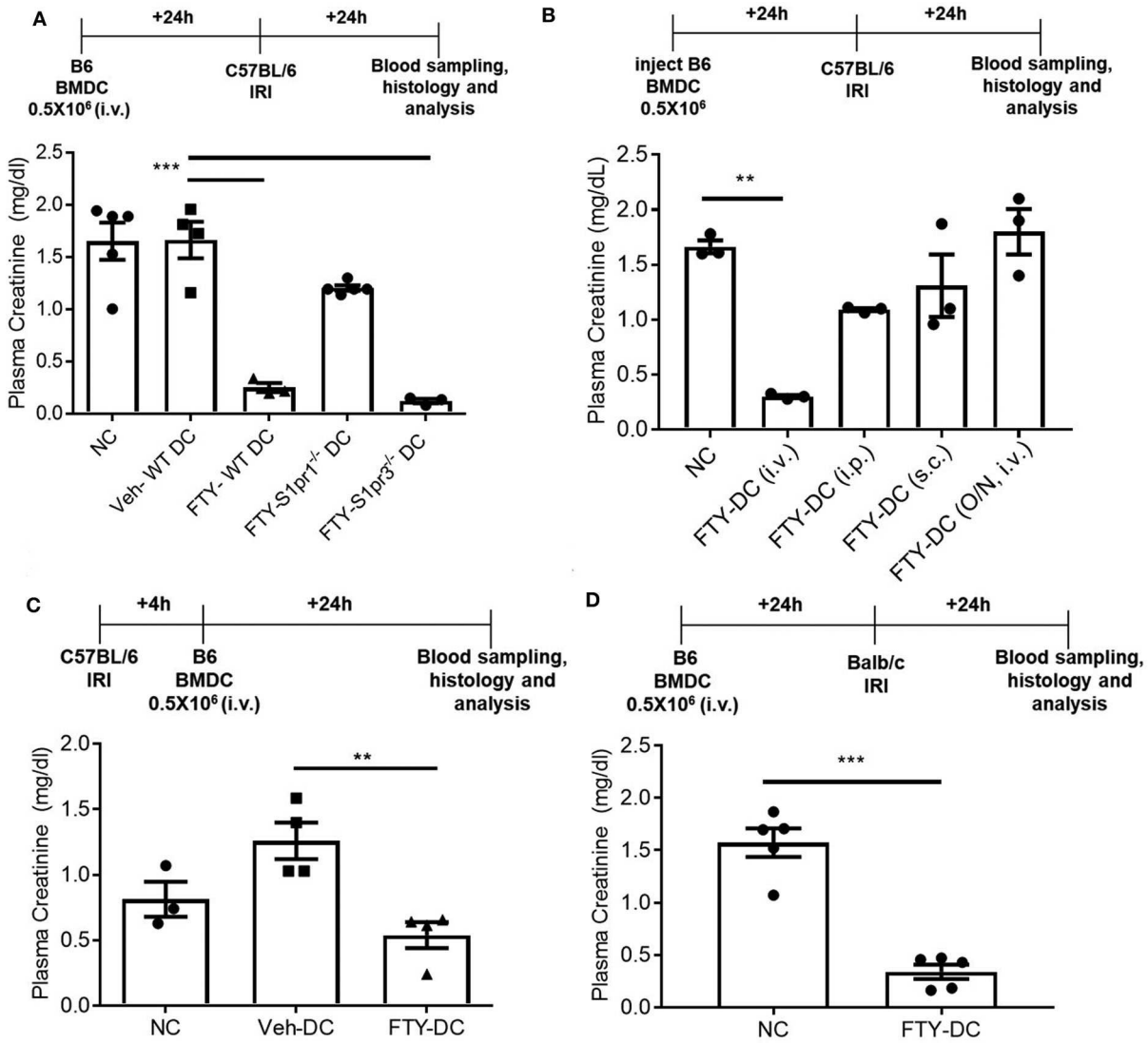

FIGURE 5 | FTY-DC require S1pr1 on BMDC to protect kidneys from IRI. **(A)** Protocol for experimental setup. FTY720 is a ligand for four out of five S1P receptors. Plasma creatinine (PCr, mg/dL) was measured 24 h after IRI. We tested if S1pr1 or S1pr3 were requited for FTY720 dependent regulatory DC phenotype. BMDCs were propagated from either C57BL/6 WT, *CD11cCreS1pr1*$^{fl/fl}$ (*S1pr1*$^{-/-}$ DC), or *S1pr3*$^{-/-}$ and treated with FTY720. FTY-*S1pr1*$^{-/-}$ DC do not protect kidneys from IRI. As demonstrated in our earlier published studies and again confirmed, transfer of *S1pr3*$^{-/-}$ DC with or without FTY720 significantly protect kidney from IRI. These studies suggest only *S1pr1* are necessary for FTY720 dependent regulatory DC phenotype. Next, we tested if the route of delivery was important for FTY-DC induced protection from kidney IRI. **(B)** Protocol for experimental setup. Plasma creatinine was measured 24 h after IRI. FTY-DC were injected either intravenous (i.v.), intraperitoneal (i.p.), subcutaneous (s.c.) or as i.v. using Veh-DC that were acutely treated with FTY720 (overnight along with LPS). These studies suggest that FTY-DC only protect kidneys from IRI if injected via i.v. and the FTY-DCs must be propagated in presence of FTY720 at start of the BMDC culture. $^{**}p \leq 0.01$. **(C)** Protocol for experimental setup. Plasma creatinine was measured 24 h after IRI. In all studies presented we injected FTY-DC 1 day before kidney IRI. We also tested if FTY-DC could be injected after injury. Mice were treated with either NC, Veh-DC or FTY-DC 4 h after bilateral IRI. Mice injected with Veh-DC had higher PCr compared to NC mice and FTY-DC treated mice were significantly protected. **(D)** Protocol for experimental setup. Plasma creatinine (mg/dL) was measured 24 h after IRI. C57BL/6 FTY-DC induce protection in BALB/c mice and protect kidneys from ischemic injury. Data represent means ± SEM, Unpaired *t*-test [D], $^{***}p \leq 0.001$; $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$, one-way ANOVA followed by Tukey's post-test.

determine the mechanisms mediating downstream effects of FTY720, *CD11cCreS1pr1*$^{fl/fl}$ (*S1pr1*$^{-/-}$-DC), and *S1pr3*$^{-/-}$ mice were used to harvest BMDCs and propagate in presence of FTY720. C57BL/6J mice were injected with half million LPS activated FTY-*CD11cCre* (WT) DC, FTY-*S1pr1*$^{-/-}$ DC or FTY-*S1pr3*$^{-/-}$ DCs 1 day before bilateral kidney IRI. FTY-*CD11cCre* (WT) DC and as expected from our previous studies (12, 13) *S1pr3*$^{-/-}$ DCs treated with FTY protected mice kidneys from injury. The protection was abrogated in mice treated with FTY-*S1pr1*$^{-/-}$ DC (**Figure 5A**).

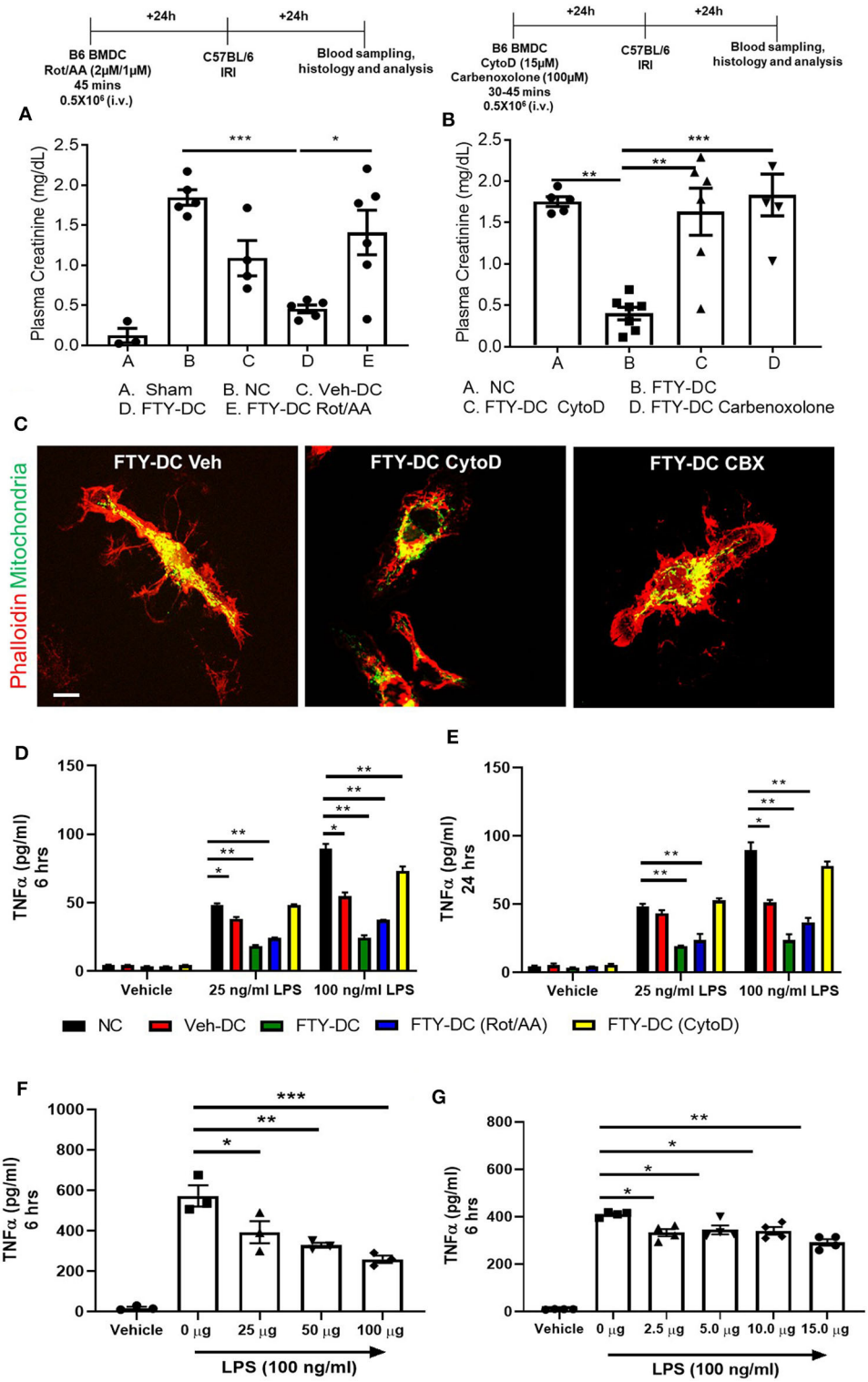

FIGURE 6 | Inhibition of FTY-DC mitochondrial function abrogates protection from kidney IRI. Protocol for experimental setup. **(A)** Plasma Creatinine (PCr). Mice were i.v. injected with 0.5 × 10^6 DCs (Veh-DC or FTY-DC) and as control no cells (NC) 1 day before bilateral kidney IRI. A group of mice were injected with FTY-DC that were treated with rotenone and antimycin A (Rot/AA). **(B)** Protocol for experimental setup. Plasma Creatinine (PCr). Mice were i.v. injected with 0.5X10^6 DCs (FTY-DC) and as control no cells (NC) 1 day before bilateral kidney IRI. Two additional group of mice were injected with FTY-DC that were treated with cytochalasin D (Cyto D) or carbenoxolone (CBX). **(C)** Immunofluorescence of *CD11cCrePham*$^{fl/fl}$ FTY-DC (green, mitochondria) and labeled with phalloidin (red, actin) that were treated with

(Continued)

FIGURE 6 | Cyto D or CBX. Scale bar, 20 μm. **(D,E)** Mice were i.v. injected with 0.5 × 10^6 DCs (Veh-DC or FTY-DC) or (FTY-DC treated with Rot/AA or Cyto D) and as control no cells (NC) 1 day before splenocytes were harvested and treated *ex vivo* with LPS (25 or 100 ng/ml) for 6 or 24 h and supernatant was analyzed by Elisa for TNFα. **(F)** Mice were i.v. injected with various amounts of isolated mitochondria (0–100 μg/mouse). Spleen was harvested 1 day after mitochondria injections and single cells suspensions were treated *ex vivo* with 100 ng/ml LPS for 6 h and supernatant was analyzed by Elisa for TNFα. **(G)** Spleen from (0 μg mito) treated mouse was harvested and incubated with various amounts of isolated mitochondria (0–15 μg/well) for 1 day before stimulating with 100 ng/ml LPS for additional 6 h and supernatant was analyzed for TNFα. Data represent means ± SEM, $^*p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$, one-way ANOVA followed by Tukey's post-test.

Interestingly, the protection by FTY-DCs is lost if BMDCs are administered either through intraperitoneal or subcutaneous injections and if DCs are only treated acutely (overnight) with FTY720 (**Figure 5B**). Therapeutic use of FTY-DCs is maintained even if given 4 h after kidney ischemia (**Figure 5C**) or if tested in allogeneic transfer experiments (C57BL/6J DCs to BALB/c) in mice (**Figure 5D**). In allogeneic transfer studies the BMDCs were not activated with LPS prior to transfer and equally protect mice kidneys from IRI. This could be due to involvement of adaptive immunity with FTY-DC.

Mitochondria Function Is Critical in FTY-DC Dependent Protection

Next, we tested if transferred FTY-DC (1) actively participate in transferring mitochondria to recipient splenic cells through actin polymerization, (2) required intact functional mitochondria, or 3) if active production of mitochondria (ATP) was involved in FTY-DC dependent protection from injury. LPS treated FTY-DC were treated with Rotenone (inhibitor of mitochondrial electron transport) and Antimycin A (inhibitor of cellular respiration) (Rot/AA; 2 μM/1 μM). As control LPS treated Veh-DC and FTY-DC were treated with equivalent volumes of DMSO. Treating FTY-DC with Rot/AA to reduce mitochondrial functions abrogated the protection from kidney IRI (**Figure 6A**). Similarly, treating FTY-DC with either Cytochalasin D (CytoD; 15 μM, an inhibitor of actin polymerization) or carbenoxolone (CBX; C4790; Sigma-Aldrich; 100 μM, non-specific inhibitor of gap junctions) abrogated the protection compared to FTY-DC treated group (**Figure 6B**). *CD11cCrePham$^{fl/fl}$* BMDCs were used to check if FTY-DC treated with either Rot/AA, CytoD, or CBX had differences in homing *in vivo* after injection. There were no statistically significant changes in number of cells found in the recipient spleen with either of the three inhibitors (data not shown). Additionally, no changes in cell viability (Annexin V and 7-AAD) were observed in FTY-DC treated with either Rot/AA, CytoD or CBX (data not shown). The structural changes in FTY-DC were also analyzed after CytoD and CBX treatment after 24 h. Compared to Veh treated FTY-DC (**Figure 6C**, CytoD treated cells have changes in actin (**Figure 6C**,) and no changes were observed in actin with CBX treatment (**Figure 6C**, right panel). To test if the FTY-DC-dependent transfer of mitochondria induced a change in cellular responses in spleen we harvested spleen from mice that were treated with either NC (1x PBS), Veh-DC, FTY-DC, FTY-DC (Rot/AA), or FTY-DC (CytoD) 24 h after injection. Total splenic single cell suspensions (∼500,000/well) were treated with LPS (25 ng/ml or 100 ng/ml) for either 6 or 24 h from the 5 groups. Splenocytes from FTY-DC (green) treated mice had significantly lower levels of TNF-α at 6 h compared to NC (black) or Veh-DC (red) treated mice (**Figures 6D,E**). Splenocytes cultures from mice treated with FTY-DC (Rot/AA) (blue) or FTY-DC (CytoD) (yellow) had higher levels of TNF-α compared to FTY-DC treated mice. Possibly due to less mitochondria transfers from [(Rot/AA) or (CytoD)] treated FTY-DC to splenocytes. We noted that splenocytes that were isolated from mice treated with Veh-DCs had significantly less production of TNFα with 100 ng/ml LPS, suggesting that Veh-DCs also donate mitochondria to splenocytes (as also shown in **Figure 3**) although to lesser extent compared to FTY-DCs. To test the hypothesis that increasing mitochondria numbers as expected with FTY-DCs are responsible for inhibitory effect on TNFα production from LPS treated splenocytes, we treated mice with different doses of healthy isolated mitochondria. To test if dose dependent uptake of mitochondria was responsible for inducing an anti-inflammatory phenotype in splenocytes, we injected mice with various amounts of isolated labeled mitochondria and found that injected mitochondria signal was mainly found in splenic macrophages as early as 30 min after injection (data not shown). In order to check if injected mitochondria are found in recipient mouse spleen, we either used *CD11cCrePham$^{fl/fl}$* BMDCs or human HEK293 cells to isolate mitochondria in separate experiments. Mice injected with mitochondria isolated from *CD11cCrePham$^{fl/fl}$* BMDCs were imaged after 24 h after injection (i.v.). As shown in **Figure 3**, in these mice, systemically injected labeled mitochondria in spleen was predominantly found in F4/80$^+$ and CD169$^+$ macrophages (data not shown). However, unlike data shown in **Figure 3**, signal associated with systemically injected mitochondria was also found in various other tissues including kidneys (data not shown). Using 50 μg mitochondria isolated from human HEK293 cells we were able to check with RTPCR the expression using human and mouse mtDNA primers in various tissues over time. In spleen relative levels of h-mtDNA/m-mtDNA expression increased as early as 30 min after injected and was higher at 24 h after injection compared to uninfected mice (data not shown). Mice were intravenously injected with various amounts (0–100 μg/mouse) of isolated mitochondria isolated from mouse liver. Splenocytes from mitochondria treated mice were cultured 24 h after injection and stimulated with LPS for 6 h. Total splenic single cell suspensions (∼100,000/well) were treated with 100 ng/ml LPS for 6 h. Mice treated with mitochondria have a significant dose dependent decrease in TNFα production compared to control (0 μg mitochondria) treated mice (**Figure 6F**). Additionally, treatment of control splenocytes (0 μg mitochondria) with isolated mitochondria *ex vivo* (0–15 μg/well) 1 day before treatment with LPS also significantly reduced TNFα in 6 h cultures in a dose dependent manner (**Figure 6G**).

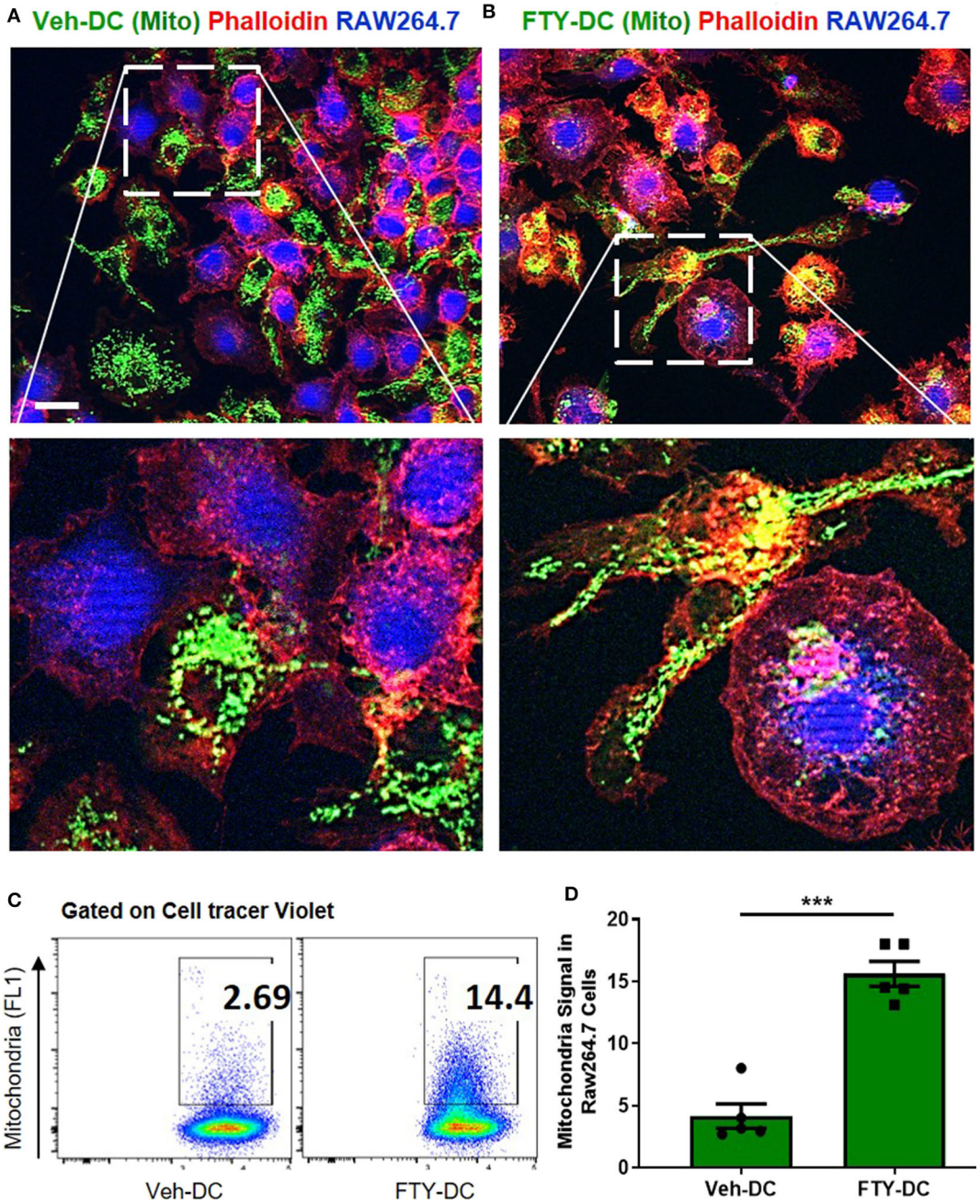

FIGURE 7 | Dendritic cells transfer mitochondria to macrophages. **(A)** Co-culture of 1:1 of macrophages (RAW264.7, blue, cell tracer violet) and *CD11cCrePham*$^{fl/fl}$ BMDCs (Veh-DC, green, mitochondria) and labeled with phalloidin (red, actin) was done after 24 h. **(B)** Co-culture of 1:1 of macrophages and *CD11cCrePham*$^{fl/fl}$ BMDCs (FTY-DC). Scale bar, 20 μm. **(C,D)** Flow cytometry of co-culture and semi-quantitative analysis after 24 h was done by gating on cell tracer violet positive RAW264.7 cells. Data represent means ± SEM, ***$p \leq 0.001$. One of three experiments is shown.

FTY-DC Are More Efficient Mitochondria Donors Compared to Veh-DC

Using coculture of BMDCs and RAW264.7cells (mouse macrophage cell line), we tested the efficiency of FTY- vs. Veh-DCs to donate mitochondria. Prior to setting up the co-culture, RAW264.7 cells were labeled blue using CellTrace^{TM} Violet (CT-Violet) proliferation dye. All analysis (imaging and flow cytometry) was done after 24 h. Compared to Veh-DCs, coculture of RAW264.7 cells with FTY-DCs had more transfer of mitochondria by immunofluorescence (**Figures 7A,B**) and quantification by gating on CT Violet (RAW264.7) and evaluating the amount of donor (Pham) green mitochondria

signal (**Figure 7C**). Co-culture of RAW264.7 (Blue, CT Violet) cells with FTY-DC (green mitochondria) have significantly more mitochondria donation compared to Veh-DC co-cultures (**Figure 7D**).

Uptake of Healthy Mitochondria by Macrophages Induce a Less Immunogenic Phenotype

To determine if uptake of healthy mitochondria by RAW264.7 cells changes their responses to LPS, we repeated the above study with isolated mitochondria rather than DC-dependent donation. BMDCs were again propagated from *CD11cCrePham*$^{fl/fl}$ mice and mitochondria was isolated from 8 day old Veh-DCs. RAW264.7 cells were treated with 10 μg/well of isolated mitochondria for 24 h. Some of the treated RAW264.7 cells were used for seahorse analysis and rest were treated with 100 ng/ml LPS for additional 24 h for gene analysis. As control, equal amounts of sonicated (Son) mitochondria (Son-Mito) were added in separate wells for 24 h. Treatment of RAW264.7 cells with healthy mitochondria significantly induced an increase in basal oxygen consumption rate (OCR) and ATP production compared to vehicle treated cells and use of Son-Mito abrogated these effects (**Figures 8A–C**). Mitochondria treated RAW264.7 cells were also analyzed for uptake of labeled mitochondria 24 h after incubation, the added mitochondria signal appears perinuclear in location (white arrows, **Figure 8D**). We next tested if addition of mitochondria regulated gene expression in RAW264.7 cells stimulated with LPS. Compared to untreated RAW264.7 cells (Veh/LPS), cells treated with healthy mitochondria had lower mRNA expression levels for *Nos2, Tnfa*, *Il1b,* and *Il16* after LPS stimulation (Mito/LPS). However, treatment of RAW264.7 cells with Son-Mito abrogated these inhibitory changes compared to healthy functional mitochondria (**Figures 8E–H**) treated cells. Data was calculated as relative fold changes compared to Veh/LPS treated cells (dash line, **Figures 8E–H**).

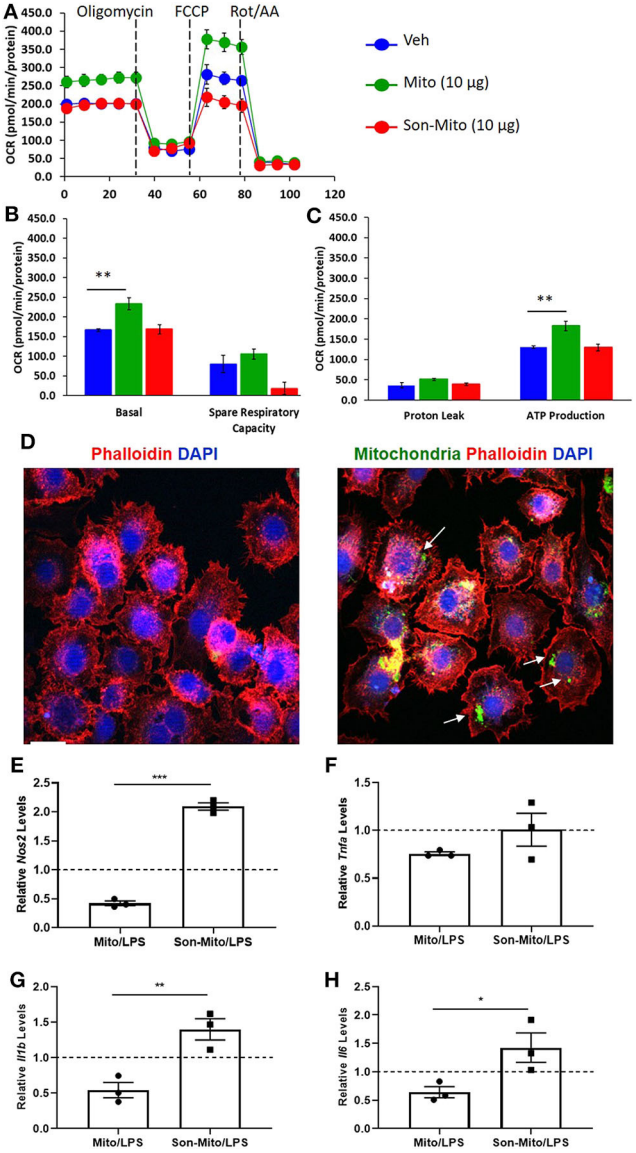

FIGURE 8 | Mitochondria donation from DCs induce anti-inflammatory phenotype in macrophages. **(A)** Seahorse analysis of RAW264.7 cells treated with healthy mitochondria (Mito) or structurally unhealthy (Son-Mito) for 24 h. **(B,C)** Oxygen consumption rate of RAW264.7 cells measure basal, spare respiratory capacity, proton leak and ATP production. The assay was normalized to total protein. **(D)** RAW264.7 cells were treated with 0 or 10 μg/well mitochondria isolated from 8 day old *CD11cCrePham*$^{fl/fl}$ BMDCs. White arrows point to labeled mitochondria that appears perinuclear in RAW264.7 cells. Scale bar, 20 μm. **(E–H)** 10 μg/well mitochondria (with and without sonication) was added to RAW264.7 cells for 24 h prior to treatment with 100 ng/ml LPS for additional 24 h. Gene expression of *Nos2, Tnfa, Il1b*, and *Il6* was analyzed. Data calculated as fold increase over Veh/LPS treated cells, shown as dash line. Data represent means ± SEM of triplicates, $^{**}p \leq 0.01$, one-way ANOVA followed by Tukey's post-test and Unpaired *t*-test **(E–H)**, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. One of three experiments is shown.

DISCUSSION

In the current study we demonstrated that immunosuppression and protection from kidney IRI induced by adoptive transfer of FTY-DC is dependent on the recipient spleen, DC-S1P1 and functional viability of transferred DC mitochondria. Furthermore, the protective effects of FTY-DC involve donation of mitochondria to splenic macrophages making them less immunogenic. In addition, our study for the first time to our knowledge demonstrates that BMDCs like mesenchymal stem cells (63) have the potential to donate mitochondria to induce an immunosuppressive phenotype in recipient cells.

Dendritic Cells in Acute Kidney Injury

AKI is a major health burden without major pharmacological advances in its prevention or treatment (64). Additionally, current therapies for allograft rejection, cancer, or autoimmune diseases use non-specific immunosuppressive drugs that are associated with adverse side effects and are limited due to lack of antigen specific tolerance. DCs are a heterogeneous group of cells important in immunity or tolerance, and the idea of using tolerized DCs in cell-based therapy of cancer,

autoimmune disease, and transplantation has been under investigation for the past 2 decades (65). However, most studies have focused on the induction of T cell–tolerogenic responses. Immune regulation of innate immune response via tolerogenic DCs is critically important in bridging innate and adaptive immunity and provides the foundation for use in transplant tolerance of allograft injury (66). Cell-based therapy using regulatory immune cells [Tregs (67), myeloid cells (68), or DCs (69, 70)] is a strategy that induces potential antigen-specific tolerance. Pharmacological or biological strategies induce regulatory or tolerogenic DCs (Tol-DC) (71), which are immature, maturation-resistant or alternatively activated cells that express low levels of MHC and co-stimulatory molecules. Compared with mature DCs, immature DCs interact actively with T cells and direct them into a regulatory response. Depletion of DCs significantly protects mouse kidneys from IRI (6, 13) and a dose-dependent increase in BMDC numbers exacerbates kidney injury (13), suggesting that DCs play a major role in inducing AKI. As our current and previously published studies demonstrate injected BMDCs accumulate in the spleen (**Figure 3**) after systemic infusion (72) and can persist for two weeks post-injection (73). In kidney IRI, DCs tolerized with an $A_{2A}R$ agonist (74) or DCs deficient of *S1pr3* (13) attenuated AKI. Our current study further demonstrates using *CD11cCrePham*$^{fl/fl}$ mice to harvest DCs that transferred DCs can donate their mitochondria to recipient cells thus making them less immunogenic.

Role of S1P Receptor Agonist (FTY720) in Kidney Injury and Dendritic Cells

S1P1 activation is important for maintaining cell viability; global deletion is embryonically lethal (75). We have previously demonstrated that the protective effect of S1P1 agonists FTY720 or SEW2871 in IRI (54) and cisplatin-induced nephrotoxicity (14, 49) was mediated by activation of S1P1 expressed on PT cells, independent of lymphopenia (14). Others have also shown that FTY720 can act as innate immune system immunomodulator that involves a role beyond its prominent effects on lymphocyte recirculation (76). In another study, using a mixed lymphocyte reaction (MLR), FTY720-treated human DCs exhibited reduced antigen presentation and altered cytokine production (77) and systemic injection with FTY720 was also found to block DC trafficking (78). Our current data (**Supplemental Figure 1**) and others have previously demonstrated that FTY720 alone does not affect the surface of BMDCs surface markers CD11c, MHCII, CD40, CD86, and indicates there is no change in viability. However, BMDCs propagated in presence of FTY720 do have immunosuppressive phenotype upon stimulation (LPS, CD40L or mixed lymphocyte reactions) and transfer of these immunosuppressive BMDCs confirms protection in various models CD80 (56, 77, 79, 80). In many of these studies the protective effects of FTY720 treated BMDCs was due to infusion of these immunosuppressive cells to block T cell responses. FTY-DCs in our study are immature compared to Veh-DCs after LPS stimulation and transfer of these FTY-DCs could potentially have regulatory responses on adaptive immunity. In our experiments we did observed a higher number of Tregs in spleen of FTY-DC treated mice but the exact mechanism of this is yet to be determined.

Role of Mitochondria in Dendritic Cells and Macrophages

DC and macrophage functions are regulated by mitochondrial metabolism. Type 1 macrophages (81, 82) and immunogenic DCs have high glycolytic rates (62). The activation of DCs or macrophages by several TLR agonist (LPS or CpG) leads to rapid increase in glycolysis followed by decrease in OXPHOS and mitochondrial membrane potential (62, 83, 84). Some role for mitochondria has been demonstrated with DCs treated *in vitro* with vitamin D, these DCs have increased OXPHOS, mitochondrial mass and mROS production (85), similar to what we observe with FTY-DCs. Although the mechanistic details of how glycolysis and mitochondria metabolism controls DC function are unknown our data suggests FTY-DC continue to rely of mitochondria OXPHOS and this could contribute to their less immunogenic phenotype. Multiple mechanisms may exist by how FTY-DC protect kidneys from IRI, one possible mechanism is through mitochondria donation. Our current data indicates that transferred FTY-DCs are more efficient at transferring mitochondria to recipient splenocytes, mainly macrophages ($CD169^+$ or $F4/80^+$). The exact mechanism of how mitochondria get transferred to macrophages is unknown, however use of either actin polymerization inhibitor Cyto D or non-specific gap junction inhibitor CBX abrogated FTY-DC dependent protection. Treatment with Cyto D or CBX did not change the viability or trafficking of FTY-DC *in vivo* (data not shown). *in vivo* transferred Veh-DC also donate mitochondria to splenocytes however to a lesser extent compared to FTY-DC. This was clear as stimulation of splenocytes isolated from mice 24 h after various DC infusion [Veh-DC, FTY-DC, FTY-DC (Rot/AA), or FTY-DC (CytoD)] had less TNF-α levels after *ex vivo* stimulation with LPS compared to NC splenocytes. TNFα was significantly lower in Veh-DC treated splenocytes compared to NC splenocytes at 6 h after LPS stimulation. Splenocytes that were from FTY-DC treated mice displayed most suppression in TNFα production, an effect that was partially lost if FTY-DC were pretreated with Rot/AA or Cyto D demonstrating a dependence upon mitochondrial function. These data indicate that uptake of naked/free mitochondria or DC-derived mitochondria in a dose dependent manner induces an anti-inflammatory phenotype, although the exact mechanism is currently unknown.

Our current findings indicate that DCs have the potential to donate mitochondria to induce immunological changes in the recipient cells a protective mechanism previously shown to be employed by mesenchymal stem (86) and bone-marrow-derived stromal cells (87). As demonstrated in our earlier studies (12), the injected bone-marrow-derived DCs are predominantly found in the recipient spleen as early as 30 min and signal persist up to 72 h. More importantly using transgenic mice (that contain labeled Pham mitochondria) to propagate DCs (*CD11cCrePham*$^{fl/fl}$) our study is the first to demonstrate

in addition to homing to the spleen, injected DCs donate mitochondria to splenic macrophages. Compared to naïve DC, DCs propagated in presence of FTY720 (FTY-DC) are more efficient at donating mitochondria to recipient splenocytes mainly to macrophages. The exact mechanism of how FTY-DC donate mitochondria is currently unknown but does involve gap junctions and actin polymerization as treatment with inhibitors (Cyto D or CBX) abrogates the protection by FTY-DCs. Our current study analyzed the involvement of macrophage dependent innate adaptive immunity in FTY-DCs dependent protection. However, we did note that in spleen of FTY-DC treated mice there was an increase in labeling of white pulp $CD4^+FoxP3^+$ cells that was in addition to disrupted $CD169^+$ labeling of the MZ, similar to as previously demonstrated using *S1pr3*$^{-/-}$ DCs (12). Thus, in addition to donating mitochondria to splenic macrophages FTY-DCs could also regulate adaptive immune responses resulting in higher Treg cells.

Limitation of our current study using mouse kidney IRI model is that this model is acute (<2 days), thus it is possible that if mice are followed for longer time periods after infusion of FTY-DCs especially in allogenic transfers (C57BL/6J BMDCs→ BALB/c mice or inverse), we might have a significant change in adaptive immune responses including higher Treg numbers. Since FTY-DCs are immunogenically immature (low CD80, CD86, MHCII, and higher IL10) after LPS stimulation and injection of these FTY-DCs increases splenic Tregs, we are in process of testing if FTY-DCs can be used to delay rejection using allogenic mouse model of heterotopic heart transplant. Lastly, if higher mitochondria numbers in FTY-DCs indeed induce the protection we observed, it would be interesting to test if artificially increasing DC mitochondria numbers (mitochondria transplant) also have similar therapeutic advantage. This is especially important since as current study demonstrates, we must propagate DCs in presence of FTY720 from start of BMDC cultures, as acute (overnight) treatment of DCs with FTY720 does not protect kidneys from IRI.

In summary we have demonstrated that BMDCs can regulate innate immune response by donating mitochondria. The anti-inflammatory responses induced by FTY-DC are dependent on the spleen and presence of S1P1 receptors. In the spleen, FTY-DC donate mitochondria more efficiently compared to Veh-DC to splenic macrophages ($F4/80^+$ and $CD169^+$). Dose dependent uptake of mitochondria by splenic and RAW264.7 macrophages induces metabolic reprogramming that is a key driver of anti-inflammatory phenotype. We conclude that regulatory FTY-DC may be useful in kidney IRI as well as in other inflammatory states such as transplantation and autoimmune disorders.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Tennessee Health Science Center and University of Virginia Institutional Animal Care and Use Committees.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

AB conceived the idea, designed the experiments, analyzed data, and wrote the manuscript. AB, TR, LH, and KS supervised the kidney ischemia experiments and critically reviewed the data and manuscript. AB, TR, LH, KS, CeK, and CaK performed experiments and prepared the figures. MN helped with western blots. AB, VM, DM, JE, and LM reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01278/full#supplementary-material

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Bendamustine Conditioning Skews Murine Host DCs Toward Pre-cDC1s and Reduces GvHD Independently of Batf3

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Graft-versus-host disease (GvHD) remains the second leading cause of death in allogeneic hematopoietic stem cell transplantation recipients, highlighting the need for improved preventative strategies. Our laboratory has previously demonstrated in an experimental bone marrow transplantation (BMT) model that bendamustine combined with total body irradiation (BEN+TBI) is a safer alternative to cyclophosphamide with TBI (CY+TBI). The biological mechanisms of action of BEN have not been fully elucidated and likely involve multiple cell populations. Host dendritic cells (DCs) can prime naïve donor T-cells immediately following transplantation, making host DCs critical for the initiation phase of GvHD. We hypothesized that BEN+TBI conditioning favorably alters host DC composition to reduce GvHD. We demonstrate that host DCs treated with BEN+TBI induce less allogeneic T-cell proliferation than those conditioned with CY+TBI. We further show that BEN+TBI conditioning results in greater total numbers of all host DC subsets but with a more favorable composition compared to CY+TBI with significantly larger proportions of type 1 conventional DCs (cDC1), a highly regulatory DC subset capable of suppressing GvHD. Our studies using recipient Batf3 KO mice indicate that CD8α+ cDC1s are largely dispensable for the reduced GvHD following BEN+TBI conditioning. We found a higher frequency of host pre-cDC1s with BEN+TBI conditioning in both wild-type (WT) and Batf3 KO mice, which was inversely associated with GvHD. Additionally, we observed that BEN treatment results in greater expression of Flt3 receptor (CD135) on host DCs compared to CY, potentially contributing to the skewing of host DCs toward cDC1s. Further, BEN+TBI conditioning results in host cDCs with greater expression of PIR-B, an inhibitory receptor capable of preventing lethal GvHD. We conclude that BEN+TBI is a safer alternative to CY+TBI, resulting in a greater frequency of host pre-cDC1s and limiting GvHD.

Keywords: graft-vs.-host disease, BMT, conditioning, dendritic cells, bendamustine

INTRODUCTION

Graft-versus-host disease (GvHD) remains a significant complication of allogeneic hematopoietic cell transplantation (alloHCT). Efforts to limit GvHD have primarily focused on depleting or modulating donor T-cells through the use of prophylactic post-transplant T-cell suppressing agents. However, these approaches may be associated with risks of allograft rejection and reduced graft-versus-leukemia (GvL) activity (1). There remains a critical need to develop new strategies that limit GvHD without compromising engraftment or GvL. Pre-transplant conditioning regimens not only have direct anti-cancer effects, but can also influence long-term GvHD and GvL. Still, modification of these preparative chemotherapy regimens as a means to limit GvHD and enhance GvL has received little attention.

Previous work from our laboratory has resulted in an ongoing phase I clinical trial investigating the use of bendamustine (BEN) following haploidentical transplant (2–5). The most widely used conditioning regimen in alloHCT for acute lymphoblastic leukemia (ALL) is cyclophosphamide (CY) with total body irradiation (TBI) (6). BEN is traditionally used clinically in chemotherapy-based conditioning regimens for autologous (7–9) and allogeneic (10, 11) transplants, but not in combination with TBI. Our laboratory has shown that replacing CY+TBI with BEN+TBI as pre-transplant conditioning reduces GvHD and improves survival in an MHC-mismatched murine bone marrow transplantation (BMT) model (12). We have previously reported that this difference in GvHD is not due to graft rejection, or a difference in engraftment kinetics. Moreover, we have excluded the possibility that the difference in GvHD is due to conditioning regimen-related toxicity by performing syngeneic BMT, wherein neither BEN nor CY groups exhibit clinical or histological evidence of GvHD (12). Further, we have determined that there are no differences in the intestinal epithelial barrier integrity with BEN vs. CY conditioning, with both groups showing comparable early histological evidence of GvHD (12). Our laboratory has also found that BEN+TBI conditioning results in tolerant T-cells while preserving T-cell dependent GvL (13). Thus, we have reasoned that there are unique immunomodulatory effects of BEN conditioning that provide advantages over CY. Like CY, BEN is an alkylating agent, but is unique in that it also contains a purine analog, conferring anti-metabolite functions that are currently unexplored in an HCT setting (14, 15). We have also shown in a haploidentical mouse model that replacing post-transplant CY with BEN as GvHD prophylaxis limits GvHD while maintaining GvL, further suggesting favorable immunomodulation by BEN (2).

We previously reported that myeloid-derived suppressor cells (MDSCs) are partially responsible for the decreased GvHD seen with BEN+TBI conditioning (12). However, other cell types are likely involved. Host cells are often overlooked in the context of alloHCT due to the fact that they are eliminated by the conditioning regimen. However, host dendritic cells (DCs) persist long enough to stimulate naïve donor T-cells immediately following transplantation and are, therefore, critical in the pathogenesis of GvHD, particularly the initiation phase of GvHD (16–22). There are two main lineages of DCs, plasmacytoid (pDCs) and conventional (cDCs). Of the two lineages, only persistent host cDCs are capable of directly presenting host antigens (Ag) to donor T-cells (23). cDCs exist as either type 1 (cDC1) or type 2 (cDC2) subsets, which primarily prime CD8+ T-cells and CD4+ T-cells, respectively. Batf3-dependent cDC1s have been linked to suppression of GvHD through activation-induced clonal deletion of allospecific donor T-cells (24) as well as superior GvL due to the specialized ability of these cells to capture, process and present tumor Ag to CD8+ T-cells (25–27). Studies using the administration of exogenous Flt3 Ligand (Flt3L) prior to transplantation have highlighted the role that host DCs, particularly host cDC1s, play in limiting GvHD (24, 28). Additionally, on the various DC subsets, greater expression of the stimulatory markers CD80 and CD86 allows for stronger engagement of naïve T-cells (29–36). Equally important in naïve T-cell priming is the expression of inhibitory markers, such as paired immunoglobulin-like receptor B (PIR-B) which has been shown to control lethal GvHD (37). Overall, it appears the relative proportions, numbers, and expression profiles of stimulatory and inhibitory markers of each DC subset in the peri-transplant period are central to the pathogenesis of GvHD.

In this study, we sought to characterize the effect of BEN+TBI on host DC populations to further elucidate the mechanisms by which BEN+TBI conditioning limits GvHD compared to CY+TBI. We demonstrate that BEN+TBI results in a skewed host DC subset composition at the time of transplant toward pre-cDC1s and Batf3-dependent cDC1s *in vivo* and results in DCs with reduced ability to stimulate allogeneic T-cell proliferation *ex vivo*. We reveal that while host Batf3-dependent CD8α+ and CD103+ cDC1s contribute to improved GvHD with BEN+TBI compared to CY+TBI, they are largely dispensable. Reduced GvHD observed with BEN+TBI is associated with higher proportions and absolute numbers of host pre-cDC1s, a Batf3-independent immediate precursor to CD8α+ cDC1s. We further found that BEN treatment results in greater expression of the receptor tyrosine kinase Flt3 (CD135), as well as greater expression of the GvHD-suppressing inhibitory receptor Paired Immunoglobulin-like Receptor B (PIR-B). Altogether, we conclude that BEN+TBI compared to CY+TBI conditioning results in greater murine host pre-cDC1s in a Batf3-independent manner and reduces GvHD.

MATERIALS AND METHODS

Mice

All strains of mice used (BALB/c, C57BL/6 and C.129S-*Batf3*^{tm1Kmm}/J) were age-matched 6–10-week-old females purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in specific pathogen-free conditions and cared for according to the guidelines of the University of Arizona's Institutional Animal Care and Use Committee.

Drug Preparation and Administration

Cyclophosphamide (Sigma-Aldrich, St. Louis, MO) and bendamustine (SelleckChem, Houston, TX) were reconstituted and diluted as described previously (2). Cyclophosphamide

was reconstituted in ddH_2O to a stock concentration of 50 mg/mL then diluted with sterile saline (General Laboratory Products, Yorkville, IL) for i.p. injection. Bendamustine was reconstituted in dimethyl sulfoxide (Sigma-Aldrich) to a stock concentration of 75 mg/mL, and diluted with sterile phosphate-buffered saline (GE Healthcare Life Sciences) containing 0.2% carboxymethylcellulose and 0.25% polysorbate 80 (Sigma-Aldrich) for i.v. injection.

BMT Models

BALB/c or Batf3 KO (C.129S-*Batf3*^{tm1Kmm}/J) recipients (H-2^{d}) received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. day −2 and 400 cGy TBI day −1 using a Cesium 137 irradiator, as previously described (12). Day 0, mice received 10^7 C57BL/6 (H-2^{b}) bone marrow (BM) or T-cell depleted bone marrow (TCD-BM) cells with 3×10^6 purified total T-cells (tT) i.v.. Mice were monitored daily, weighed twice weekly and percentage of starting weight was calculated. Additionally, mice were scored clinically on skin integrity, fur texture, posture and activity, and cumulative GvHD scores were calculated (38). Moribund mice, including mice with a cumulative score of ≥8 after day +8, were euthanized.

Preparation of Total T-Cells and T-Cell Depleted BM for BMT

Total T-cells were isolated from naïve C57BL/6 spleens by negative selection using the mouse Pan T-cell Isolation Kit II (Miltenyi Biotec, Auburn, CA), with a purity of >97%. Where TCD-BM is indicated, T-cells were depleted from BM cells using the CD3ε MicroBead Kit (Miltenyi Biotec), with <0.3% CD3ε+ cells remaining (data not shown).

Isolation of Dendritic Cells for Analysis

Spleens were processed to single cell suspension and splenocytes were counted. Splenic DCs were isolated from conditioned BALB/c spleens by negative selection using the Pan Dendritic Cell Isolation Kit (Miltenyi Biotec) or by positive selection using the CD11c+ MicroBead Kit (Miltenyi Biotec), with purity and yield matching that of the manufacturer's specifications. The Pan DC population isolated includes both CD11c+ and CD11c- fractions (data not shown). CD8α+ cDC1s were isolated from conditioned BALB/c spleens by negative selection to deplete T, B, and NK cells, and then by positive selection using direct labeling of CD8α using the CD8+ Dendritic Cell Isolation Kit (Miltenyi Biotec).

Flow Cytometry

For engraftment flow, blood was collected by tail tipping. Spleens were processed to single cell suspension and dendritic cells were isolated as described above then counted. Red blood cells were lysed with BD Pharm Lyse buffer (BD Biosciences, San Jose, CA) and flow cytometry was performed as previously reported (39). Fluorescence data were collected using an LSRFortessa cell analyzer (BD Biosciences) and analyzed using FlowJo 2 (Tree Star, Ashland, OR). Antibodies used were anti-mouse H2K^{b} PerCP-eFluor 710 (AF6-88.5.5.3), CD8α PE-Cy7 (53-6.7), CD103 PE (2E7), PIR-B APC (10-1-PIR) (Thermo Fisher, Carlsbad, CA); CD11c FITC (N418), CD11c VioBlue (REA754) (Miltenyi Biotec); B220 Brilliant Violet 510 (RA3-6B2), SIRPα APC/Cy7 (P84), CD24 Pacific Blue (M1/69), CD80 APC (16-10A1), CD86 Alexa Fluor 700 (GL-1), CCR7 PE-Cy5 (4B12) (Biolegend, San Diego, CA); and CD135 PE-CF594 (A2F10.1) (BD Biosciences). Host-type cells are defined as H2K^{b}-; plasmacytoid DCs are defined as CD11c+B220+; conventional DCs are defined as CD11c+B220-; cDC1s are defined as CD11c+B220-CD8α+; cDC2s are defined as CD11c+B220-SIRPα+; CD103+ cDC1s are defined as CD11c+B220-CD103+CD8α±; pre-cDC1s are defined as CD11c+B220-CD24^{high}CD8α-.

Mixed Leukocyte Reactions

Mixed leukocyte reactions (MLRs) were conducted and analyzed as previously reported (2). In assays using tritiated-thymidine, T-cells were isolated from spleens of naïve C57BL/6 mice and stimulated with isolated DCs or CD3/CD28 beads as a positive control (Thermo-Fisher Scientific, Waltham, MA). Splenic DCs from naïve or BEN or CY conditioned BALB/c mice with and without TBI were isolated and co-incubated with T-cells for 3 days at 37°C in 7.5% CO_2 in a 96-well plate. 0.5 μCi of tritiated thymidine was added to each well on day 3 of culture. After an additional 18 h of culture, plates were harvested using a Brandel wash pump harvester (Gaithersburg, MD). T-cell proliferation was measured as counts per minute (CPM) using a MicroBeta2 counter (PerkinElmer, Waltham, MA).

Suppression Assays

Suppression assays were conducted and analyzed as previously reported (2). In assays using tritiated-thymidine, T-cells were isolated from spleens of naïve C57BL/6 mice and stimulated with CD3/CD28 beads (Thermo Fisher Scientific, Waltham, MA). Splenic DCs from naïve or BEN+TBI or CY+TBI conditioned BALB/c mice were isolated and co-incubated with pre-stimulated T-cells for 3 days at 37°C in 7.5% CO_2 in a 96-well plate. 0.5 μCi of tritiated thymidine was added to each well on day 3 of culture. After an additional 18 h of culture, plates were harvested using a Brandel wash pump harvester. T-cell proliferation was measured as CPM using a MicroBeta2 counter. For supplemental experiments, T-cells were stained with CellTrace Violet (Invitrogen) immediately following isolation from the spleen and pre-stimulated with CD3/CD28 beads then co-cultured with naïve or BEN+TBI or CY+TBI conditioned DCs. After 4 days of co-incubation, flow cytometry was performed and data was analyzed using Modfit Software (Verity Software House, Topsham, ME) to determine the T-cell proliferation index (PI).

Flt3 Ligand ELISA

Blood was collected via cardiac puncture, spun down at 10,000 g for 10 min and plasma was collected and stored at −20°C. Plasma was used in an ELISA per the manufacturer's protocol to determine plasma levels of Flt3 Ligand (R&D Systems, Minneapolis, MN).

Statistical Analysis

Kaplan-Meier curves were analyzed by Mantel-Cox log-rank test to determine survival percentages and differences between groups. Mann-Whitney tests were used to determine significant

differences in cell counts, percentages, proliferation, weight, and GvHD scores. One-way ANOVA and Tukey's *post-hoc* tests were used to determine fold-change differences among DC populations. *P*-values < 0.05 were considered statistically significant.

RESULTS

BEN+TBI Results in Host DCs Less Stimulatory of Alloreactive T-Cells and Improves GvHD Compared to CY+TBI Conditioning

To mimic clinical BMT, recipient mice were conditioned with BEN or CY supplemented with TBI as outlined in **Figure 1A**. In a severe GvHD model (10^7 TCD-BM + 3×10^6 total T-cells), BEN+TBI conditioning results in significantly improved survival (**Figure 1B**) with lower clinical GvHD scores and reduced weight loss (**Figures 1C,D**) compared to CY+TBI. Complete engraftment was achieved with both conditioning regimens (**Supplemental 1**). We have previously shown that syngeneic controls exhibited complete engraftment and survival with no signs of GvHD, indicating that deaths are not due to toxicity from the conditioning regimens (2). As host APCs are well-known to play a significant role in GvHD pathogenesis, we hypothesized that BEN+TBI conditioning alters host DCs in a way that attenuates acute GvHD pathogenesis. To test this, we performed an MLR using DCs from BEN- or CY-conditioned mice with or without TBI as stimulators of allogeneic T-cells. We found that BEN-conditioned DCs have reduced capacity to stimulate allogeneic T-cell proliferation compared to naïve or CY-conditioned DCs (**Figure 1E**), with or without the addition of TBI, although this was not statistically significant. To exclude the possibility that conditioned DCs may have been differentially necroptotic or apoptotic in culture, we determined viability at the beginning of culture by Trypan blue staining and performed a Propidium Iodide and Annexin V staining at the end of co-culture and determined that there were no differences in viability between BEN+TBI and CY+TBI conditioned DCs at either timepoint (data not shown). These results support our hypothesis that BEN+TBI conditioning attenuates the capacity of host DCs to stimulate alloreactive T-cell proliferation, albeit mildly.

A Higher Ratio of Host Plasmacytoid to Conventional DCs Remain After BEN+TBI Conditioning Compared to CY+TBI Conditioning

We next sought to characterize the overall composition of host DC subsets and their activation status following BEN+TBI compared to CY+TBI. Conditioning results in significant and extensive epithelial tissue necrosis (data not shown), making isolation of DCs from GvHD target-tissues such as the intestines at these early time points technically difficult. Therefore, to assess the relative abundance of the two major host DC lineages in the peri-transplant period, mice were conditioned with BEN+TBI or CY+TBI and splenic DCs were collected either on day 0 (prior to transplant) or post-BMT on day +1 or +3, as depicted in **Supplemental 2**. Isolated splenic DCs were counted and analyzed by flow cytometry. DC subset data shown herein (**Figures 2–6**) are from the same subjects. There were no significant differences in the absolute number or percent yield of DCs isolated from spleen between BEN+TBI and CY+TBI groups on day 0 (**Supplemental 3**). Flow cytometric analysis confirmed that $H2K^b$- host cells comprised >50% of all splenic DCs through day +3 post-transplant (**Supplemental 4**). The identifying markers used to characterize each DC subset and accompanying gating strategies are shown in **Supplementals 5, 6** (29, 40). Among isolated splenic DCs, there was no difference in percent host CD11c+ DCs between BEN+TBI and CY+TBI treated groups on day 0, +1 or +3 (data not shown). The mice receiving BEN+TBI conditioning had a significantly higher percentage of host pDCs (**Figures 2A,B**) and a significantly lower percentage of host cDCs (**Figures 2A,C**) compared to CY+TBI on day 0 and day +3.

Conventional DCs Have a Higher Expression of CD80 and CD86 After BEN+TBI Compared to CY+TBI Conditioning

We were additionally interested in assessing host cDC expression of the co-stimulatory molecules CD80 and CD86 and chemokine receptor CCR7 on day 0. BEN+TBI conditioning yielded a significantly higher percentage of CD80+ and CD86+ cDCs compared to CY+TBI, with a significantly greater CD80 MFI (**Figures 2D,E**) of CD80+ cDCs. BEN+TBI conditioning additionally resulted in a significantly greater CCR7 MFI on CCR7+ cDCs compared to CY+TBI (**Figure 2F**). In summary, our results indicate that BEN+TBI conditioning results in proportionally more pDCs compared to cDCs than CY+TBI, and in more highly activated cDCs with greater potential to migrate to secondary lymphoid organs compared to CY+TBI.

BEN+TBI Compared to CY+TBI Conditioning Results in Higher Proportions of CD8α+ cDC1s

cDCs resistant to conditioning are capable of priming naïve donor T-cells and guiding their effector functions (23, 40). In mice, CD8α+ cDC1s are adept at cross-presentation and priming of CD8+ T-cells while the SIRPα+ cDC2s prime CD4+ T-cells. Several reports have found that CD8α+ cDC1s play an important role in suppressing alloreactive T-cell responses and limiting GvHD through activation-induced clonal deletion of allospecific donor T-cells (25–27). Therefore, we evaluated the two host cDC subsets in BEN+TBI vs. CY+TBI conditioning. Representative flow plots of cDC1 and cDC2 populations on day 0 are depicted in **Figure 3A**, demonstrating differences between BEN+TBI and CY+TBI. The CD8α-SIRPα- DCs are considered pre-cDCs that have not yet committed to either of the two cDC lineages. Quantification of these flow cytometry plots shows significantly more CD8α+ cDC1s with BEN+TBI conditioning than with CY+TBI (**Figure 3B**), and

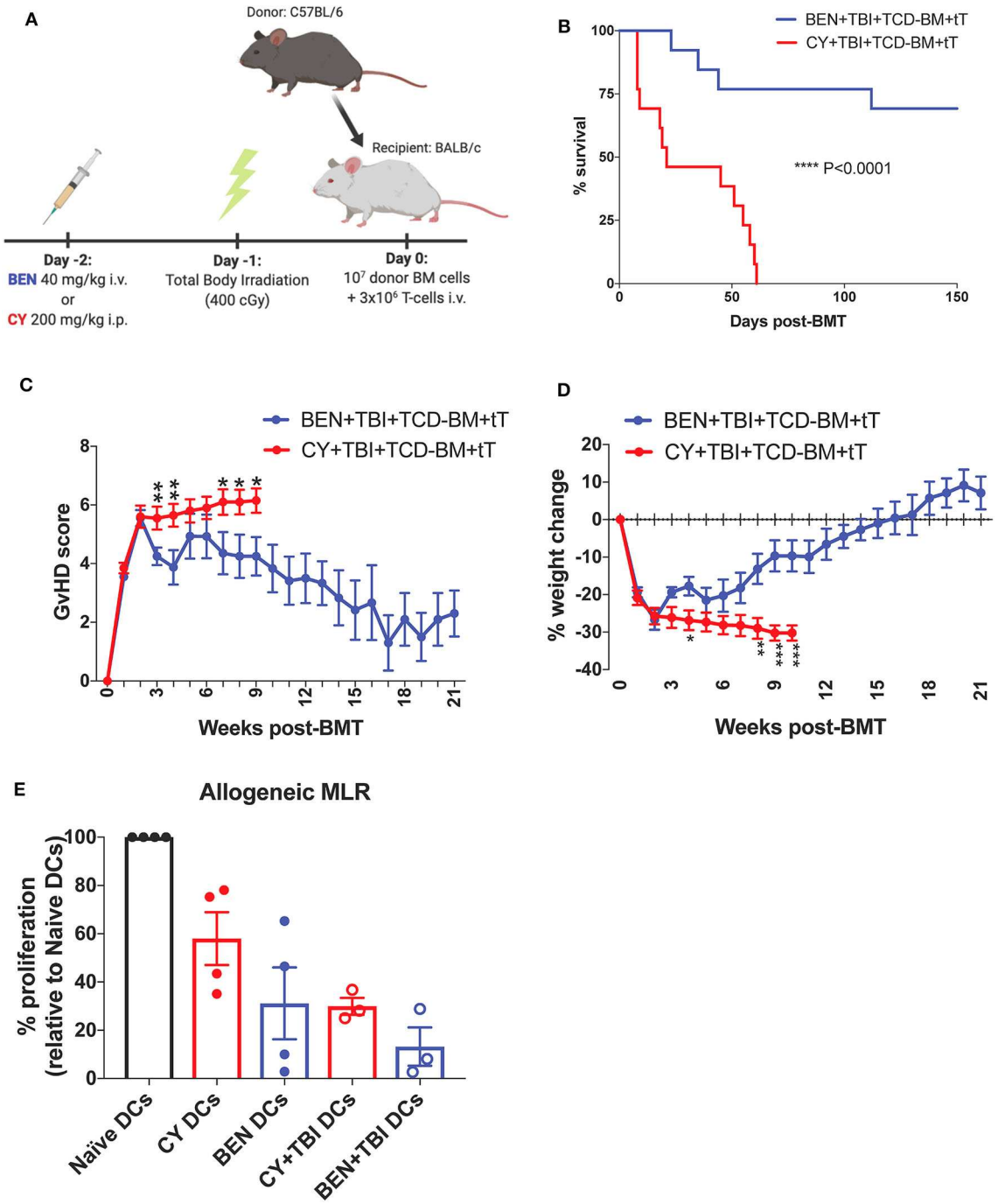

FIGURE 1 | BEN+TBI compared to CY+TBI conditioning results in host DCs less stimulatory of alloreactive T-cells and improves GvHD. **(A)** BALB/c recipient mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day −2, 400 cGy TBI on day −1 and 10^7 T-cell depleted bone marrow (TCD-BM) with 3×10^6 purified T-cells (tT) on day 0. Created with Biorender.com. **(B)** Pooled survival data from 3 experiments are shown, $n = 15$ mice/group. A log-rank Mantel-Cox test was used to determine significance. $^{****}P < 0.0001$. **(C)** The weekly average of the mean clinical GvHD score per group is shown with SEM. **(D)** The weekly mean percent weight change from the starting weight with SEM is shown. Pooled data from 3 experiments are shown, $n = 15$ mice/group. Multiple t-tests were used to determine significance. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(E)** BALB/c recipient mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day −2 and 400 cGy TBI on day −1. Splenic DCs from naïve or BEN or CY treated mice with or without TBI were isolated by MACS negative selection on day 0 and used as stimulators of allogeneic T-cells. MLRs were plated at a stimulator to responder ratio of 1:10. T-cell proliferation was assessed by tritiated-thymidine uptake after 4 days of co-culture and shown as percent proliferation (relative to Naïve DCs) with SEM. Pooled data from 4 experiments are shown, $n = 3$–4 mice/group. Mann-Whitney unpaired t-tests were used to determine significance (BEN vs. CY $P = 0.34$; BEN+TBI vs. CY+TBI $P = 0.40$).

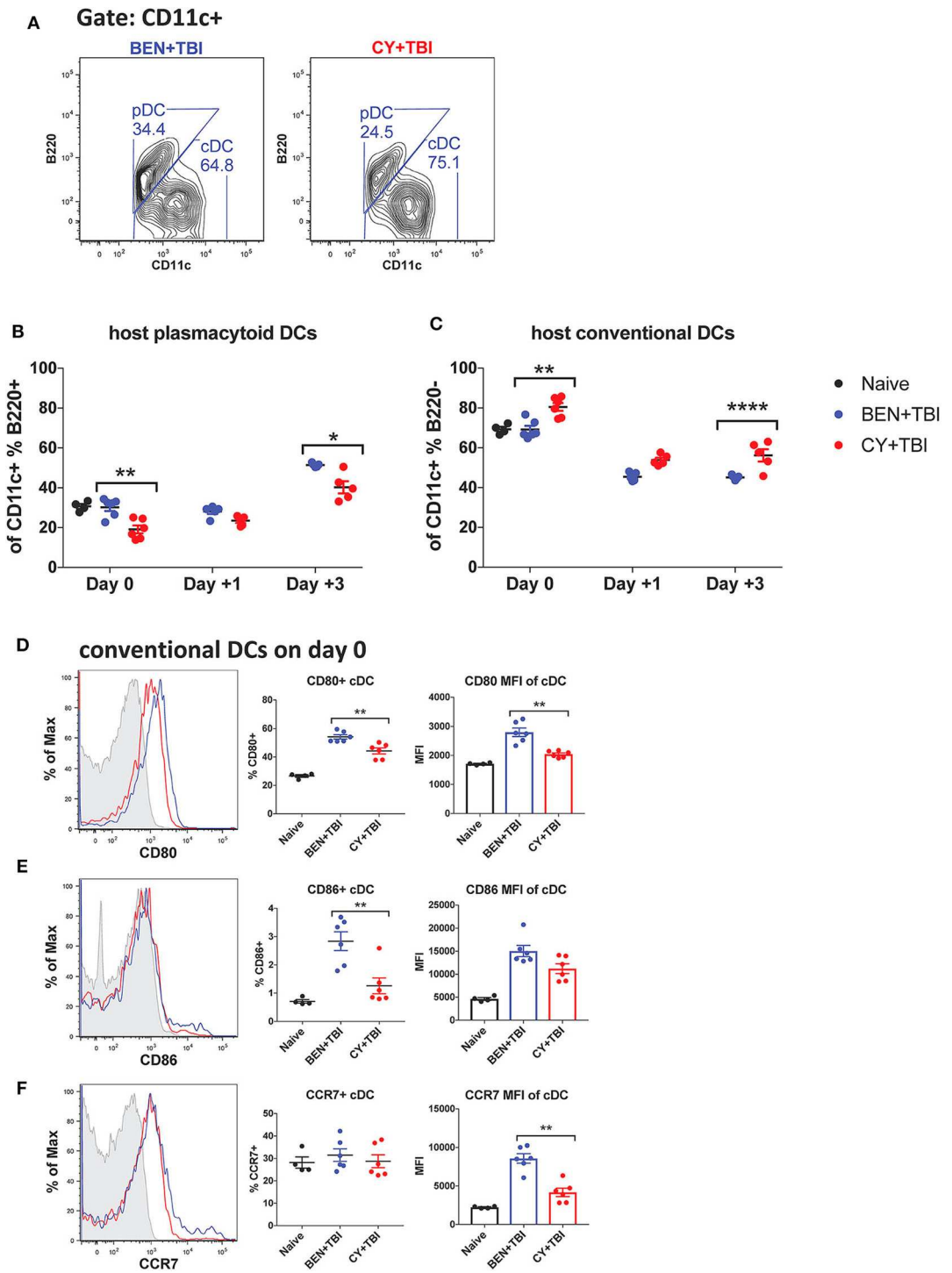

FIGURE 2 | Higher ratios of host plasmacytoid to conventional DCs remain and conventional DCs have a higher expression of CD80 and CD86 after BEN+TBI compared to CY+TBI conditioning. BALB/c mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. day −2 and 400 cGy TBI on day −1. Some groups were transplanted on day 0 with 10^7 BM + 3 × 10^6 total T-cells for post-transplant analysis of DCs. On day 0, +1 or +3, spleens were collected and DCs were isolated by MACS negative selection and counted before analysis by flow cytometry. Naïve mice were used as controls. **(A)** Representative flow cytometry gating of conventional (CD11c+B220–) and plasmacytoid (CD11c+B220+) DC subsets are shown. Of total host CD11c+ DCs, **(B)** percent pDCs and **(C)** percent cDCs were quantified on day 0, +1 and +3. Representative histograms, percent and MFI among cDCs positively expressing the activation markers **(D)** CD80, **(E)** CD86 and **(F)** CCR7 are shown, with fluorescence minus one (FMO) control shown in gray, BEN+TBI shown in blue and CY+TBI shown in red. Pooled data from 2 experiments are shown, *n* = 4–6 mice per group per time point. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

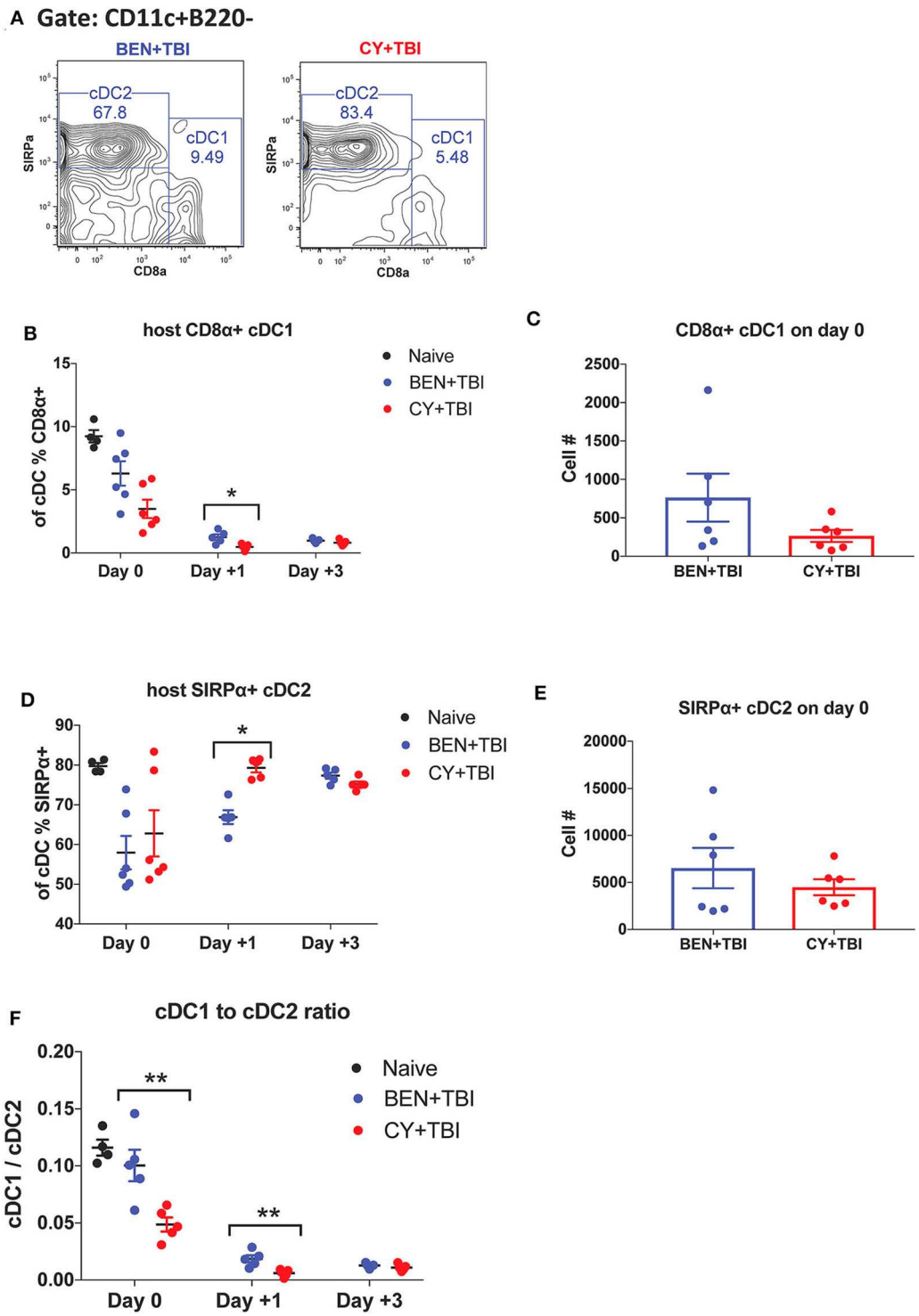

FIGURE 3 | BEN+TBI compared to CY+TBI conditioning results in higher proportions of CD8α+ cDC1s. Data shown is from the same mice as in **Figure 2**. **(A)** Representative flow cytometry gating of splenic CD8α+ cDC1 and SIRPα+ cDC2 populations on day 0 with BEN+TBI or CY+TBI. Quantification of **(B)** percent CD8α+ cDC1s on days 0, +1 and +3 and **(C)** total number of CD8α+ cDC1s on day 0 are shown for BEN+TBI and CY+TBI groups. Quantification of **(D)** percent SIRPα+ cDC2s on days 0, +1 and +3 and **(E)** total number of SIRPα+ cDC2s on days 0 are shown for BEN+TBI and CY+TBI groups. Naïve mice were used as controls. **(F)** Ratio of favorable CD8α+ cDC1s to unfavorable SIRPα+ cDC2s on day 0, +1 and +3 is shown. Pooled data from 2 experiments are shown, $n = 4–6$ mice per group per timepoint. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. $^{*}P < 0.05$, $^{**}P < 0.01$.

significantly fewer SIRPα+ cDC2s (**Figure 3D**) on day +1. The absolute numbers of CD8α+ cDC1s (**Figure 3C**) and SIRPα+ cDC2s (**Figure 3E**) were not different on day 0. Given that total cDCs have been reported to exacerbate GvHD, whereas CD8α+ cDC1s are highly effective suppressors of GvHD, we evaluated the ratio of CD8α+cDC1 to SIRPα+cDC2 in each mouse conditioned with BEN+TBI or CY+TBI. We found that the ratio of favorable cDC1s to unfavorable cDC2s was significantly higher in BEN+TBI mice on days 0 and +1 (**Figure 3F**).

BEN+TBI Compared to CY+TBI Conditioning Results in More Highly Activated cDCs With Greater Migratory Capacity

Both BEN+TBI conditioned cDC subsets trended toward higher percent positive and MFI of the co-stimulatory molecules CD80 and CD86 on day 0 (**Figures 4A–F**), with cDC2s demonstrating a significantly higher percent of CD86+ cells (**Figure 4E**). Additionally, both BEN+TBI conditioned cDC subsets trended toward a higher percent positive and MFI of CCR7, reaching significance in cDC1 percent positive (**Figure 4C**) and in cDC2 MFI (**Figure 4F**). Overall, we observed more activated, migratory cDCs and a significantly greater proportion of CD8α+ cDC1s in BEN+TBI conditioned mice compared to CY+TBI.

BEN+TBI Compared to CY+TBI Conditioning Results in Higher Proportions of CD103+ cDC1s

We next sought to evaluate the mobile, non-lymphoid-residing counterpart of CD8α+ cDC1s, CD103+ cDC1s. CD103+ cDC1s are functionally equivalent to CD8α+ cDC1s, with both subsets requiring the Batf3 transcription factor for development, expressing the same set of pattern-recognition receptors and displaying the same Ag-processing and presentation capabilities (40). The major difference is that CD8α+ cDC1s reside within secondary lymphoid organs while CD103+ cDC1s reside primarily in skin and intestines and migrate to secondary lymphoid organs upon activation (31, 40). Therefore, we evaluated CD103+ cDC1s in the spleen, bearing in mind that CD103+ cDC1s may co-express CD8α+ and may overlap in our percentage analyses. We observed a significantly greater percentage of CD103+ cDC1s in BEN+TBI conditioned mice compared to CY+TBI on day 0, +1 and +3 (**Figures 5A,B**), with a trend toward higher absolute number of CD103+ cDC1s in BEN+TBI compared to CY+TBI conditioning (**Figure 5C**). BEN+TBI conditioning resulted in a trend toward increased expression of CD80 and CD86 that reached significance in the CD86 MFI of CD86+ CD103+ cDC1s on day 0 (**Figures 5D,E**). Further, when we evaluated the migratory capacity of these CD103+ cDC1s we found that BEN+TBI conditioning resulted in a significantly greater percent expression and MFI of CCR7 compared to CY+TBI (**Figure 5F**). Altogether, these results suggest that BEN+TBI conditioning favors the persistence,

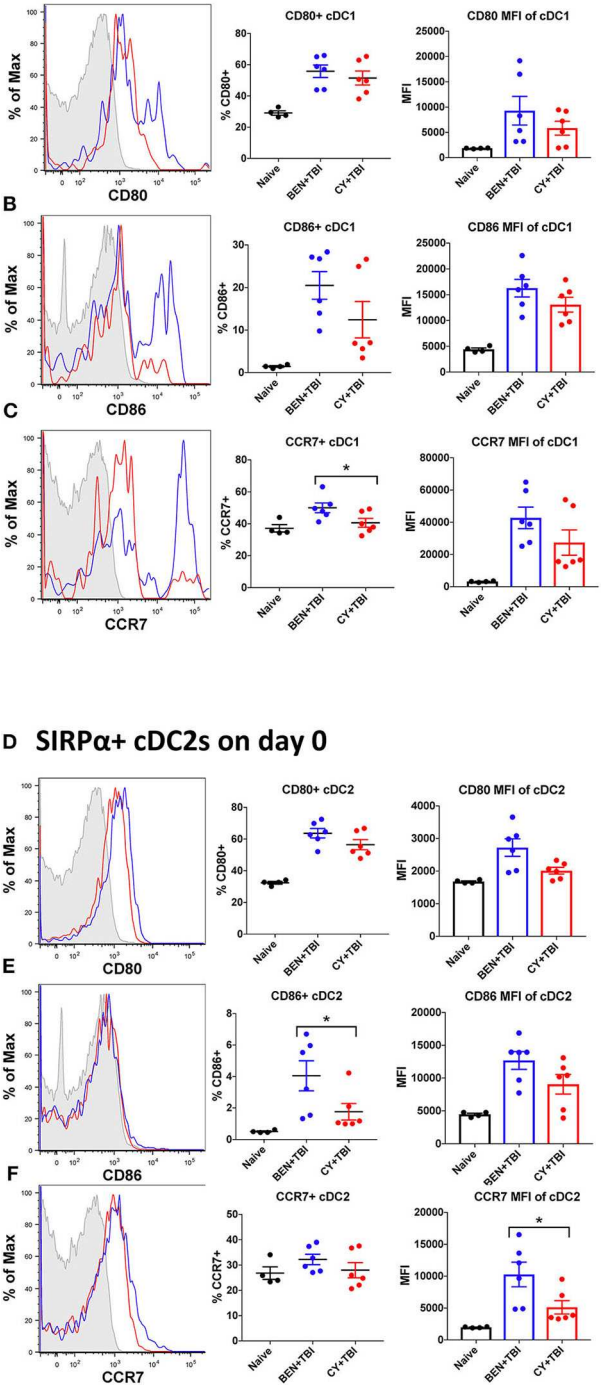

FIGURE 4 | BEN+TBI compared to CY+TBI conditioning results in more highly activated cDCs with greater migratory capacity. Data shown is from the same mice as in **Figure 2**. Representative histograms, percent and MFI among cells positively expressing the activation markers CD80, CD86 and CCR7 are shown for **(A–C)** splenic CD8α+ cDC1s and **(D–F)** splenic SIRPα+ cDC2s, with FMO control shown in gray, BEN+TBI shown in blue and CY+TBI shown in red. Pooled data from 2 experiments are shown, $n = 4$–6 mice per group per timepoint. Mann-Whitney unpaired t-tests were used to determine significance with SEM shown. $^*P < 0.05$.

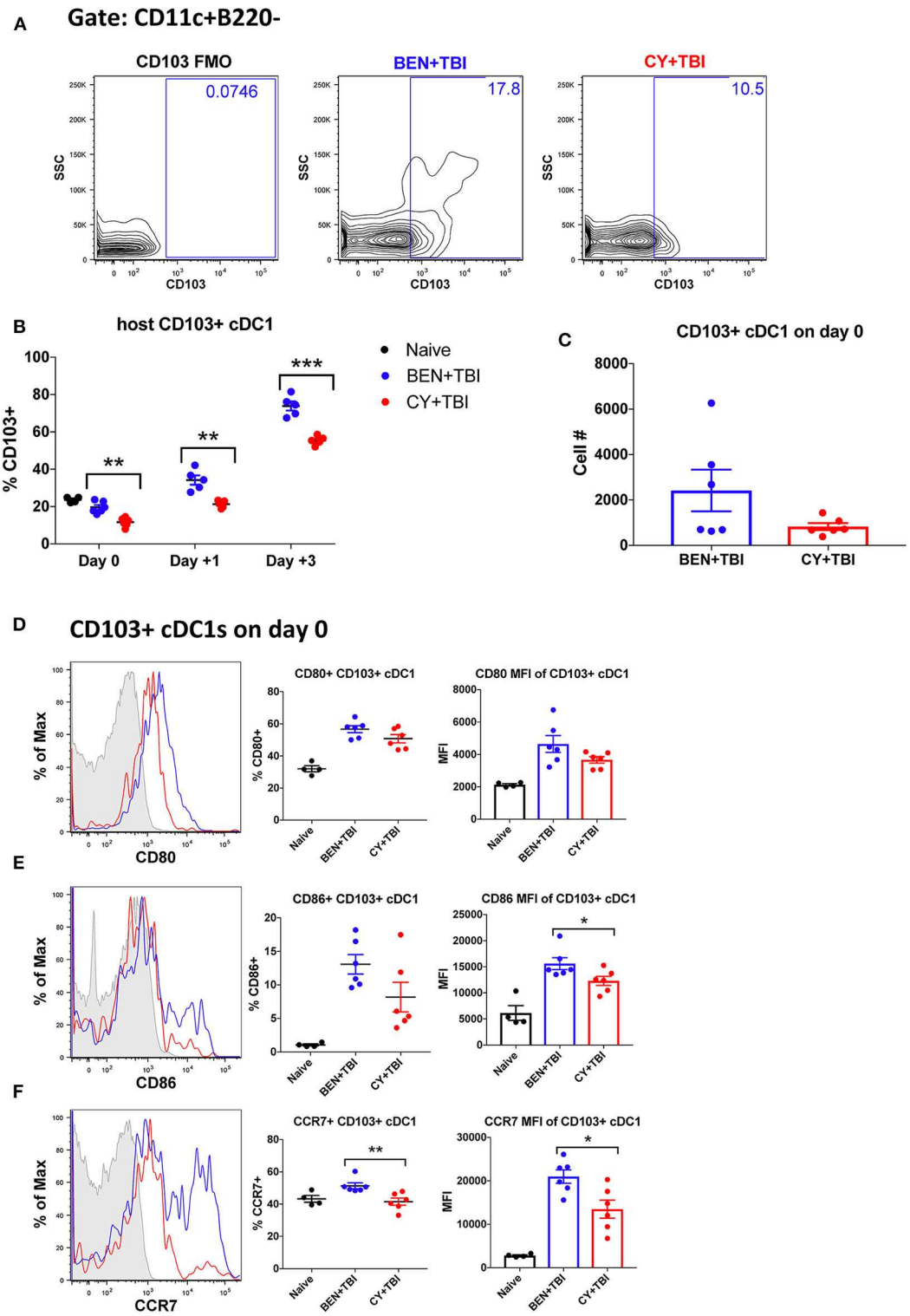

FIGURE 5 | BEN+TBI compared to CY+TBI conditioning results in higher proportions of CD103+ cDC1s and robust accumulation of pre-cDC1s. Data shown is from the same mice as in **Figure 2**. **(A)** Representative flow cytometry gating of the splenic CD103+ cDC1 population on day 0, showing the FMO control. **(B)** Quantification of percent CD103+ cDC1s on days 0, +1 and +3 are shown from BEN+TBI or CY+TBI conditioned mice. Naïve mice were used as controls. **(C)** Total cell numbers of CD103+ cDC1 on day 0 are shown. Representative histograms, percent and MFI among cells positively expressing the activation markers **(D)** CD80, **(E)** CD86 and **(F)** CCR7 are shown for CD103+ cDC1s, with FMO control shown in gray, BEN+TBI shown in blue and CY+TBI shown in red. Pooled data from 2 experiments, $n = 4$–6 mice per group per time point. Mann-Whitney unpaired t-test was used to determine significance with SEM shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

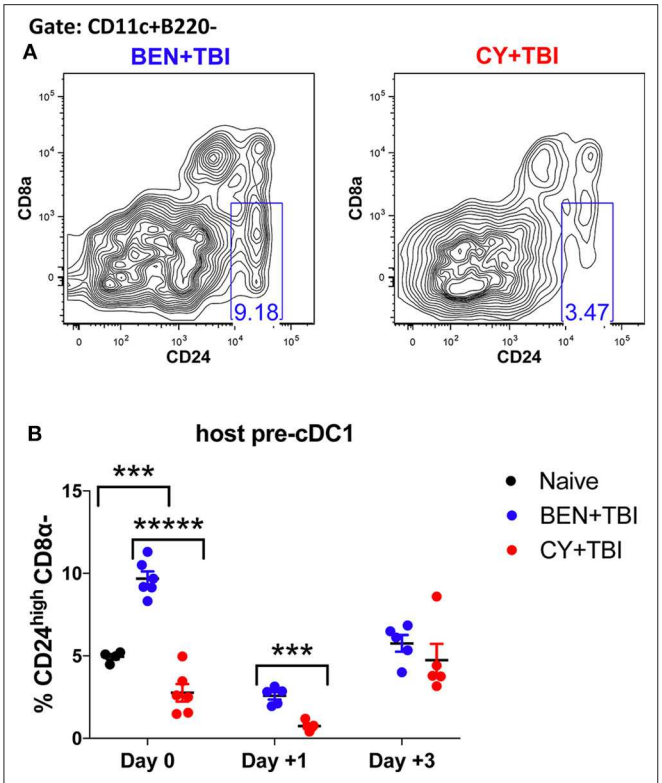

FIGURE 6 | BEN+TBI conditioning results in robust accumulation of pre-cDC1s. Data shown is from the same mice as in **Figure 2**. **(A)** Representative flow cytometry gating of the splenic CD24^{high}CD8α- pre-cDC1 population on day 0 is shown. **(B)** Quantification of percent pre-cDC1s on days 0, +1 and +3 are shown for BEN+TBI and CY+TBI groups. Naïve mice were used as controls. Pooled data from 2 experiments, $n = 4$–6 mice per group per time point. Mann-Whitney unpaired *t*-test was used to determine significance with SEM shown. ***$P < 0.001$, *****$P < 0.00001$.

activation and migration of host Batf3-dependent CD8α+ and CD103+ cDC1s compared to CY+TBI.

BEN+TBI Conditioning Results in Robust Accumulation of Pre-cDC1s

It remains unclear whether BEN+TBI conditioning spares host cDC1s, alters their recruitment to or from the spleen, or alters the host microenvironment to increase the differentiation of cDC1s *in situ*. To try to evaluate the possibility that BEN+TBI is promoting cDC1 development, we quantified the immediate precursor to CD8α+ cDC1s, termed "pre-cDC1s", in the spleen. The pre-cDC1s become committed to the CD8α+ cDC1 lineage in the bone marrow and then mobilize to the spleen and other peripheral tissues to differentiate into mature DCs (29, 41). This precursor population is identified by a lack of CD8α and the high expression of CD24, which is a membrane glycoprotein that senses damage-associated molecular patterns (DAMPs) (42). Representative flow plots show distinct differences in pre-cDC1 populations between BEN+TBI and CY+TBI on day 0 (**Figure 6A**). There are significantly higher proportions of pre-cDC1s with BEN+TBI compared to CY+TBI conditioning, as well as naïve mice, on day 0 (**Figure 6B**). This significant difference was maintained through day +1 post-transplant. In contrast, we did not observe a consistent difference or trend in pre-cDC2s following BEN+TBI and CY+TBI conditioning (data not shown). These findings indicate that BEN+TBI conditioning may promote the commitment of DC progenitors to the cDC1 lineage.

BEN+TBI Compared to CY+TBI Conditioned cDC1s Have a More Suppressive Function *ex vivo*

We next sought to investigate the function of the CD8α+ cDC1 subset more closely in our model. We performed suppression assays to examine whether BEN+TBI conditioned CD8α+ cDC1s exhibited a regulatory function *ex vivo*. Naïve or BEN+TBI or CY+TBI conditioned CD8α+ cDC1s were used as suppressors of T-cells stimulated with CD3/CD28 beads. Isolation purity of CD8α+ cDC1s is shown (**Supplemental 7A**). When BEN+TBI conditioned cDC1s were added to the stimulated T-cell cultures, there was significantly less T-cell proliferation, measured by tritiated-thymidine uptake, compared to either naïve or CY+TBI conditioned cDC1s (**Figure 7A**). A similar suppression assay performed by flow cytometric analysis of CellTrace Violet dilution corroborates this finding (**Supplemental 7B**). These data indicate that BEN+TBI conditioning results in CD8α+ cDC1s with regulatory abilities, capable of suppressing T-cell proliferation to a greater extent than CD8α+ cDC1s conditioned with CY+TBI.

Host Batf3-Dependent cDC1s Contribute to but Are Not Required for the Reduction of GvHD Following BEN+TBI Conditioning

To better understand the requirement of host cDC1s as a mechanism by which BEN+TBI reduces GvHD, we utilized Batf3 KO mice as BMT recipients alongside wild-type (WT) BALB/c mice. Consistent with reports, Batf3 KO mice are devoid of CD8α+ cDC1s (**Supplemental 8A**) and significantly deficient in the non-lymphoid residing CD103+ cDC1s (**Supplemental 8B**) (43, 44). We hypothesized that BEN+TBI promotes regulatory host cDC1s to achieve reduced GvHD and, therefore, expected BEN+TBI conditioned Batf3 KO recipients to exhibit significantly worse GvHD than BEN+TBI conditioned WT BALB/c recipients. Surprisingly, we did not observe significant differences in survival, clinical GvHD score or weight loss between WT and Batf3 KO groups in either BEN+TBI or CY+TBI conditioned groups (**Figures 7B–D**), with complete donor engraftment confirmed in this model (**Supplemental 8C**). This indicates that BEN+TBI conditioning remains a safe regimen with limited GvHD mortality and morbidity, even in the absence of cDC1s. However, the difference in survival between BEN+TBI and CY+TBI was trending but no longer significant when utilizing the Batf3 KO recipients ($P = 0.1449$) (**Figure 7B**). Although the Batf3-dependent DC subsets appear to contribute to the overall advantage of BEN+TBI over CY+TBI, these results indicate that host-type CD8α+ cDC1s are unessential for

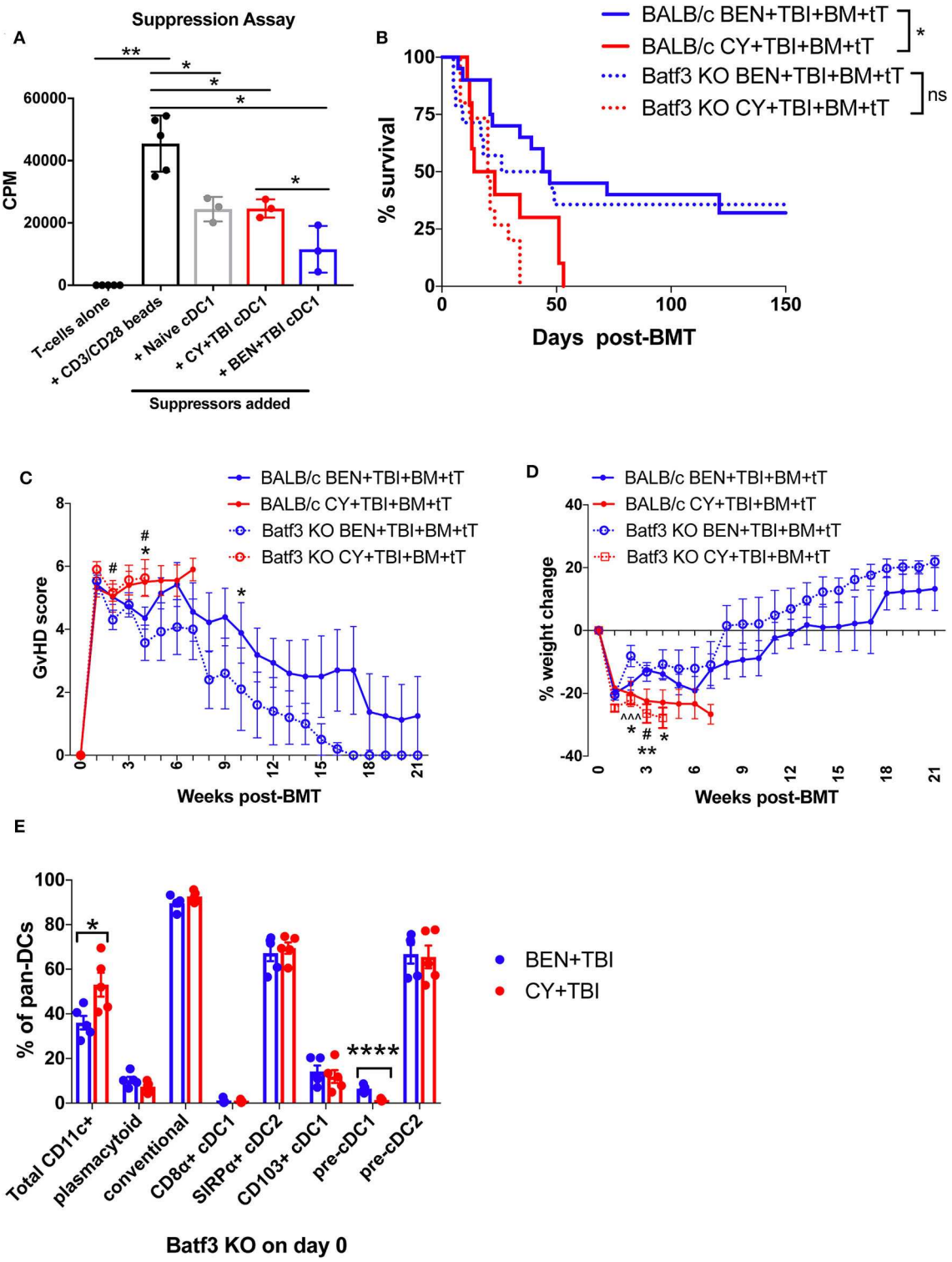

FIGURE 7 | Batf3-dependent CD8α+ cDC1s are more suppressive following BEN+TBI compared to CY+TBI conditioning, but are not the only cell type contributing to reduced GvHD. BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day −2 and 400 cGy on day −1. **(A)** On day 0, splenic CD8α+ cDC1s were isolated by magnetic bead isolation and plated in a suppression assay in a 96-well plate with T-cells pre-stimulated with CD3/CD28 beads. 0.5 μCi of tritiated-thymidine was added to each well of cultures on day 3 and T-cell proliferation was quantified as counts per minute (CPM) on day 4. CD8α+ cDC1s from naïve mice were used as a control. Data is from 1 experiment, $n = 3$ mice per group. Mann-Whitney unpaired t-tests were used to determine significance with SEM shown. $^*P < 0.05$;

(Continued)

FIGURE 7 | $^{**}P < 0.01$. **(B–D)** BALB/c or Batf3 KO recipient mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day −2, 400 cGy TBI on day −1 and 10^7 BM with 3×10^6 total T-cells (tT) on day 0. **(B)** Pooled survival data from 3 experiments are shown, $n = 15$–20 mice/group. A log-rank Mantel-Cox test was used to determine significance. $^{*}P < 0.05$. **(C)** The weekly average of the mean clinical GvHD score per group is shown with SEM. **(D)** The weekly mean percent weight change from the starting weight is shown with SEM. Multiple *t*-tests were used to determine significance. *indicates significance between BALB/c BEN+TBI and BALB/c CY+TBI. #Indicates significance between Batf3 KO BEN+TBI and Batf3 KO CY+TBI. ^indicates significance between BALB/c BEN+TBI and Batf3 KO BEN+TBI. **(E)** Batf3 KO mice received 40 mg/kg BEN i.v. or 200 mg/kg CY i.p. on day −2, 400 cGy TBI on day −1 and splenic pan-DCs were isolated by magnetic separation on day 0 for flow cytometry analysis. The percentage of total CD11c+ DCs among pan-DCs and each DC subset within their respective parent gate, as indicated by the gating strategy in **Supplemental Material**, in BEN+TBI and CY+TBI conditioned Batf3 KO mice on day 0 is shown. Data is from 1 experiment, $n = 5$ mice per group. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. $^{*}P < 0.05$, $^{****}P < 0.0001$.

improved GvHD with BEN+TBI over CY+TBI, indicating that other host cell subsets also play a role.

Given these unexpected results, we sought to characterize the effect of BEN+TBI conditioning on host DC composition in Batf3 KO mice. Batf3 KO mice had overall fewer absolute numbers of DCs compared to BALB/c WT mice. However, we did not find statistical differences between BEN+TBI and CY+TBI conditioning in the total number or percent yield of isolated splenic DCs on day 0 (data not shown). Flow cytometry revealed that BEN+TBI conditioning results in significantly lower percent total CD11c+ cells compared to CY+TBI in the isolated pan-DCs (**Figure 7E**). As expected, we found negligible percentages of CD8α+ cDC1s. Among the DC subsets in conditioned Batf3 KO mice, only the pre-cDC1 population was significantly higher in BEN+TBI compared to CY+TBI (**Figure 7E**). This finding is consistent with our results in BEN+TBI conditioned BALB/c mice in **Figure 6B** in which the pre-cDC1 population was the most significantly elevated on day 0 compared to CY+TBI.

BEN+TBI Results in Greater Numbers of Pre-cDC1s in Both BALB/c and Batf3 KO Mice Compared to CY+TBI Conditioning

To further evaluate the similarities between the effects of BEN+TBI conditioning on BALB/c and Batf3 KO mice, we calculated the ratios of each DC subset with BEN+TBI conditioning compared to CY+TBI. In both BALB/c (**Figure 8A**) and Batf3 KO mice (**Figure 8B**) there was a striking ∼5-fold higher ratio of pre-cDC1s, the immediate precursor to CD8α+ cDC1s compared to CY+TBI. Pre-cDC1s do not require the Batf3 transcription factor, and are therefore present in our Batf3 KO recipient mice (45). Their role in GvHD and alloreactivity has not been explicitly investigated, though they are reported to function similarly to the CD8α+ cDC1s that have been identified as suppressors of GvHD (24–26, 28). To determine the prevalence and biological relevance of this DC subset in BEN+TBI conditioned mice compared to CY+TBI, we calculated the total cell number as well as the ratio of pre-cDC1s to all other DCs in the spleen on day 0. We observe that BEN+TBI conditioning results in a significantly greater number of pre-cDC1s in the spleens of BALB/c (**Figure 8C**) and a trend toward more in Batf3 KO mice (**Figure 8D**). We also found that in both BALB/c (**Figure 8E**) and Batf3 KO mice (**Figure 8F**), BEN+TBI conditioning results in a significantly higher ratio of pre-cDC1s to all other DCs compared to CY+TBI. Ultimately, these results indicate a strong association between reduced GvHD and higher numbers of pre-cDC1s, which may play a previously unreported role in suppressing GvHD.

BEN Compared to CY Treatment Results in Greater Expression of Flt3 Receptor and the Inhibitory Receptor PIR-B on cDCs

We next sought to determine the mechanism by which BEN+TBI conditioning promotes the prevalence of cDC1s independently of the Batf3 transcription factor. The DC subset composition observed with BEN+TBI conditioning, associated with increased cDC1s compared to other DC subsets, is consistent with enhanced Flt3 signaling following administration of exogenous Flt3 Ligand (Flt3L) (24, 28). We therefore sought to measure circulating levels of Flt3L to test the hypothesis that BEN achieves greater proportions of cDC1s by enhancing Flt3L signaling. However, when we measured plasma levels of Flt3L on day 0 we found no difference between BEN and CY treated mice that would explain the phenotype (**Figure 9A**). We additionally evaluated plasma levels of Flt3L on day −1 and again found no difference (data not shown). We, therefore, measured expression levels of the receptor Flt3 (CD135) by flow cytometry and observed that BEN treatment results in significantly greater percent expression of Flt3 among CD11c+ DCs compared to CY conditioning (**Figure 9B**). Further, we show that conventional DCs have significantly greater expression of Flt3 by percent and MFI with BEN treatment compared to CY treatment (**Figure 9C**), and this is largely driven by higher expression among CD8α+ cDC1s (**Figure 9D**).

Administration of exogenous Flt3 Ligand prior to transplant has been shown to significantly reduce GvHD *in vivo*, an effect attributed to robust expansion of CD8α+ cDC1s (24, 28). Murine DCs generated *in vitro* with Flt3L have been shown to closely resemble steady-state DCs and exhibit regulatory function with reduced production of inflammatory cytokines (46). This suggests that Flt3L-driven DCs may acquire a unique transcriptional program compared to DCs receiving other survival and differentiation signals. We therefore sought to determine whether BEN+TBI conditioned DCs exhibited evidence of regulatory function as a result of enhanced Flt3 receptor expression. Paired Immunoglobulin Like Receptor B (PIR-B) delivers inhibitory signals by binding to CL-I on T-cells and is highly expressed on regulatory DCs (37). DCs transfected with PIR-B have been shown to prevent lethal GvHD, and deficiency of PIR-B significantly exacerbates GvHD (37). We therefore measured the expression of PIR-B on conditioned DCs by flow cytometry on day 0. While we found no consistent difference or pattern among CD8α+ cDC1s or pre-cDC1s (data not shown), we found that conventional DCs from BEN+TBI conditioned mice have greater expression of PIR-B by percent and MFI, though this was not significant (**Figure 9E**).

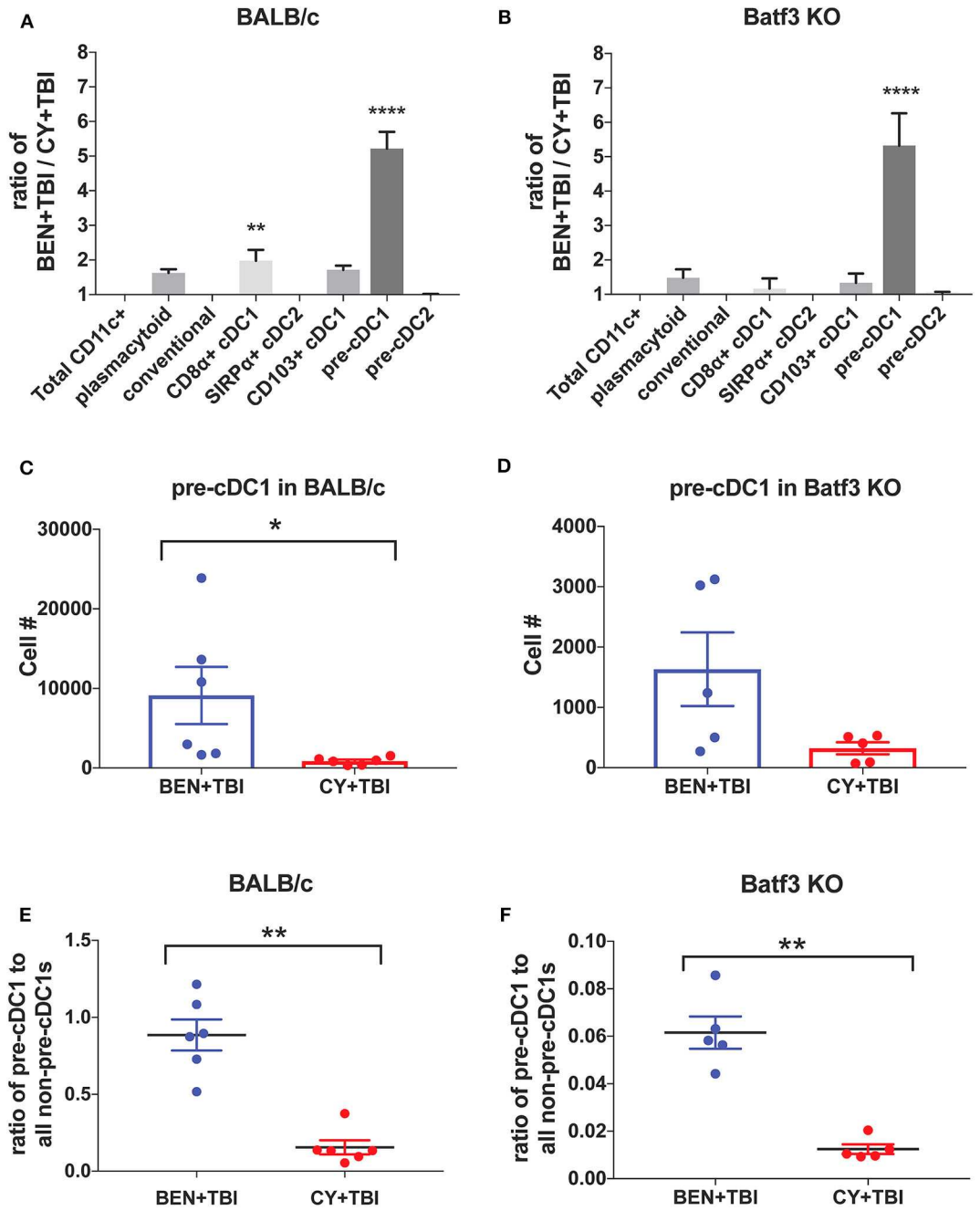

FIGURE 8 | BEN+TBI compared to CY+TBI conditioning results in greater numbers of pre-cDC1s in both BALB/c and Batf3 KO mice. **(A)** The ratio change of each DC subset within BEN+TBI conditioned BALB/c mice compared to CY+TBI conditioned BALB/c mice on day 0 is shown. Data is from 2 experiments, $n = 6$ mice per group. One-way ANOVA and Tukey's *post-hoc* test was used to determine significance compared to total CD11c+ DCs with SEM shown. $^{****}P < 0.0001$. **(B)** The ratio change of each DC subset within BEN+TBI conditioned Batf3 KO mice compared to CY+TBI conditioned Batf3 KO mice on day 0 is shown. Data is from 1 experiment, $n = 5$ mice per group. One-way ANOVA and Tukey's *post-hoc* test was used to determine significance compared to total CD11c+ DCs with SEM shown. $^{****}P < 0.0001$. **(C)** The total number of splenic pre-cDC1s in BEN+TBI and CY+TBI conditioned BALB/c mice on day 0 is shown. Data is pooled from 2 experiments, $n = 6$ mice per group. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. $^{*}P < 0.05$. **(D)** The total number of splenic pre-cDC1s in BEN+TBI and CY+TBI conditioned Batf3 KO mice on day 0 is shown. Data is pooled from 2 experiments, $n = 6$ mice per group. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown ($P = 0.098$). **(E)** The ratio of pre-cDC1 to all non-pre-cDC1s in BEN+TBI and CY+TBI conditioned BALB/c mice on day 0 is shown. Data is pooled from 2 experiments, $n = 6$ mice per group. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. $^{**}P < 0.01$. **(F)** The ratio of pre-cDC1 to all non-pre-cDC1s in BEN+TBI or CY+TBI conditioned Batf3 KO mice on day 0 is shown. Data is pooled from 2 experiments, $n = 6$ mice per group. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. $^{**}P < 0.01$.

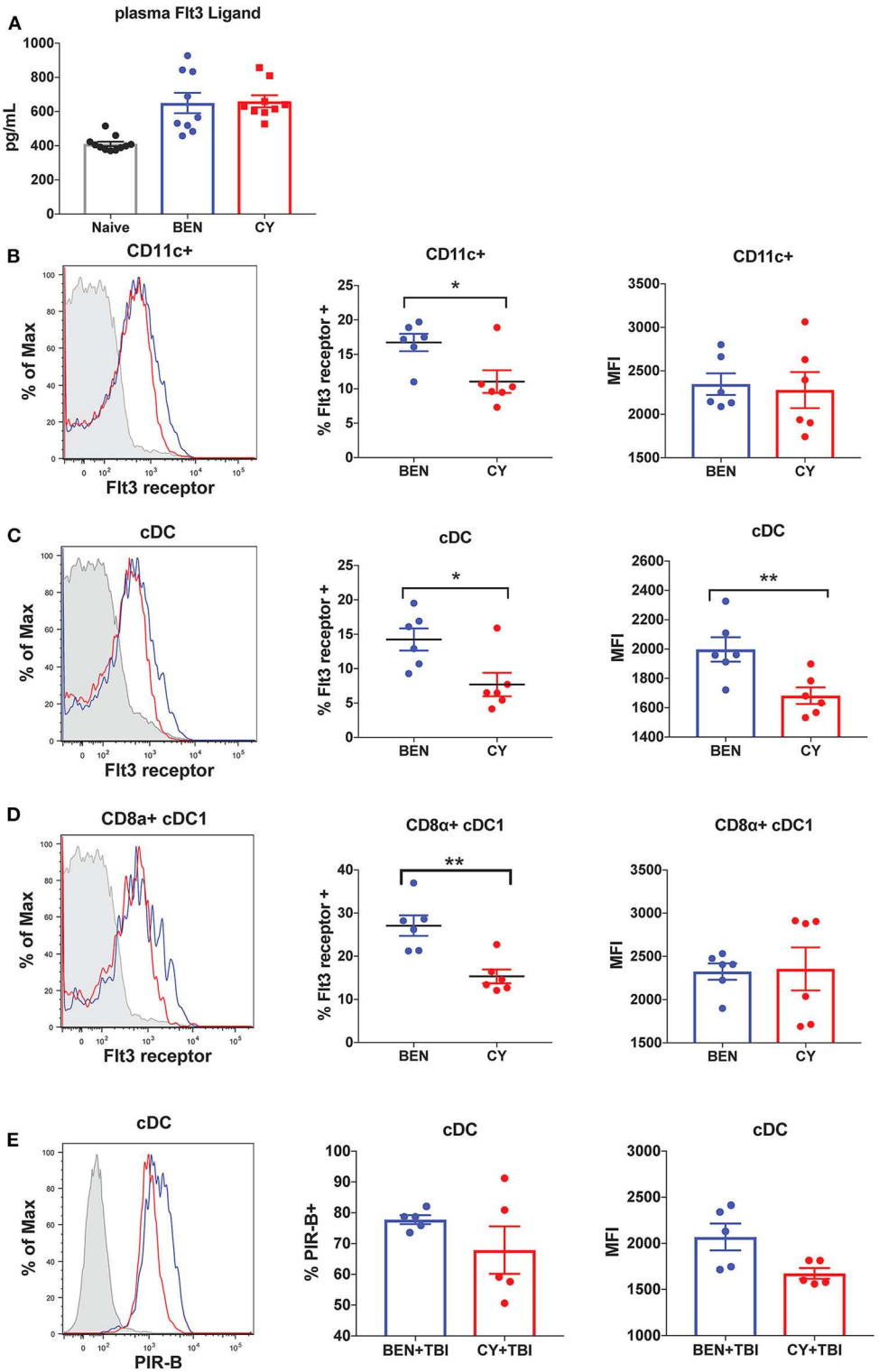

FIGURE 9 | BEN compared to CY treatment results in greater expression of Flt3 receptor and the inhibitory receptor PIR-B on cDCs. BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day −2. **(A)** On day 0, blood plasma was collected and used in an ELISA to determine circulating levels of Flt3 Ligand. Naïve mice were used as a control. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. **(B,C)** On day 0, splenic DCs were isolated by MACS negative selection and analyzed by flow cytometry. Representative histograms, percent and MFI of Flt3 receptor on **(B)** CD11c+ DCs **(C)** cDCs (CD11c+B220-) and **(D)** CD8α+ cDC1s positively expressing Flt3 receptor are shown for BEN (blue) and CY (red) treated mice, with FMO control shown in gray. Data is pooled from 2

(Continued)

FIGURE 9 | experiments, $n = 6$ mice per group. Mann-Whitney unpaired *t*-tests were used to determine significance with SEM shown. $^{*}P < 0.05$, $^{**}P < 0.01$. **(E)** BALB/c mice received 40 mg/kg BEN or 200 mg/kg CY on day −2 and 400 cGy TBI on day −1. On day 0, splenic DCs were isolated by MACS negative selection and analyzed by flow cytometry. Representative histograms, percent positive ($P = 0.50$) and MFI ($P = 0.092$) of PIR-B on cDCs (CD11c+B220-) are shown for BEN+TBI and CY+TBI conditioned mice. Data is pooled from 2 experiments, $n = 5$ mice per group. Mann-Whitney unpaired *t*-test was used to determine significance with SEM shown.

Overall, these data indicate that BEN+TBI conditioning results in host DCs that are more receptive to Flt3L and exhibit greater expression of GvHD-suppressing receptors.

DISCUSSION

We have extensively studied the C57BL/6 → BALB/c MHC-mismatched BMT model evaluating pre-transplant conditioning regimens. We have previously determined that comparable doses of BEN and CY, based on their respective maximum tolerated dose, induce similar levels of epithelial barrier damage and other host organ toxicities (12). We have also excluded graft rejection and conditioning regimen-induced toxicity as potentially confounding factors by monitoring blood engraftment and using syngeneic BMT controls (12). Both BEN and CY have short half-lives of <4 h, and are eliminated from circulation prior to the time of transplantation (47–49). Therefore, donor cells are not directly exposed to these chemotherapeutic agents, and the improvement in GvHD with BEN+TBI is attributable in part to its effects on host antigen-presenting cells (APCs), particularly host DCs (12). Pre-transplant conditioning regimens result in gradual elimination of host DCs, allowing them to play a critical role in initiation of GvHD immediately following transplantation (19–23). Donor DCs require time to develop from bone marrow progenitors into differentiated DCs, and therefore do not participate in the early induction phase of GvHD (19). It should be noted that our use of $H2K^{b}$- to define host cells on days +1 and +3 post-transplant would not exclude donor DCs that may have lost expression of MHC. In our pre-clinical model, majority of DCs in the spleen on day +3 post-transplant are host-type, offering a window in which they can prime naïve donor T-cells. Further, donor T-cells can generate long-term synapses with host DCs in the spleen and lymph nodes (50), indicating that the composition of splenic host DCs during the peri-transplant period is critical to donor T-cell activation and long-term GvHD outcomes.

We have found that BEN+TBI has a distinct effect on host DCs compared to CY+TBI. We demonstrated that BEN+TBI conditioned DCs stimulate less allogeneic T-cell proliferation *in vitro* compared to CY+TBI conditioned DCs. The differences in splenic host DC composition observed between BEN+TBI and CY+TBI conditioning are summarized in **Table 1,** along with the reported effect of each DC subset on GvHD upon transfer into recipients. While we observed greater absolute numbers of every DC subset with BEN+TBI conditioning compared to CY+TBI, we found significant changes in the overall composition by percentage. Briefly, we observed higher proportions of pDCs after BEN+TBI conditioning. pDCs are reported to induce GvHD, yet induce tolerance when they are CCR9+ (51, 52). We did not, however, observe any difference in the number of CCR9+ pDCs between BEN+TBI and CY+TBI groups (data not shown). We also observed lower percentage but greater numbers of total cDCs after BEN+TBI conditioning, which are reported to induce GvHD (52). However, when we distinguished between cDC1 and cDC2, we found greater numbers and percentages of both CD8α+ and CD103+ cDC1s in mice receiving BEN+TBI compared to CY+TBI conditioning. While host CD103+ cDC1s have not been explicitly evaluated in the context of GvHD, their lymphoid-residing counterpart, CD8α+ cDC1s, have been widely acknowledged as suppressors of GvHD (24–28). We also observed lower cDC2s in BEN+TBI mice, which can induce GvHD (24, 26, 28, 52). We further documented larger numbers of pre-cDC1s, the immediate precursor to CD8α+ cDC1s, following BEN+TBI conditioning, which have not previously been explicitly evaluated in the context of BMT and GvHD. We also demonstrate numerous incidences of greater expression of the activation markers CD80 and CD86 in BEN+TBI conditioned DCs compared to CY+TBI, indicating that BEN+TBI conditioning results in host cDCs capable of stronger engagement and presentation of host antigen to donor T-cells. We additionally found several incidences of increased expression of CCR7, suggesting CD8α+ and CD103+ cDC1s with greater potential to migrate to lymphoid tissues. However, it is not technically feasible to retrieve sufficient numbers of DCs from lymph nodes or target tissues as there are too few cells following conditioning, limiting our ability to provide direct evidence of enhanced migration. It should also be noted that DCs were isolated without chemical dissociation of spleens, potentially impacting our DC yield and biasing our results.

Murine CD8α+ cDC1s have been widely acknowledged as suppressors of GvHD via activation-induced clonal deletion and exhaustion of allospecific donor T-cells (24–28), warranting

TABLE 1 | Murine DC subset effect on GvHD and summary of results presented as mean cell number and mean percentage.

Host DC subset	Reported effect on GvHD	BEN+TBI # (%)	CY+TBI # (%)
pDC	↑ GvHD (39)	5,403 (30.23%)	1,830 (19.12%)
cDC	↑ GvHD (39)	12,180 (69.2%)	7,003 (80.52%)
CD8α+ cDC1	↓ GvHD (17, 40, 41)	763.3 (6.29%)	265.1 (3.49%)
cDC2	Unknown	6,528 (57.97%)	4,490 (62.82%)
CD103+ cDC1	Unknown	2,420 (19.6%)	830.5 (11.66%)
Pre-cDC1	Unknown	9,132 (9.94%)	867 (2.76%)

↑ Indicates exacerbation of GvHD; ↓ Indicates amelioration of GvHD.

further investigation of this subset in the context of BEN+TBI conditioning. We demonstrated that CD8α+ cDC1s conditioned with BEN+TBI exhibit greater suppressive function than those conditioned with CY+TBI. Several groups have shown that Batf3 KO recipients exhibit more severe GvHD (24, 26, 53), while others found that GvHD is unaffected (24, 26, 54). Using our model, survival and clinical GvHD score were comparable in Batf3 KO and WT BALB/c mice receiving BEN+TBI conditioning, indicating that Batf3-dependent CD8α+ cDC1s are expendable to the mitigation of GvHD seen with BEN+TBI. Batf3-dependent CD8α+ cDC1s do appear to play a role in reducing GvHD, however, as there was no longer a significant difference in survival between BEN+TBI and CY+TBI in Batf3 KO mice that was otherwise observed in WT BALB/c mice. Overall, while Batf3-dependent cDC1s do contribute to BEN+TBI conditioning's reduction of GvHD, there appear to be other cellular mechanisms at play.

We also found significantly greater proportions and absolute numbers of pre-cDC1s, the immediate precursor to CD8α+ cDC1s, with BEN+TBI compared to CY+TBI in both WT BALB/c and Batf3 KO mice. This ∼5-fold increase in pre-cDC1s with BEN+TBI conditioning was the most striking difference found in any of the host DC subsets and was observed in both strains of mice. Pre-cDC1s are committed to the cDC1 lineage but require the Batf3 transcription factor for their terminal differentiation into mature CD8α+ cDC1s (45). To our knowledge, the role of pre-cDC1s has not been reported in the context of GvHD. However, they express the same pattern recognition receptors as CD8α+ cDC1s, yet have an enhanced lifespan compared to CD8α+ cDC1s (55). Pre-cDC1s have been shown to induce stronger priming of viral-specific CD8+ T-cells compared to mature CD8α+ cDC1s (55). This effect may be attributed to their high expression of CD24, a sensor for damage-associate molecular patterns (DAMPs) that dictates CD8+ T-cell differentiation into effector or memory fates (42). These facets of pre-cDC1 function may prove beneficial in the context of GvHD whereby early priming of CD8+ T-cells against DAMPs may promote tolerance to host antigens. We demonstrate that the prevalence of pre-cDC1s is strongly associated with significant improvements in GvHD and survival achieved with BEN+TBI conditioning, and therefore postulate that pre-cDC1s may have a previously unreported role in limiting GvHD.

There may be an additional advantage of BEN+TBI conditioning's resultant prevalence of pre-cDC1 and CD8α+ cDC1 in the context of viral reactivation. Reactivation of latent viruses, particularly human cytomegalovirus (HCMV), following HSCT is a major cause of morbidity and non-relapse mortality among transplant recipients (56, 57). Numerous studies have determined that Batf3-dependent cDC1s play a critical role in mounting CD8+ T-cell responses against a variety of viruses, including cytomegalovirus (CMV) (58), West Nile virus (WNV) (43), influenza (59, 60), cow pox virus (61), and herpes simplex virus (HSV) (62, 63). Further studies depleting XCR1+ DCs, which include both pre-cDC1s and CD8α+ cDC1s, determined a critical role for these DCs in priming naïve CD8+ T-cell responses and in reactivating memory CD8+ T-cells (64, 65). Therefore, the prevalence of cDC1s in the peri-transplant period with BEN+TBI conditioning could provide the added benefit of protecting against viral reactivation and opportunistic infections. In support of this hypothesis, interim results from an ongoing phase I clinical trial (NCT02996773) to determine the safety of replacing post-transplant CY with post-transplant BEN in haploidentical BMT have shown significantly lower incidence of CMV reactivation and lower CMV viral load with BEN compared to CY (5).

The DC composition differences that we observed with BEN+TBI mirror that of administration of exogenous Flt3L, which robustly expands all DC populations but preferentially increases cDC1s (24, 28). While we did not find elevated plasma levels of Flt3L with BEN+TBI conditioning, we did find a greater expression of the Flt3 receptor, CD135, among DCs conditioned with BEN compared to CY. The Flt3 signaling pathway is intimately involved in the homeostasis, commitment and differentiation of steady state DCs (40, 66, 67). Notably, numerous pre-clinical studies have found that administration of Flt3L *prior* to transplant can alleviate GvHD and enhance GvL, while administration of Flt3L *after* transplant significantly exacerbates GvHD by stimulating donor stem cells to proliferate (24, 28, 68, 69). Thus, BEN+TBI conditioning may prove advantageous over CY+TBI in that it results in enhanced Flt3 signaling, specifically among host cells. Further, the effect of pre-transplant Flt3L has been largely attributed to increased numbers of host CD8α+ cDC1s capable of eliminating antigen-specific donor T-cells (24, 70–72). However, Flt3L also greatly expands pre-cDC1s to the point that nearly half of CD8α- DCs are pre-cDC1s (55). Given the fact that this pre-cDC1 population was only recently characterized, it is possible that the effects of Flt3L on GvHD have been inequitably attributed to CD8α+ cDC1s alone, when perhaps pre-cDC1s also play a significant role. Further investigation is required to fully understand the potential role of pre-cDC1s in alloreactivity and GvHD.

In summary, BEN+TBI conditioning results in a greater number and proportion of murine host pre-cDC1s in a Batf3-independent manner, which is associated with reduced GvHD. We demonstrate that BEN treatment results in host DCs with greater expression of Flt3 receptor, potentially contributing to the skewing of host DCs toward cDC1s. BEN may prove to have significant advantages as a pre-transplant conditioning agent over CY to reduce GvHD and potentially limit viral reactivation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Arizona Institutional Animal Care and Use Committee.

AUTHOR'S NOTE

MM is a Ph.D. Candidate at the University of Arizona. This work is submitted in partial fulfillment of the requirements for the Ph.D.

AUTHOR CONTRIBUTIONS

MM designed and performed experiments, analyzed and reviewed data, and wrote the manuscript. JS and EH helped design and perform experiments, reviewed data, and revised the manuscript. JE performed experiments and revised the manuscript. YZ and RS contributed to the experimental design, data interpretation and discussion, and revised the manuscript. EK designed the project, supervised and advised on the implementation and conduction of experiments, reviewed and interpreted data, and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01410/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Metabolic Pathways in Alloreactive T Cells

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Allogeneic hematopoietic stem cell transplantation (aHSCT) is a curative therapy for a range of hematologic illnesses including aplastic anemia, sickle cell disease, immunodeficiency, and high-risk leukemia, but the efficacy of aHSCT is often undermined by graft-versus-host disease (GVHD), where T cells from the donor attack and destroy recipient tissues. Given the strong interconnection between T cell metabolism and cellular function, determining the metabolic pathways utilized by alloreactive T cells is fundamental to deepening our understanding of GVHD biology, including its initiation, propagation, and potential mitigation. This review summarizes the metabolic pathways available to alloreactive T cells and highlights key metabolic proteins and pathways linking T cell metabolism to effector function. Our current knowledge of alloreactive T cell metabolism is then explored, showing support for glycolysis, fat oxidation, and glutamine metabolism but also offering a potential explanation for how these presumably contradictory metabolic findings might be reconciled. Examples of additional ways in which metabolism impacts aHSCT are addressed, including the influence of butyrate metabolism on GVHD resolution. Finally, the caveats and challenges of assigning causality using our current metabolic toolbox is discussed, as well as likely future directions in immunometabolism, both to highlight the strengths of the current evidence as well as recognize some of its limitations.

Keywords: alloreactive T cells, GVHD biology, immunometabolism, glycolysis, fatty acid oxidation (FAO), mammalian target of rapamycin (mTOR), AMPK

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a potentially curative therapy for a wide range of hematologic maladies ranging from genetic diseases to aggressive leukemias and lymphomas (1–3). An unintended and potentially deadly consequence of aHSCT is graft-versus-host disease (GVHD), where donor T cells primed to react against alloantigens attack host tissues in the skin, gastrointestinal tract, and liver (4). While corticosteroid-induced immunosuppression can treat GVHD, corticosteroids are an imperfect therapy and durable remissions only occur in 50% of patients (5). Furthermore, the broad immunosuppression necessary to treat GVHD often limits physiologic immunity and impairs T cell mediated clearance of leukemia [aka the graft-versus-leukemia (GVL) effect (6, 7)]. Thus, common complications following the increased immunosuppression surrounding GVHD treatment include infection (8, 9) and cancer relapse (9), making development of GVHD especially dangerous. In fact, while absolute T cell depletion can minimize GVHD risk, the ensuing increase in infection, and disease relapse result in comparable

overall survival (10). Thus, there is a need for novel approaches to treat and prevent GVHD while still preserving physiologic immunity and maintaining aHSCT efficacy.

It is broadly accepted that metabolism and immune cell function are linked, with immunologic differentiation influencing immune cell metabolism and metabolic pathways impacting immune responses (11, 12). It is therefore imperative to understand how metabolism influences T cell function in specific environmental contexts. This is particularly true during GVHD, where high levels of chronic antigen stimulation result in robustly activated T cells with a sharply increased metabolic demand. Understanding the unique metabolic profile of alloreactive T cells will enhance our ability to improve current therapeutic options and advance our contextual knowledge of *in vivo* T cell biology. This review will highlight our current understanding of alloreactive T cell metabolism in light of the major metabolic pathways, present evidence for involvement of various pathways at distinct stages of the process, define key metabolic regulators that influence substrate choice, and integrate multiple lines of evidence into a cohesive overarching hypothesis. We close by highlighting examples of additional ways in which metabolism can influence GVHD and discuss challenges to the interpretation of metabolic data.

OVERVIEW OF CELLULAR METABOLISM

Cellular metabolism is a complex interplay between multiple different enzymes, substrates, intermediates, and end products. Classically, glycolysis, and oxidative phosphorylation (OXPHOS) have been studied as the primary pathways that supply cellular energy. Glycolysis consists of a series of enzymatic steps that convert glucose into pyruvate. Depending on the intrinsic and extrinsic needs of the cell, pyruvate can then either be converted into lactate and excreted from the cell or channeled into acetyl-coA and further oxidized via OXPHOS. While lactate fermentation classically occurs in oxygen poor environments, T cells can perform glycolysis and lactate fermentation in oxygen replete environments, referred to as aerobic glycolysis. Although glycolysis might not be the best choice based solely on energy production, glycolytic intermediates can also act as substrates for anabolic pathways including amino acid synthesis, nucleotide synthesis, and the pentose phosphate pathway (PPP) (13), all processes necessary in actively proliferating cells.

Oxidative phosphorylation is a more efficient process used to generate cellular energy. Specifically, the tricarboxylic acid (TCA) cycle uses the end products of glycolysis, fatty acid oxidation, and glutamine metabolism to generate the reducing intermediates, NADH, and $FADH_2$ (14). NADH and $FADH_2$, in turn, fuel the electron transport chain (ETC) by donating electrons to Complex I and II (14), a process which results in ATP production and concurrent consumption of oxygen (14).

METABOLIC PATHWAYS CONTRIBUTING TO ALLOREACTIVE T CELL EFFECTOR FUNCTION

Classically, naïve T cells are considered largely quiescent, catabolically relying on OXPHOS to meet their modest energy demands. Upon activation, naïve T cells switch to anabolic metabolism (15) and despite the availability of oxygen, increase aerobic glycolysis in a process known as the Warburg effect (16, 17). Aerobic glycolysis produces less energy per molecule of substrate than oxidative pathways but has the advantage of maintaining redox balance (18) and allowing for the bulk of cellular machinery to be used in the production of biomolecules required for proliferation and T cell function, including cytokines (19, 20). In contrast to recently activated T cells, memory and regulatory T cells (T_{regs}) rely on oxidation of fatty acids and glucose to maintain their energetic balance (21–25). This classic view has recently been challenged, where effector T cells have been demonstrated to increase oxidative metabolism *in vivo* and be less reliant on glycolytic metabolism compared to *in vitro* activated cells (26). How alloreactive T cells meet their energetic demands during GVHD remains a work in progress, but evidence supports the adoption of both aerobic glycolysis and OXPHOS during early stages of T cell activation and disease initiation.

The studies highlighted in this review compare the profile of allogeneic T cells to either syngeneic or naïve T cell controls. While both syngeneic and naïve T cells are less activated than alloreactive cells, syngeneic T cells are the preferred negative control because they experience the inflammatory milieu of pre-transplant conditioning and most accurately reflect the lymphopenia-driven reconstitution of the immune system seen in human transplants. In contrast, naïve T cells are relatively inert. Thus, comparing alloreactive and naïve T cells risks identifying differences that are not unique to alloreactive T cells, but are instead characteristic of any proliferating T cell. Since the aim is to distinguish alloreactive from homeostatically proliferating T cells, syngeneic T cells remains the better negative control.

Since aerobic glycolysis is critical for physiologic T cell activation (16, 27, 28), it is natural to consider the role of glucose in alloreactive cells. However, discussions on glucose metabolism are challenging given that pyruvate, a critical glycolytic intermediate, can either be converted into lactate for fermentation or alternatively channeled through pyruvate dehydrogenase into the TCA cycle for oxidation. For the purposes of this review, we will consider both forms of glucose metabolism under one discussion. In two pre-clinical models of GVHD, glucose uptake increased in donor T cells 14 days post-transplant (allogeneic > syngeneic), accompanied by increased expression of glucose transporters Glut1 and Glut3 (29). Expression of the key glycolytic enzymes hexokinase (isoforms 1 and 2) and lactate dehydrogenase also increased in allogeneic cells, while donor T cell numbers decreased following treatment with the pan-glycolysis inhibitor 2-deoxyglucose (2DG) (29). In this same study, recipients experienced improved

survival when treated with 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO), an inhibitor of phosphofructokinase (30), the rate-limiting step in glycolytic metabolism (29, 31, 32). Consistent with glucose transport playing an important role in GVHD development, T cells lacking *Glut1*, which experienced a decreased glycolytic rate *in vitro*, were unable to induce GVHD *in vivo*. In this case, post-transplant weight loss and survival outcomes were similar between recipients of Glut1$^{-/-}$ T cells and those transplanted with bone marrow alone (33). In addition to its therapeutic potential, increased glucose uptake may also have diagnostic implications. Positron emission tomography using ^{18}F-fluorodeoxyglucose (FDG-PET), a glucose analog, identifies increased tracer uptake in the gastrointestinal tract during GVHD in both mice and humans (34). Thus, inhibiting glycolytic metabolism, either genetically or pharmacologically, might constrain alloreactive T cell effector (T_{eff}) function, while increased glucose trafficking may be of potential diagnostic value. The caveat is that glycolytic metabolism may also prove essential to the function of non-GVHD T cells.

A key metabolic regulator that could connect glycolytic metabolism and alloreactive T cell function is the mammalian target of rapamycin (mTOR), a serine/threonine kinase belonging to the phosphoinositol 3-kinase (PI3K) family. mTOR is the catalytic subunit for either of two distinct protein complexes; mTORC1 (with scaffolding protein Raptor) and mTORC2 (with scaffolding protein RICTOR) (35). mTORC1 and mTORC2 each have unique roles within cells. mTORC1 promotes protein and lipid synthesis while mTORC2 promotes cytoskeletal rearrangement (36). In a variety of settings, mTOR integrates environmental signals into regulation of immune cell metabolism, differentiation, and effector function. In particular, mTOR and has been shown to play a key role in T cell activation and cell fate (37–39). Metabolically, T cell receptor stimulation activates mTOR via PI3K/Akt signaling, which then promotes glycolysis, glutaminolysis, and activation of the PPP (37, 40, 41). mTOR promotes glycolysis in part through the activation of HIF1α and c-Myc, transcription factors which drive expression of glycolytic proteins including pyruvate dehydrogenase kinase 1, hexokinase 2, and lactate dehydrogenase A (40, 42–44). Furthermore, the PI3K/Akt/mTORC1 pathway has been implicated in T cells as a key regulatory step for the expression and trafficking of the glucose transporter, Glut1, with mTORC inhibitors preventing Glut1 expression (33, 45). Thus, mTOR contributes to metabolic reprogramming following T cell activation by promoting both glycolysis and general anabolic pathways. mTOR signaling also influences T cell differentiation. T cells deficient in mTOR are unable to differentiate into T-helper type 1 (Th1), Th2, or Th17 cells under *in vitro* skewing conditions but still readily differentiate into FoxP3^{+} T_{reg} cells (46). Furthermore, mTORC1, and mTORC2 promote differentiation of specific T cell subsets. mTORC2 promotes Th2 differentiation (47), while mTORC1 promotes development of Th17 cells (47, 48). The role played by mTORC1 in Th1 differentiation remains uncertain, with evidence both for (47), and against (48) mTORC1 involvement in Th1 responses.

mTOR has also been implicated in promoting the pathogenicity of alloreactive T cells. In pre-clinical models, mTOR activity increases in alloreactive T cells and both the pharmacologic inhibition of mTOR, and its genetic deletion, inhibit glycolysis without impacting OXPHOS (29, 49). These interventions also improve outcomes in animal models of GVHD (29). In the clinic, targeting mTOR using the inhibitors sirolimus (rapamycin) and everolimus is well-established and has been found to be efficacious for both GVHD prophylaxis and treatment (50, 51). Thus, inhibition of mTOR improves GVHD, in part through its inhibition of glycolysis.

In addition to glucose metabolism, there is ample evidence that alloreactive T cells rely on the energy and by-products of OXPHOS. This necessity of OXPHOS, in addition to an increase in lactate fermentation, is likely necessary due to the greater energetic demands experienced by T cells undergoing constant exposure to high levels of antigen in a near continuous manner. In allogeneic cells, oxygen consumption increased markedly compared to either naïve T cells or T cells recovered from syngeneic recipients (52). Mitochondrial activity also increased in allogeneic T cells with a corresponding increase in mitochondrial superoxide production (52, 53). Consistent with increased mitochondrial activity, alloreactive T cells upregulated expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc1α), a regulator of mitochondrial biogenesis (54). Finally, administration of BZ-423, an F1-F0 ATPase inhibitor that targets cells with increased mitochondrial respiration, improved survival, lowered clinical scores, and decreased lymphocytic infiltration into GVHD target organs in a murine model of GVHD (53). These results suggest that alloreactive T cells are preferentially susceptible to ETC inhibition, in part because of their increased reliance on mitochondrial respiration (53). Taken together these findings demonstrate an increased dependence on both OXPHOS and glycolytic metabolism in alloreactive T cells, making them metabolically distinct from other T cell populations.

Although the mechanisms that influence OXPHOS are complex, the cellular energy sensor AMP-activated protein kinase (AMPK) has been implicated as a driver of oxidative metabolism and could play a role. AMPK is heterotrimeric protein complex consisting of a serine/threonine kinase α subunit, a stabilizing β subunit, and a regulatory γ subunit. The γ subunit detects low intracellular energy levels by sensing the AMP/ATP ratio (increased during low energy states) and responds by facilitating activation of the AMPKα kinase domain (55). AMPK activation conserves energy by inhibiting anabolic pathways (e.g., fat and protein synthesis), while increasing energy production via catabolic pathways, including OXPHOS and autophagy (56, 57). In part, AMPK restricts anabolism by antagonizing mTORC1 via phosphorylation of Raptor as well as upstream regulator tuberous sclerosis complex 2 (28, 58). AMPK also directly promotes OXPHOS and fatty acid catabolism. In skeletal muscle, acetyl-coA carboxylase (ACC) produces malonyl-coA, which allosterically inhibits carnitine palmitoyl transferase 1 (CPT1a), a key enzyme in fat oxidation, and thereby blocks FAO (55, 59). AMPK inhibits ACC through phosphorylation, which decreases malonyl-CoA levels and thus increases FAO. In addition, ACC

is a key enzyme in fatty acid synthesis (FAS) so that AMPK inhibition of ACC not only increases FAO but also reduces the anabolic process of FAS.

Given that AMPK is key to promoting both OXPHOS and FAO, it follows that AMPK would be integral to T cell homeostasis. However, the exact role for AMPK in T cells continues to evolve. Systemic ablation of AMPKα1 increased lymphocyte susceptibility to mitochondrial inhibition (i.e., treatment with oligomycin) but did not impact T cell development or differentiation (60). Later studies noted an increase in T cell glycolysis following global deletion of AMPKα1 and an increase in T cell production of the pro-inflammatory cytokines interferon gamma (IFN-γ) and interleukin 17a (IL-17a) (61). In a *Listeria monocytogenes* infection model, T cell-specific deletion of AMPKα1 impaired memory CD8 cell generation compared to wildtype T cells without impacting primary immune responses (62). More recently, AMPK was shown to be necessary for maximal T_{eff} generation during both viral and bacterial challenges *in vivo* (63) and has been implicated in driving oxidative metabolism in T cell acute lymphoblastic leukemia (64). AMPK has also been suggested to be necessary for T_{reg} development, with increased AMPK phosphorylation in cultured T_{reg} cells and an increase in T_{reg} percentages following *in vivo* administration of metformin, an indirect AMPK activator (24). Metformin also increases T_{reg} number at the expense of Th17 cells in models of autoimmune arthritis (65, 66). However, whether AMPK is directly responsible for these changes, or they are driven by actions of metformin independent of AMPK, remains to be determined.

Despite characterization of AMPK in activated T cells, how AMPK contributes to GVHD pathogenicity is poorly understood. In the only paper to date investigating AMPK and GVHD, 5 days of metformin administration ameliorated GVHD severity and decreased disease lethality, with fewer Th17 and Th1 and increased T_{reg} cells recovered from metformin-treated recipients (49). However, given the fact that metformin is a direct inhibitor of Complex I of the ETC (67–69), there is a high likelihood that metformin directly inhibits oxidative metabolism, a process necessary in alloreactive cells. In our hands, transplantation of donor T cells lacking both AMPK α1 and α2 improved GVHD-related lethality with decreased recovery of AMPK-deficient donor T cells, reduced T cell homing to target organs, and an improved T_{reg} to T_{eff} cell ratio (70). Indeed, these later results are in line with studies demonstrating an important role for AMPK in T_{eff} cell survival and recovery (63). Thus, on balance, metformin treatment improves GVHD, but likely in an AMPK independent manner, as genetic elimination demonstrates that AMPK is necessary in donor T cells for maximal GVHD severity. In both cases, more work needs to be done to determine the exact mechanism behind the observed effects.

Given the increased oxidative metabolism of donor T cells during GVHD, an ongoing question becomes which substrate or substrates fuel this pathway. In fact, glucose utilization, fat oxidation, and glutamine metabolism have all been shown to be crucial for T cell proliferation and survival in various allogeneic contexts (15, 71, 72). T cells isolated on day 7 post-allogeneic transplant show elevated levels of fat import, higher acylcarnitine concentrations, and increased fatty acid oxidation (53, 54). These changes are supported by increased expression of CPT1a and CPT2, enzymes necessary for transport of long and very-long chain fatty acids into the mitochondria for β-oxidation. Furthermore, treatments with the FAO inhibitor etomoxir improved GVHD severity while simultaneously decreasing T cell proliferation and the number of donor T cells (54). This dependence on FAO may be most prevalent at early times post-transplant, as metabolic interrogation at later time points, in a distinct model of GVHD, demonstrated fat transport in allogeneic cells at an intermediate level between unstimulated and syngeneic T cells (29). Thus, the timing of FAO in alloreactive T cells, as well as its absolute necessity, remains unresolved.

In addition to lipids and glucose, glutamine is another common metabolic substrate for T cells. From the beginning, glutamine uptake and metabolism have been shown to increase following T cell activation and glutamine is required for both Th1 and Th17 differentiation (71, 73). Glick et al. demonstrated that glutamine can act as an anaplerotic nutrient source in alloreactive T cells to replenish TCA cycle intermediates and provide substrates for the PPP (74). However, in contrast to the glutamine dependence seen in alloreactive cells, which would suggest worsening disease with glutamine supplementation, there is strong evidence that systemic glutamine administration facilitates therapeutic recovery following aHSCT. In a murine model of GVHD, systemic glutamine administration increased T_{reg} numbers and decreased serum levels of tumor necrosis factor α, limiting pro-inflammatory immune responses and improving recipient survival by 30% (75). In human patients, glutamine supplementation improved post-transplant survival with a trend toward decreased rates of GVHD (76). Thus, while there is much to be learned regarding glutamine metabolism in individual cell types, systemic glutamine administration appears to be protective.

METABOLISM IN REGULATORY T CELLS

T_{regs} provide crucial inhibitory signals to T_{eff} and are key to dampening immune responses and promoting tolerance (77). In GVHD, T_{regs} are of immense interest because of their potential ability to correct the balance between inflammation and immunosuppression. In fact, the frequency of T_{reg}, as marked by $CD4^+CD25^+$ expression, was lower in patients with chronic GVHD than in healthy controls or in patients post-transplant without chronic GVHD (78). Furthermore, enhancing T_{reg} frequency, either through ultra-low dose IL-2 administration, or adoptive transfer of *ex vivo* expanded T_{reg}, is an effective way to improve GVHD (79–81). Thus, increasing T_{reg} frequency could be an essential component for GVHD prevention and treatment and metabolic interventions could play a large role in this effort.

In addition to filling a unique immunologic niche, T_{regs} have a metabolic profile distinct from T_{eff} cells. T_{regs} generated *in vitro* increase their reliance on lipid and mitochondrial metabolism (24) while transgenic expression of the Glut1 receptor increases glycolysis, which impedes suppressive function. Opposing this signaling is the transcription factor Foxp3, which reprograms

T_{reg} metabolism toward OXPHOS and away from glycolysis (82, 83), driving up T_{reg} suppressive capacity. Foxp3 is thought to achieve these results by inhibiting Myc (82), a transcription factor that promotes both glycolysis and glutamine metabolism (43). Thus, changes in suppressive function are metabolically dependent, with a loss of OXPHOS decreasing suppressor activity (84). This tuning of T_{reg} suppressive capacity may be integral to the propagation and subsequent waning of an immune response. At times of high stimulation (e.g., infection) T_{reg} increase in number but are minimally suppressive, allowing effector responses to proceed unabated. As the infection subsides, inflammatory signals decrease and FoxP3 levels stabilize, leading to the adoption of OXPHOS, which decreases T_{reg} proliferation but increases suppressive function, limiting the effector response and restoring a state of tolerance (83).

Multiple studies have also demonstrated an integral link between mitochondrial metabolism and T_{reg} mediated suppression both *in vitro* and *in vivo* (84–86). Deletion of complex III specifically in T_{reg} led to development of a fatal early inflammatory disease (87) and transfer of Complex III deficient T_{reg} was unable to protect recipients in a model of T-cell driven colitis (85). T_{reg} also depend upon the mitochondrial transcription factor A (Tfam), which controls mitochondrial DNA copy number and is integral to ETC activity (88, 89). Loss of Tfam in T_{reg} decreased mitochondrial respiration, blunted expression of inhibitory markers ICOS and CTLA4, and resulted in a severe inflammatory disorder (86). Interestingly, although mTOR signaling is known to drive T cell glycolysis (which is expected to decrease T_{reg} function), mTOR is also required for proper T_{reg} function and development. Compared to conventional T cells, T_{reg} have higher mTORC1 activity (90, 91) and T_{reg}-specific deletion of Raptor, an obligate component of the mTORC1 complex, resulted in a fatal inflammation and loss of T_{reg} suppressor function (91). Similar results were found in mice lacking the mTOR protein, where Th2 responses increased significantly in the lungs and gastrointestinal tract of knock-out mice (86).

Despite our increased working knowledge of T_{reg} metabolism, little is known about T_{reg} metabolism in the alloreactive environment. Sirtuin-1 (Sirt1) is a class III histone deacetylase whose expression influences multiple metabolic pathways. Donor T cells lacking Sirt1 show increased FoxP3 stability in inducible T_{reg} (iT_{reg}) with a subsequent decreased conversion to pathogenic IFN-γ producing cells and a loss of follicular helper T cell development (92). In other studies, human iTregs propagated *in vitro* via pSTAT3 inhibition prevented xenogeneic GVHD yet spared donor antileukemia immunity. Metabolically, pSTAT3 inhibition shifted iTreg metabolism from OXPHOS to glycolysis, with a reduction in ETC activity. However, this metabolic impairment could be corrected by treating pSTAT3-inhibited T_{reg} with coenzyme Q10, which restored OXPHOS and augmented their suppressive potency (93). In other work, adoptive transfer of T_{reg} lacking vimentin, or pre-treated with the phosphokinase C inhibitor AEB071, improved GVHD survival, clinical scores, and weight loss to a greater degree than WT T_{reg}. Mechanistically, absence or inhibition of vimentin enhanced oxidative metabolism within the T_{reg} compartment and concomitantly increased their suppressive capacity (94). Finally, transplantation of adenosine producing $CD150^{+}$ T_{reg} into allogeneic animals decreased the severity of immune cell infiltration into the intestine (95). Thus, T_{reg} function is a finely tuned process, accomplished through integration of multiple inputs including mTOR signaling, intracellular energy sensing, metabolic pathways, and the influence of local environmental cues including danger signals. Furthermore, T_{reg}-associated metabolic changes found in other contexts appear to hold following aHSCT, in particular the association between increased OXPHOS and enhanced suppressive capacity.

EFFECTS OF METABOLIC INHIBITION ON GRAFT-VS.-TUMOR RESPONSES

A major indication for allogeneic transplantation is relapsed or refractory leukemia and lymphoma. aHSCT's benefit in this setting derives from donor T cells reactivity against foreign tumor cells, the so-called Graft-versus-tumor (GVT) effect. It is expected that anything that interrupts T cell alloreactivity, or impairs allogeneic T cell number, might disrupt the therapeutic efficacy of allogeneic transplantation. And yet metabolic manipulation does not appear to be universally detrimental to anti-cancer responses. Donor T cells that lack AMPK induce less severe GVHD but continue to demonstrate preserved or even enhanced cytotoxic potential post-transplant (70). Similarly, mice treated with recombinant Thioredoxin at the time of transplantation exhibit decreased GVHD severity while simultaneously preserving GVT effects (96). In some cases, preserved cytotoxicity results from a preservation in cytokine responses in remaining T cells (70), coupled with a decrease in T cell dysfunction due to lower rates of GVHD (97). In other cases, better tumor control may result from the dual impact of metabolic modulation on both alloreactive T cells and the underlying malignancy (98, 99). It has also been argued that T cell activation, and hence metabolic demands, operate on a continuum, with GVHD-causing T cells at the far end of the activation and metabolic spectrum (100). In this case, highly active T cells would be more susceptible to metabolic or similar perturbations than anti-tumor T cells with more modest energy requirements. Indeed, Treg transfer experiments support this concept of differential sensitivity, as exogenous Treg administration sufficiently controls alloreactive T cell expansion without compromising GVT activity (101).

METABOLIC INFLUENCE BEYOND T CELLS

It was been known for some time that T_{reg} induction in the gastrointestinal tract is influenced by production of short chain fatty acids (SCFAs), commonly produced by commensal bacteria, and primarily in the forms of butyrate and propionate. SCFAs induce Foxp3 expression (102, 103) by either inhibiting histone deacetylases or by activating G-protein receptor 43 (GPR43) (103). Butyrate levels were found to be excessively low in intestinal tissues following allogeneic transplantation

and administration of exogenous butyrate increased these levels back to normal while decreasing GVHD severity and improving weight loss and clinical scores (104). However, these beneficial effects were found to be independent of T_{reg} and instead resulted from direct salutary effects of butyrate on the intestinal epithelial cells (IECs), essentially improving the host response to injury. In a follow-up study, GPR43 expression was found to be necessary to realize the GVHD-protective effects of butyrate and because of this necessity GVHD severity increased in mice lacking GPR43 (105). Ultimately, GPR43 signaling increased inflammasome activation in IECs and enhanced IEC integrity and epithelial repair secondary to increases in local cytokine secretion including IL-18. Thus, metabolites and metabolic pathways beyond those utilized directly by T cells can have a profound effect on GVHD pathobiology, in part by influencing host cell responses.

TOWARD A UNIFYING THEORY OF ALLOREACTIVE T CELL METABOLISM

As highlighted thus far, alloreactive T cell metabolism is complex, with studies implicating a role for multiple and sometimes opposing metabolic pathways and substrates. While some contradictory findings might relate to minor differences in animal models, or the time point tested, an additional possibility is that the metabolic pathways being considered are not mutually exclusive and alloreactive T cells might upregulate multiple pathways at the same time. To this point, data from our lab demonstrates that CD8 T cells isolated from allogeneic recipients on day 7 post-transplant simultaneously increased both OXPHOS, as measured by oxygen consumption rates, and glycolysis, as measured by extracellular acidification (**Figure 1**). These data argue that OXPHOS and glycolysis are not mutually exclusive pathways within T cells and instead hint that alloreactive T cells might increase both aerobic glycolysis and OXPHOS simultaneously to meet their increased energy needs.

CURRENT CHALLENGES

Inherently, our understanding of T cell metabolism is limited by the availability of current methods—namely pharmacologic manipulation and genetic deletion. While both tools can determine the role of a particular target in a specific pathway or disease process, pharmacologic manipulation and genetic deletion both come with limitations. For pharmacologic activators and inhibitors, this limitation is often an unintended effect of the drug, whereas genetic knockouts are often compromised by timing of the deletion in relation to when the effects are being measured.

Looking more closely at pharmacologic manipulation, activators and inhibitors can work indirectly, have off-target effects, or mediate on-target effects in off-target cells/tissues. The use of metformin as an AMPK activator is a perfect example of this challenge (106–108). Because metformin inhibits complex I of the ETC, it activates AMPK *indirectly* by increasing the AMP:ATP ratio (109–111). Thus, it is often difficult to determine if metformin-related changes are due to AMPK activation or instead to metformin's direct effects on the ETC. Inhibitors can also cause off target effects. Many foundational studies used the CPT1a inhibitor etomoxir to study FAO (112, 113) and connect FAO with specific changes in T_{reg} and/or memory T cell (T_{mem}) populations (24, 25, 114). Recently, however, it was shown that the higher concentrations of etomoxir used in most studies not only inhibited long-chain FAO, but also broadly inhibited OXPHOS secondary to a decreased abundance of TCA cycle intermediates (115). Thus, given the potential off-target effects of etomoxir, interpretation of many seminal findings have come under increased scrutiny. Lastly, pharmacologic administration can cause unwanted effects in off-target tissues, resulting in toxic side effects and limiting the utility of the drug. For example, the glutamine antagonist, 6-Diazo-5-oxo-L-norleucine (DON) (116), promotes tolerance in combination with glycolytic inhibitors (117). However, its use has been limited because of severe on target, off-tissue toxicity, particularly to the gastrointestinal tract (118, 119). This challenge has led to the development of a DON pro-drug to more specifically deliver glutamine inhibition directly to tissues of interest (120).

Regarding genetic knockouts, while enhanced technologies can target a specific gene of interest within a given tissue type, timing of the deletion, particularly vis-à-vis measurement of effect, becomes extraordinarily important. In most cases of T cell specific-deletion, Cre recombinase is expressed under control of either the CD4 or lymphocyte protein kinase (Lck) promoter. In both cases, genetic deletion occurs during T cell development, leaving a long time for T cells to utilize alternative compensatory pathways, akin to taking the back-roads into work when the highway is unavailable. This point is important to keep in mind,

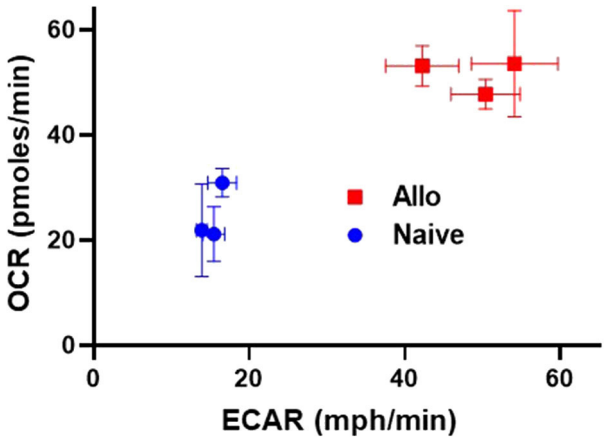

FIGURE 1 | Alloreactive $CD8^+$ T cells simultaneously increase both OXPHOS and glycolysis. 2×10^6 $CD45.1^+$ B6 T cells and 5×10^6 B6 bone marrow (BM) cells were transplanted into irradiated, allogeneic (B6 × DBA F1) recipients. On day 7 post-transplant, donor $CD8^+$ T cells ($CD8^+CD45.1^+TCR\text{-}\beta^+$) were flow-sorted and 2×10^5 cells placed into a Seahorse metabolic analyzer. Values for both oxygen consumption rate (OCR) and extracellular acidification of the media (ECAR), a proxy for glycolysis, were simultaneously increased in day 7 $CD8^+$ donor T cells.

as experiments using genetic knockouts might easily yield results confounded by the adoption of compensatory pathways.

In the example cited earlier, Raud et al. (115) used CD4 promoter driven Cre expression to delete CPT1a specifically in T cells, allowing them to conclude that T_{mem} and T_{reg} cells developed *in vitro* and *in vivo* in the absence of CPT1a (presumably lacking FAO). While these findings appear to contradict a necessity for FAO in T_{reg} and T_{mem} cells (24, 25, 114), alternative explanations exist. A distinct possibility is that an extended loss of CPT1a allowed T cells to become dependent on other metabolic pathways (121). In fact, Raud et al. (115) concede that their experiments did not limit medium or short-chain FAO, leaving open the possibility of this alternative pathway. In this one example, acute inhibition of FAO (as would be the intent with pharmacologic inhibitors) might give a very different outcome than prolonged absence of fat oxidation using genetic models, highlighting that timing of genetic deletion vis-à-vis measurement of effect must be strongly considered in every situation.

The potential for metabolic flexibility also influences our approach in treating T cell-driven pathogenesis. For example, treatment efficacy could improve by targeting multiple metabolic pathways simultaneously, in essence restricting T cells from upregulating compensatory pathways. Akin to retroviral therapy for human immunodeficiency virus (HIV) (122, 123), where a combination of drugs targets different viral components, targeting multiple metabolic pathways concurrently may be necessary to overcome the metabolic adaptations of pathogenic cells. In the context of solid organ transplantation, the combination DON (glutamine inhibition), 2-DG (glycolytic inhibition), and metformin (targeting OXPHOS) effectively promoted tolerance in fully mismatched skin and heart allograft models (117). Alternatively, inhibiting a mediator central to the metabolic reprogramming of multiple pathways might also be feasible. During GVHD, PD-L1 was shown to be central to reprogramming multiple T cell pathways and PD-L1 deficient T cells reduced glycolysis, OXPHOS, and FAO, improving GVHD outcomes in the process (124).

Finally, careful study of T cell metabolism as well as secondary/tertiary compensatory pathways will improve the timing and specificity of our inhibition. In this regard, aHSCT offers the distinct advantage of having a period in which donor cells are manipulated *ex vivo* prior to transplantation into recipients. Leveraging this advantage, metabolic inhibitors or activators could be applied exclusively to donor cells, sparing tissues from systemic administration of the compound. Furthermore, it may be possible to inhibit a first pathway through *ex vivo* manipulation (e.g., FAO), followed by subsequent inhibition of a compensatory pathway *in vivo* through systemic administration of a second inhibitor (e.g., glutamine metabolism). Cells lesioned in the first pathway would be expected to be more sensitive to secondary inhibition, while T cells arising *de novo* (and not having experienced the primary inhibition) would be spared. Ultimately, precise metabolic modulation and the potential for simultaneous inhibition of multiple metabolic pathways, will enhance the efficacy of metabolism-based therapies.

FUTURE DIRECTIONS

The breadth of research highlighted thus far lays a strong foundation for an increased understanding of T cell metabolism during aHSCT. However, outstanding questions remain regarding the heterogeneity of T cells recovered post-transplant and whether murine findings are translatable to humans. Like many complex disease processes, T cells collected and analyzed post-aHSCT represent a heterogenous population of cells including those driving the alloreactive response and bystanders simply responding to the inflammatory milieu. Studying metabolism in whole T cells during GVHD thus glosses over differences between individual T cell subsets and complicates the understanding of metabolism in the most highly alloreactive T cells. One approach to clarify this issue has been to use GVHD models in which donor T cells respond to a specific antigen. For example, transgenic CD8 T cells that recognize the SIINFEKL peptide of ovalbumin (i.e., OT-1 T cells) can be injected into CAG-OVA recipients which express ovalbumin as a self-antigen. Using this model, T cells transplanted into CAG-OVA recipients expressed increased levels of ROS and PD-1 compared to OT-1 T cells responding to immunization with CAG-OVA expressing dendritic cells (125). In a different study, OT-1 T cells isolated during GVHD increased fat transport while bystander (non-OVA reactive) T cells did not (54). However, the use of transgenic systems for the study of metabolism in alloreactive T cells has been limited.

Another approach is to use single-cell metabolomics to determine the individual metabolic profiles of donor T cells isolated from allogeneic recipients, a breakthrough that has the potential to revolutionize the study of immunometabolism during GVHD. While there are many groups working to develop single-cell metabolic technologies, the approach remains relatively new and faces numerous challenges, including the technical hurdle of how to collect cells without altering their metabolite abundance (126–129). To circumvent these issues, Miller et al. (130) developed a system that measures the activity of five key metabolic enzymes in conjunction with cell marker analysis to measure metabolic activity with single-cell resolution. Although this alternative approach is promising, there is no measure of global metabolic activity and the process is not high throughput. Thus, measuring metabolic activity at the single cell level, although highly promising for the study of GVHD, is currently limited by available technology.

Finally, the studies highlighted in this review have primarily utilized models using murine T cells and known strain combinations. While animal models have been integral to building foundational knowledge, their inherent limitations, including differences in murine and human immunology, pathogen free housing conditions, and genetic homogeneity can limit the translatability of the findings. Humanized murine models of xenogeneic GVHD (xGVHD) improve upon existing animal models by injecting human peripheral blood mononuclear cells (hPBMCs) into immunodeficient mice (131), allowing for the expansion and activation of human T cells *in vivo*. Increased use of xGVHD models will undoubtedly increase the likelihood that experimental findings will apply to human

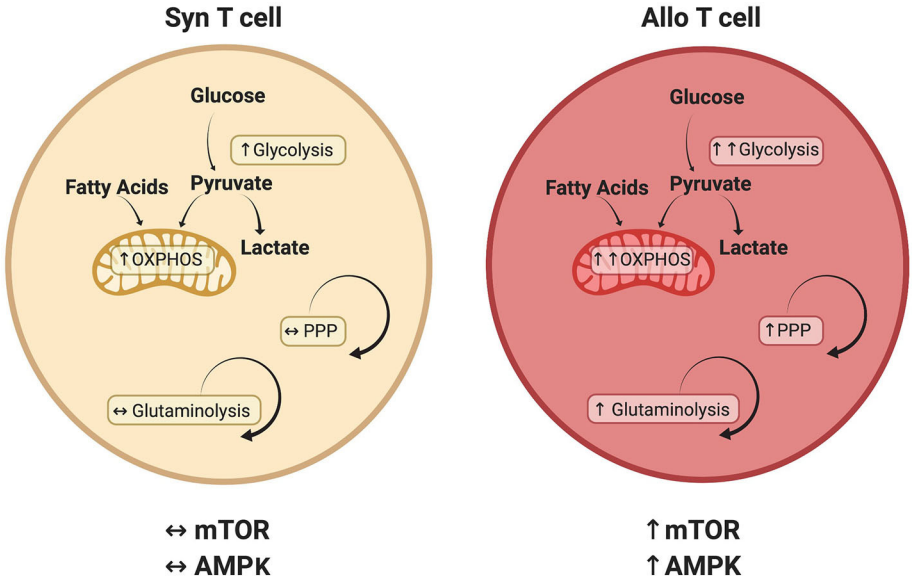

FIGURE 2 | Metabolic pathways that distinguish alloreactive and syngeneic T cells. T cells isolated from allogeneic recipients have a unique metabolic profile including a greater increase in both glycolysis and oxidative phosphorylation (OXPHOS) (29, 53, 124). While various carbon sources contribute to this increased oxidative metabolism, including glucose (29, 52) and fatty acids (54), the relative contribution of each substrate remains to be determined. Glutaminolysis and the PPP are also preferentially upregulated in alloreactive T cells, as is activation of the AMPK and mTOR pathways (29, 70, 74). Because T cell metabolism is a dynamic process, this figure represents known or suspected metabolic activity in donor T cells on day 7 post-transplant. The relative contribution of each pathway is likely to change over time as discussed in the text.

patients. However, xGVHD models themselves are imperfect and do not improve entirely upon deficiencies of classical animal models. Thus, patient samples may prove to be the best way to validate laboratory findings and ensure that observations are applicable to real world scenarios. In recent years, the repertoire of available technologies for *in vivo* human work has expanded, making the *in vivo* study of T cell metabolism during GVHD more feasible than ever. For example, recent publications have used administration of non-radioactive isotope tracers to study tissue metabolism in human patients in real time (132, 133). It could be envisioned that using non-radioactive isotope tracing in GVHD patients at the time of diagnosis could help to determine the dynamics of T cell metabolism during an active alloreactive immunologic response *in vivo*.

CONCLUSION

This review has investigated recent developments in our understanding of alloreactive T cell metabolism. While evidence suggests that many metabolic pathways are active in alloreactive T cells (**Figure 2**), including glycolysis, OXPHOS, FAO, and glutamine metabolism, there is no current consensus on the relative importance of each pathway or their temporal necessity. Furthermore, while studies have examined the significance of glycolysis and OXPHOS independent from each other, it is likely that glycolysis and OXPHOS increase simultaneously in alloreactive cells to meet enhanced energetic demands. In addition, the inherent ability of T cells to exhibit metabolic flexibility, adopting a compensatory metabolism when an initial pathway is lesioned (as occurs with genetic deletions), coupled with the indirect and somewhat pleiotropic nature of metabolic activators and inhibitors, makes assignment of causality difficult in many cases. Finally, T_{reg} exhibit a distinct metabolic profile linking oxidative metabolism to T cell suppressive function, a phenomenon that appears to hold during allogeneic transplantation, yet remains the focus of intense investigation. In the end, studying T cell metabolism in the context of GVHD will help to deepen our understanding of *in vivo* T cell biology and identify novel therapies for the treatment of T cell-mediated pathologies.

AUTHOR CONTRIBUTIONS

RB reviewed the literature, drafted the manuscript, and reviewed the final product. CB reviewed and interpreted the primary literature, edited the manuscript draft, and critically revised the final manuscript. All authors contributed to the article and approved the submitted version.

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Diverse Routes of Allograft Tolerance Disruption by Memory T Cells

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Memory T lymphocytes constitute a significant problem in tissue and organ transplantation due their contribution to early rejection and their relative resistance to tolerance-promoting therapies. Memory cells generated by environmental antigen exposure, as with T cells in general, harbor a high frequency of T cell receptors (TCR) spontaneously cross-reacting with allogeneic major histocompatibility complex (MHC) molecules. This phenomenon, known as 'heterologous' immunity, is thought to be a key barrier to transplant tolerance induction since such memory cells can potentially react directly with essentially any prospective allograft. In this review, we describe two additional concepts that expand this commonly held view of how memory cells contribute to transplant immunity and tolerance disruption. Firstly, autoimmunity is an additional response that can comprise an endogenously generated form of heterologous alloimmunity. However, unlike heterologous immunity generated as a byproduct of indiscriminate antigen sensitization, autoimmunity can generate T cells that have the unusual potential to interact with the graft either through the recognition of graft-bearing autoantigens or by their cross-reactive (heterologous) alloimmune specificity to MHC molecules. Moreover, we describe an additional pathway, independent of significant heterologous immunity, whereby immune memory to vaccine- or pathogen-induced antigens also may impair tolerance induction. This latter form of immune recognition indirectly disrupts tolerance by the licensing of naïve alloreactive T cells by vaccine/pathogen directed memory cells recognizing the same antigen-presenting cell *in vivo*. Thus, there appear to be recognition pathways beyond typical heterologous immunity through which memory T cells can directly or indirectly impact allograft immunity and tolerance.

Keywords: immune memory, autoimmunity, tolerance, transplantation, infection, vaccination

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INTRODUCTION

Memory T cells constitute a formidable obstacle both for preventing early graft rejection and for the eventual induction of allograft tolerance. For example, memory CD8 T cells can trigger early aggressive rejection of cardiac allograft rejection in mice (1). Importantly, memory T cells are also relatively resistant to tolerance-promoting therapies (2–4). An important property of memory cells thought to be especially relevant for impairing allograft survival is their strong 'heterologous' reactivity to allogeneic MHC molecules. The concept of heterologous immunity originated by the observation that humoral or cellular immunity to one pathogen could impart reactivity to a

secondary, unrelated pathogen (5). This property is found in memory T cells generated in response to one virus that that can cross-react with a new unrelated viral infection (6, 7). This term has been borrowed by the transplantation field to describe a specific feature of memory T cells that imparts a high degree of cross-reactivity to allogeneic MHC molecules (8, 9). This phenomenon is almost certainly due to the high intrinsic bias of the TCR repertoire for MHC recognition (10, 11). Thus, simply by chance, any polyclonal antigen-specific T cell response would be expected to contain a significant subpopulation of allogeneic MHC-reactive T cells. This concept is strikingly illustrated by the findings from Amir et al. showing that nearly half of CD4 and CD8 virus-specific human T cell clones cross-reacted to at least one allogeneic HLA allele (12). A high degree of cross-reactivity to alloantigens by pathogen-induced T cells has also been demonstrated in mouse infection models (13–15).

AUTOIMMUNITY AS AN ENDOGENOUS SOURCE OF HETEROLOGOUS ALLOGRAFT IMMUNITY

There has been interest in the transplant field for how alloimmunity may initiate nascent autoimmunity that can impact the graft. This has been especially evident in chronic allograft reactivity in lung transplantation (16, 17). However, the converse may also be true; pre-existing autoimmunity may be a source of potential alloimmunity in the form of heterologous immunity. We have had a long-standing interest in islet transplantation using the non-obese diabetic (NOD) model of spontaneous autoimmune Type 1 diabetes. NOD mice have a multi-factorial predisposition for developing diabetes due to T and B cell dependent islet beta cell-specific autoimmunity (18–20). Importantly, diseased NOD mice destroy syngeneic (NOD) pancreas (21) or isolated islet (22) transplants through a process of recurrent disease, a phenomenon that also occured in non-immunosuppressed Type 1 patients receiving a partial pancreas transplant from a non-diseased identical twin (23). Moreover, NOD mice also show a strong response to islet allografts (22, 24, 25). As such, the NOD mouse model is highly useful for studying islet transplant autoimmunity and alloimmunity, including potential heterologous immunity, in the setting of Type 1 diabetes.

Based on the discussion above, the autoimmune T cell repertoire, like any polyclonal T cell population, would be expected to have a high degree of cross-reactivity to allogeneic MHC molecules. Interestingly, a survey of established autoreactive (islet antigen-specific) T cell clones derived from NOD mice revealed that over one third cross-reacted to one or more of three allogeneic MHC haplotypes (26), a result conceptually similar to what had been found previously for human virus-specific T cell clones (12). Based on this concept, we interrogated the TCR specificity of T cells infiltrating MHC-unrelated islet allografts grafted into spontaneously diabetic NOD mice. Consistent with results from screening autoreactive T cell clones, TCRs from islet allografts were profoundly enriched with dual autoreactive/alloreactive specificities (26). Thus, autoimmunity can be a source *endogenously* generated heterologous immunity contributing to allograft rejection.

Heterologous Autoreactive T Cells With Alloreactivity: One or Two TCRs?

While the simultaneous reactivity of individual T cells for both self-MHC-restricted cognate antigens and allogeneic MHC molecules has been apparent for many years (27), it is not always clear whether this is due to a single TCR α/β pair or due to two separate TCRs on a given cell. There is ample reason to posit that autoreactive T cells demonstrating additional alloreactivity could be due to the contribution of two separate TCRs. A significant percentage (estimated to be roughly between 1-8%) of mature mouse (28–30) and human (31) peripheral T cells express two TCRs, presumably due to a substantial frequency of developing T cells expressing two functional TCR α chains (32). Moreover, dual TCR-expressing T cells indeed have a high frequency of an alloreactive second receptor (33, 34), and these can play an important role in triggering graft-versus-host disease in mice (33). It is conceivable, then, that autoreactive T cells demonstrating cross-reactive alloreactivity could be the result of two separate TCR specificities on the same cells.

Conversely, when studying a dual TCR-expressing T cell clone with both self-MHC-restricted peptide specify (OVA) *and* alloreactvity, Malissen et al. found that only one the two TCR α/β pairs imparted this dual reactivity (35). This demonstrates that a single TCR can possess combined nominal antigen plus cross-reactive allo-specificity. This concept was supported by studies involving high-throughput sequencing of a large repertoire of TCR transcripts from T cells targeting islet allografts in spontaneously diabetic NOD mice (26). Importantly, screening the antigen reactivity of highly expressed TCRs indicated that single TCR α/β pairs conferred simultaneous dual autoantigen/alloantigen (MHC) reactivity (26). Thus, the predominant heterologous immunity identified by this approach could be accounted for by single autoreactive TCRs with clear cross-reactivity to allogeneic MHC molecules. Of course, these finds do not preclude the potential of heterologous alloimmunity emerging from autoreactive T cells being the result of a second TCR. However, results to date suggest that the most frequent source of simultaneous autoreactive/alloreactive T cells in islet transplants in the setting of autoimmunity is the result of a single, cross-reactive TCR.

Conventional Antigen-Stimulated Versus Autoimmune Heterologous Immunity: A 'Trojan Horse' Model of Allograft Immunity

One potentially key difference between memory T cells generated by past antigen challenge and ongoing autoimmunity may simply be in the activation state of antigen-experienced T cells in these two scenarios. In fact, memory T cells may not be completely resistant to tolerance induction (2). For example, naïve mice can be tolerized to tissue and organ transplants despite bearing a degree of memory T cells generated by

environmental antigen exposure. If this is the case, then the impact of immune memory on allograft rejection and tolerance may be related in part to the pre-transplant burden of pre-existing alloreactive T cell memory (8, 36). However, the activation state of memory cells may also impact their potential to be tolerized. In most cases, one would expect memory cells from past antigen exposure to be in a more quiescent state of central memory (2). However, the autoimmune T cell pool may be experiencing persistent activation/re-activation in the host, including those cells expressing cross-reactive alloimmunity. This means that the alloimmune component found in autoimmune disease may already be in a heightened activation state and potentially more challenging to tolerize. While NOD mice have a variety of tolerance defects (37, 38) the presence of alloreactvity found within the smoldering autoreactive repertoire may contribute to the dramatic resistance of NOD mice to allograft tolerance, even toward tissues/organs for which they have no apparent autoimmunity (37). It will be most interesting to test this concept in future studies.

In addition, there is a second and more unusual property of heterologous (alloreactive) autoreactive T cells that may make them especially virulent as mediators of islet rejection. In the conventional view of heterologous immunity, antigen-experienced memory cells contribute to allograft immunity and tolerance resistance due to their chance cross-reactivity to the graft, unrelated to the specificity of the original stimulating antigen. However, in the case of autoimmunity, heterologous T cells have the potential to interact with graft through two qualitatively distinct recognition pathways simultaneously (**Figure 1**). One route of islet graft interaction can be through the recognition of self MHC-restricted islet autoantigens acquired from the transplant and processed and presented by host antigen-presenting cells (APCs). We previously found that monoclonal BDC2.5 TCR transgenic CD4 T cells without allogeneic cross-reactivity could nevertheless recognize allograft-derived autoantigens processed by host APCs and destroy islet allografts though this type of indirect autoantigen recognition (39). Thus, this autoreactive specificity alone was sufficient to trigger allograft rejection. However, since polyclonal autoreactive T cells targeting the islet graft also contain cross-reactive, alloreactive T cells (26), some of these cells can *also* directly recognize the native allogeneic MHC expressed by the graft. An example of this phenomenon is a CD4 TCR (9860-A3B3) isolated from an MHC mismatched C3H (H-2^k) islet graft in NOD mice. This TCR recognizes an islet-associated Chromogranin A peptide presented by the NOD MHC class II I-A^{g7} while *also* directly recognizing allogeneic I-A^k expressed by the donor (26). Thus, this unusual situation could represent a sort of 'Trojan Horse' phenomenon in the islet graft in which the influx of T cells responding to autoantigens also ferries in a cohort of heterologous alloreactive T cells that can directly engage the allograft MHC. This simultaneous graft recognition through either auto- or allo- specificities could account for the accelerated response to MHC unrelated islet allografts in NOD mice despite the lack of intentional prior alloantigen exposure in these mice (24, 25). This property of heterologous immunity within autoimmune T cells could potentially be a general dilemma in controlling allograft rejection in the setting of autoimmune disease.

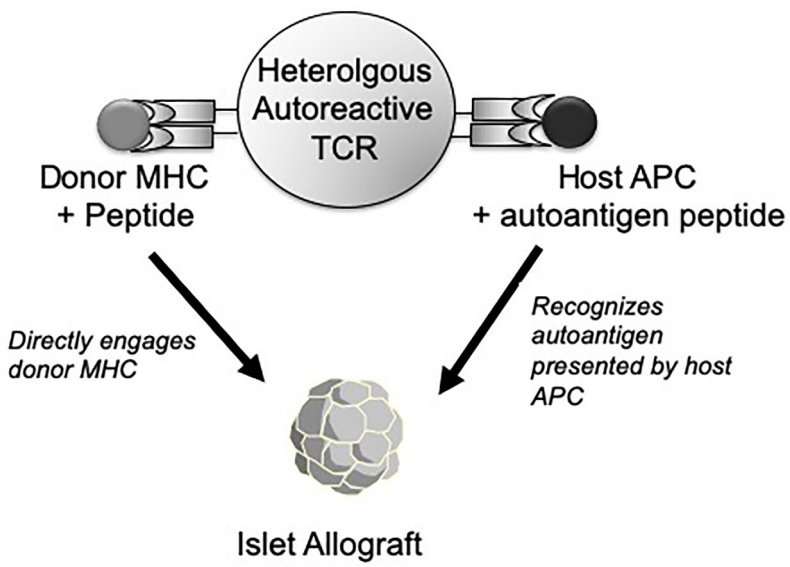

FIGURE 1 | Depiction of an autoreactive (islet-specific) T cell with a TCR with both autoantigen specificity and cross-reactivity to allogenic MHC molecules. In response to an islet allograft, this type of heterologous TCR can recognize a host MHC-restricted, graft-derived autoantigen peptide presented by host APC (right side). Alternatively, the same TCR may also directly recognize a native donor MHC molecule plus an unidentified peptide expressed by the graft (left side). As such, the same T cell has the potential to interact with the islet graft through either a host MHC-restricted (autoreactive) or donor MHC-restricted (alloreactive) recognition pathway.

AN ADDITIONAL AND LESS APPARENT ROUTE OF TOLERANCE BLOCKADE BY MEMORY T CELLS

A major ongoing goal in transplantation is to transition from chronic non-specific immunosuppression towards the induction of antigen (donor)-specific tolerance. To this point, this discussion has centered on heterologous immunity by memory T cells to allogeneic MHC molecules themselves (either from prior antigen exposure or *via* autoimmunity) as a key problem in transplant immunity and tolerance. As such, the importance of assessing pre-existing humoral or cellular immunity to donor MHC has been a major focus of screening efforts in transplantation (40–42). While such efforts are clearly warranted, there are potentially alternative routes whereby T cell memory could impair tolerance induction without a requirement for substantial heterologous immunity to the donor MHC (43). Unfortunately, the metagenome of both organ donors and recipients encode a variety of non-self antigens, such as those derived from microbiota (44, 45) or from latent infections such as CMV and EBV (46–49) that are clearly associated with impaired allograft outcomes in clinical transplantation. It is clear that the activation of anti-viral immunity can abrogate allograft tolerance (50, 51), possibly by the induction of inflammation that itself may non-specifically impair tolerance induction (52, 53).

One could assume that much of the impairment of tolerance induced by recipient responses to donor-associated pathogens is related to either heterologous immunity generated during the pathogen response and/or to the associated inflammation. We propose another more provocative form of host immunity to donor-derived non-MHC antigens that also could impair tolerance induction. This problem of donor-derived, non-self, non-MHC antigens has arguably been under-represented in most small animal studies. This being the case, we developed a model system in which the donor expressed a non-self transgenic antigen (OVA) to which the host was immune *via* vaccination (43), a scenario that could have relevance to clinical transplantation in which vaccination might protect from a donor-derived pathogen (54). Tolerance was induced using a common approach of administering a pre-transplant donor-specific transfusion (DST) in the form of donor spleen cells plus costimulation blockade (55, 56). Interestingly, host anti-OVA vaccination alone was innocuous, generating negligible anti-donor heterologous alloimmunity and had no impact on tolerance induction to wild-type allografts. Even peri-transplant re-activation of host anti-OVA reactivity did not impair tolerance induction. However, treatment with an OVA-expressing allogeneic DST in an OVA-immune recipient profoundly abrogated tolerance induction, even if the subsequent allograft did not express OVA (43).

TOLERANCE DISRUPTION OF NAÏVE T CELLS BY MEMORY T CELLS *VIA* LINKED ANTIGEN PRESENTATION

A key feature of this admittedly contrived system was that that the alloantigen and non-self (OVA) antigen had to be presented on the same APC in order to disrupt tolerance (**Figure 2**). That is, 'linked' recognition of the vaccine-directed antigen and the alloantigen was required for tolerance blockade (43). This scenario illustrates the potential for an alternative route whereby memory cells may impact the microenvironment during initial tolerance induction at the level of antigen presentation, not *via* donor MHC recognition, but rather through the recognition of another non-MHC antigen introduced by donor cells. Currently, probably the most recognizable concept involving T cells influencing one another *via* recognition of the same APC is that of 'linked suppression' in which putative regulatory T cells inhibits the function of another uncommitted T cell through interacting with the same APC (57). However, the concept of linked recognition leading to cell *activation* is actually considerably older. The original description of 'linked' antigen recognition referred to the observation of the carrier-hapten phenomenon in which the 'helper' determinant for antigen formation required physical linkage between the 'helper' determinant and the antibody specificity (58). This concept was later adapted to refer to the finding that helper T cells for the generation of cytotoxic T cells required recognition of the same APC *in vivo* (59). Three seminal studies later found that the basis of such CD4 T cell help for CD8 T cells was in the form of CD40:CD154 interactions with the APC resulting in the licensing of such APCs to activate other T cells (60–62). We had proposed that such T-T cell collaboration could be bi-directional in that CD4 and CD8 T cells could potentially influence one another through linked recognition of the APC (63). This latter concept could explain how memory CD8 T cells could disrupt T cell tolerance and promote allograft rejection instead (64). Of course, while this model of tolerance disruption required memory CD8 T cells (43), there is clear evidence that both CD4 (65–67) and CD8 (64, 65, 68) T cells can be involved in tolerance blockade in pre-clinical models. However, it is usually unclear how specific memory T cell subsets actually impair tolerance induction.

In what situation might this type of memory cell reactivity be important in transplantation? In the setting of autoimmunity or c donor pathogen infections such as CMV and EBV (46–49), the host could be immune to donor-derived, non-MHC antigens without obvious pre-transplant anti-donor MHC immunity. However, depending on the tissue distribution of autoantigens or donor pathogen-derived antigens, memory cells for these antigens could disrupt tolerance induction by diverting the naïve T cells recognizing the same APC from a tolerized fate to an effector phenotype (**Figure 2**). Because the existing host T cell memory to donor-derived, non-self antigens was self-MHC restricted in this linked recognition model (65), we would propose that the 'indirect' pathway of alloantigen recognition by host APCs was chiefly involved in disrupting tolerance. This could contrast sharply with how heterologous memory T cells (i.e., T cells with direct donor MHC reactivity) disrupt tolerance. Such donor MHC cross-reactive T cells may influence tolerance through the 'direct' pathway of antigen recognition. Future studies are needed to define the specific cellular interactions required by memory T cells to impair tolerance. Moreover, this

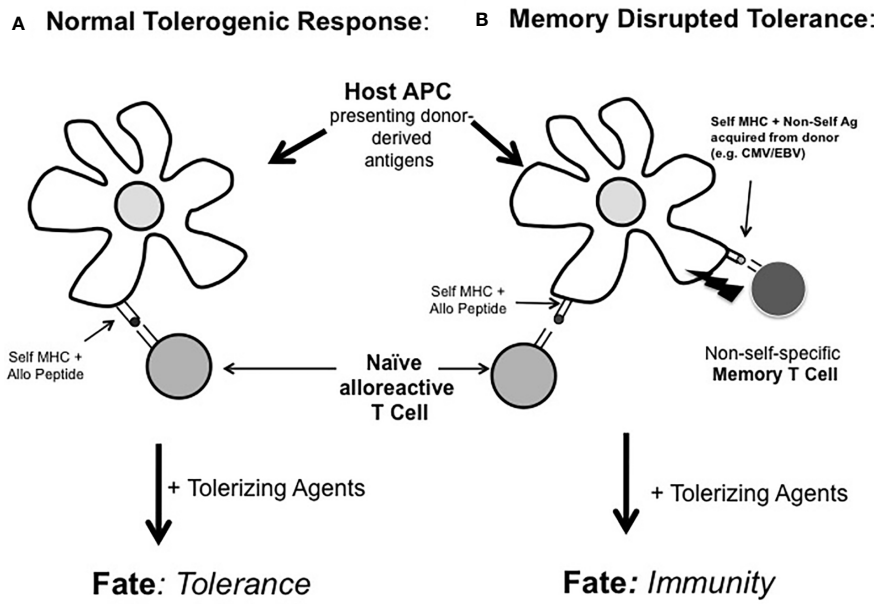

FIGURE 2 | Working model of tolerance blockade by linked recognition of alloantigens and non-MHC donor antigens. In **(A)** host naïve T cells responding to donor antigens acquired by host APCs are amendable to tolerance induction by tolerance-promoting agents. In **(B)**, if these same APCs also acquire other donor-derived antigens to which the host has pre-existing immunity (e.g., to non-self pathogen-derived antigens or autoantigens), tolerance is disrupted at the level of the APC. In this case, the fate of such uncommitted alloreactive T cells is diverted from tolerance to immunity.

tolerance blockade could occur without significant evidence of conventional heterologous anti-donor MHC reactivity. If this alternate and less apparent route of memory T cell tolerance blockade is significant, it implies that assessing pre-transplant anti-donor MHC reactivity alone may not be sufficient to predict the potential success of tolerance-promoting therapies. It may also be important to more carefully assess the presence of donor-derived pathogens and the corresponding host immunity to these antigens or to autoantigens.

CONCLUDING REMARKS

The role of memory T cells for providing resistance to allograft tolerance induction is well established. Moreover, the high degree of heterologous anti-donor MHC alloreactivity found within memory T cell populations is rightly considered a major potential source of tolerance disruption. By this view, the implied paradigm is that memory cells behave essentially as directly allo-sensitized cells that are resistant to regulation. However, there are other routes of memory cell specificity that expand and perhaps complicate this straightforward view (summarized in **Table 1**). For example, autoimmunity may constitute a form of ongoing memory T cell generation and heterologous alloreactivity that does not require exogenous antigen exposure. Also, heterologous autoreactive T cells have the unusual potential for recognizing autoantigen-expressing allografts through autoreactive and alloreactive specificities simultaneously. Alternatively, memory T cells can potentially subvert tolerance induction by recognizing donor-derived, non-MHC antigens (such as autoantigens or from pathogens) co-presented on APCs with conventional alloantigens resulting in the disruption of tolerance by naïve alloreactive T cells. Importantly, this latter form of antigen recognition could

TABLE 1 | Characteristics of differing pathways whereby memory T cells impair allograft tolerance.

Pathway of tolerance blockade	Potential source of memory-directed antigen	Direct specificity for donor MHC molecules	Reactivity with donor versus host APCs	Potential clinical scenario	Pre-clinical evidence	Clinical evidence
1. Conventional heterologous immunity	Environment/pathogens/ vaccination	Yes	Donor APCs and tissues	Recipient with common cellular immune memory	(8, 13–15, 69)	(12, 40, 67, 70–73)
2. Heterologous immunity from autoimmunity	Autoantigens	Yes	Donor *and* host APCs	Autoimmune recipient of organ transplant	(26)	Unknown
3. Linked recognition of donor-associated antigens	Pathogens or autoantigens	Not required	Host APCs	CMV+ or EBV+ organ transplanted into CMV+ EBV+ recipient	(43)	Unknown

impair tolerance even in the absence of significant anti-donor MHC reactivity. The relative significance of this latter route of tolerance blockade by memory T cells requires further clarification. Unfortunately, the clinical transplantation field currently relies on chronic non-specific immunosuppression to maintain graft survival and has not yet progressed to the point of using defined therapeutics to induce allograft tolerance in prospective trials. As such, it is challenging to determine the degree to which these or other potential pathways of tolerance impairment by immune memory pose significant barriers to achieving transplantation tolerance in humans.

AUTHOR CONTRIBUTIONS

AB and RG wrote and edited the review article. RG created the model figures and AB created **Table 1**. All authors contributed to the article and approved the submitted version.

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Targeting Interleukin-2-Inducible T-Cell Kinase (ITK) Differentiates GVL and GVHD in Allo-HSCT

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Allogeneic hematopoietic stem cell transplantation is a potentially curative procedure for many malignant diseases. Donor T cells prevent disease recurrence *via* graft-versus-leukemia (GVL) effect. Donor T cells also contribute to graft-versus-host disease (GVHD), a debilitating and potentially fatal complication. Novel treatment strategies are needed which allow preservation of GVL effects without causing GVHD. Using murine models, we show that targeting IL-2-inducible T cell kinase (ITK) in donor T cells reduces GVHD while preserving GVL effects. Both $CD8^+$ and $CD4^+$ donor T cells from $Itk^{-/-}$ mice produce less inflammatory cytokines and show decrease migration to GVHD target organs such as the liver and small intestine, while maintaining GVL efficacy against primary B-cell acute lymphoblastic leukemia (B-ALL). $Itk^{-/-}$ T cells exhibit reduced expression of IRF4 and decreased JAK/STAT signaling activity but upregulating expression of Eomesodermin (Eomes) and preserve cytotoxicity, necessary for GVL effect. Transcriptome analysis indicates that ITK signaling controls chemokine receptor expression during alloactivation, which in turn affects the ability of donor T cells to migrate to GVHD target organs. Our data suggest that inhibiting ITK could be a therapeutic strategy to reduce GVHD while preserving the beneficial GVL effects following allo-HSCT treatment.

Keywords: GVHD after blood transfusion, T cell, GvL, ITK deficiency, Eomesodermin (EOMES), JAK-STAT signalling pathway

HIGHLIGHTS

- ITK-deficient donor T cells exhibit minimal GVHD, but maintain GVL activity.
- ITK-deficient donor T cells exhibit significantly reduced production of inflammatory cytokines and migration to GVHD target organs.
- Eomes is required for the GVL effect.

INTRODUCTION

During allogeneic hematopoietic stem cell transplantation (allo-HSCT), alloreactive donor T cells are essential for the graft-versus leukemia effect (GVL) (1–3). The same donor T cells may also cause significant tissue damage to the host, known as graft-versus-host disease (GVHD) (4). Development of GVHD results in significant morbidity and mortality which complicates allo-HSCT, a potentially curative treatment for leukemia. Standard immunosuppressive therapy for GVHD is often therapeutically sub-optimal and predisposes patients to opportunistic infections such as Cytomegalovirus (CMV) and relapse of the underlying malignancy (5, 6). Thus, specific signaling pathways that can be targeted to allow the effects of GVL to persist while inhibiting GVHD need to be identified. The Tec family nonreceptor tyrosine kinase, Interleukin-2-inducible T cell kinase (ITK), regulates activation of T cells downstream of the T cell receptor (TCR). ITK is involved in the activation of intracellular calcium signaling and MAPK pathways, as well as polarization of the actin cytoskeleton, supporting an integral role for ITK in T cell activation and function (7, 8). ITK is involved in signaling which leads to cytokine production by T cell populations, and also negatively regulates the development of a distinct, innate-type cytokine-producing T cell population in the thymus (9), referred to as innate memory phenotype (IMP) T cells. These cells express significantly higher levels of CD122, CD44, and Eomes compared to T cells from WT mice. Since the activation, expansion, cytokine production, and migration of alloreactive donor T cells to target organs are hallmarks of GVHD (10, 11), and ITK is involved in these T cell activities, we examined the role of ITK in GVHD and GVL in an allo-HSCT model.

Previous studies have shown that Ibrutinib, an inhibitor of the related Tec kinase Bruton's tyrosine kinase (BTK) which can also inhibit ITK, is able to reduce chronic GVHD (12). Here we use a murine model of allo-HSCT involving allotransplant of T cells from C57Bl/6 (WT) mice or *Itk*$^{-/-}$ mice into BALB/c mice, to examine GVHD and GVL. We found that CD4^{+} and CD8^{+} T cells transplanted from ITK-signaling-deficient mice induce significantly less GVHD while retaining GVL function, compared to T cells from WT mice. We also found that this separation of GVHD from GVL was not dependent on the development of IMP T cells since T cells from IL-4 receptor-alpha and ITK-double knockout mice (*Itk/Il4ra* DKO), which lack the IMP phenotype (13), did not induce GVHD, Instead, the presence or absence of ITK separated GVHD from GVL in a cell-intrinsic manner. Furthermore, *Itk*$^{-/-}$ donor T cells exhibited cell-intrinsic reduction in proliferation, and both CD8^{+} and CD4^{+} T cells donor T cells from *Itk*$^{-/-}$ mice exhibit increased expression of perforin and significantly reduced expression of pro-inflammatory cytokines. Both CD4^{+} and CD8^{+} T cells from *Itk*$^{-/-}$ mice upregulate the key transcription factor Eomes, which we found is critical for both GVHD and GVL, since *Itk*$^{-/-}$ Eomes$^{flox/flox}$ CD4cre^{+} T cell donors (deficient in both Eomes and ITK) did not mount a cytotoxic response against primary leukemia cells or clear tumor cells, both *in vitro* and *in vivo*. Our data further demonstrate that ITK deficiency affects JAK1/2 (14) and IRF-4 (15) signaling, and CD4^{+} and CD8^{+} T cells from ITK-deficient mice show defects in T cell migration into GVHD target tissues, caused by reduced expression of chemokine receptors. This leads to decreased tissue damage during allo-HSCT. *Itk*$^{-/-}$ T cells can successfully clear leukemia cells in circulation, however they are unable to clear subcutaneously growing leukemic cells due to this migration defect. Finally, RNA sequencing data revealed that ITK deficiency impacts genes involved in cytokine production, cell adhesion, and chemokine and cytokine receptor expression. These genes are involved in the pathogenesis of GVHD. Our studies identify a specific and novel potential therapeutic target and its downstream mechanism for separating GVHD and GVL after allo-HSCT. Targeting ITK may also prove beneficial for other T cell-mediated diseases.

MATERIALS AND METHODS

Mice

Itk$^{-/-}$ mice were described previously (16). C57BL/6, C57BL/6.SJL (B6-SJL), ROSA26-pCAGGs-LSL-Luciferase, Thy1.1 (B6.PL-Thy1a/CyJ), CD45.1 (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ), and BALB/c mice were purchased from Charles River or Jackson Laboratory. Eomes$^{flox/flox}$ mice, B6.129S1mice, and CD4cre mice were purchased from Jackson Laboratory. Mice expressing Cre driven by the CMV promoter (CMV-Cre) were purchased from the Jackson Laboratory and crossed to ROSA26-pCAGGs-LSL-Luciferase mice (B6-luc). B6-luc mice were bred with *Itk*$^{-/-}$ mice to create *Itk*$^{-/-}$ luc mice. *Itk*$^{-/-}$/*Il4ra*$^{-/-}$ double knockout mice have been described (13). Mice aged 8–12 weeks were used, and all experiments were performed with age and sex-matched mice. Animal maintenance and experimentation were performed in accordance with the rules and guidance set by the institutional animal care and use committees at SUNY Upstate Medical University and Cornell University.

Reagents, Cell Lines, Flow Cytometry

Monoclonal antibodies were purchased from eBiosciences (San Diego, CA) or BD Biosciences (San Diego, CA). Antibodies used included anti-CD3, anti-CD28, anti-CD3-FITC, Anti-CD3-BV605, anti-CD8-FITC, anti-BrdU-APC, anti-IFNγ-APC, anti-TNFα-PE, anti-CD45.1-PerCPCy5.5, anti-CD122-APC, anti-CD44-Pacific blue, anti-Eomes-PE-Cy7, anti-CD25-BV421, anti-FoxP3-APC, anti-T-bet-BV421, anti-CD4-BV785, anti-CD45.1-Pacific Blue, and anti-H-2K^{d}-Pacific Blue. We performed multiplex ELISAs using Biolegend LEGENDplex kits, and some kits were custom ordered to detect both mouse and human cytokines. Luciferin was purchased from Perkin Elmer (Waltham, MA) and Gold Bio (St Louis MO). Dead cells were excluded from analysis with LIVE/DEAD Fixable Aqua Dead Cell staining. Flow cytometry was performed using a BD LSR-II or BD LSRFortessa cytometer (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

For cell sorting, T cells were purified with either anti-CD8 or anti-CD4 magnetic beads using MACS columns (Miltenyi

Biotec, Auburn, CA) prior to cell surface staining. FACS sorting was performed with a FACS Aria III cell sorter (BD Biosciences). FACS-sorted populations were typically of >95% purity. Antibodies against IRF4, STAT3, JAK2, JAK1, GAPDH, and β-Actin (for total and/or phospho proteins) were purchased from Cell Signaling Technology (Danvers, MA). All cell culture reagents and chemicals were purchased from Invitrogen (Grand Island, NY) and Sigma-Aldrich (St. Louis, MO), unless otherwise specified. The A20 cell lines (American Type Culture Collection; Manassas, VA), and primary mouse B-ALL blast cells (17) were transduced with luciferase, and cultured as described previously (18).

Allo-HSCT and GVL Studies

Lethally irradiated BALB/c mice (800 cGy) were injected intravenously with 10×10^6 T cell-depleted bone marrow ($_{TCD}$BM) cells with or without 1×10^6 FACS-sorted $CD8^+$ T cells, 1×10^6 $CD4^+$ T cells, or CD8/CD4 cells mixed at a 1:1 ratio. FACS-sorted total $CD8^+$, total $CD4^+$, or mixed donor $CD4^+$ and $CD8^+$ T cells from WT (C57Bl/6) or *Itk*$^{-/-}$ mice were used. For GVL experiments, B-cell acute lymphoblastic leukemia (B-ALL) primary blasts (17) transduced with luciferase were cultured as described previously (18), and 2×10^5 luciferase-expressing B-ALL blasts were used. Mice were evaluated twice a week from the time of leukemia cell injection for 65 days by bioluminescence imaging using the IVIS 50 Imaging System (Xenogen) as previously described (19). Clinical presentation of the mice was assessed 2–3 times per week by a scoring system that sums changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (20). Mice were euthanized when they lost ≥30% of their initial body weight or became moribund.

For chimera experiments, bone marrow cells from *Itk*$^{-/-}$ ($CD45.1^+$) or C57Bl/6 ($CD45.2^+$) mice were mixed at different ratios—1:1 (WT:*Itk*$^{-/-}$), 1:2, 1:3, or 1:4—and transplanted into lethally irradiated Thy1.1 mice. In some experiments, we used *Itk*$^{-/-}$ on a CD45.2 background and WT on a CD45.1 background as indicated in the figure legends. Mice were bled from the tail vein after 9 weeks to determine the presence of *Itk*$^{-/-}$ and WT cells. For GVHD assessment experiments, *Itk*$^{-/-}$ ($CD45.1^+$) and WT (CD45.2) T cells were FACS-sorted from Thy1.1 hosts and then transplanted to irradiated BALB/c mice carrying leukemia cells, along with $_{TCD}$BM as described above. This was followed by analysis of GVHD and GVL. In some experiments FACS-sorted $CD8^+$ T cells from WT or *Itk*$^{-/-}$ mice were mixed at a 1:1 ratio and injected into BALB/c mice (2×10^6 $CD8^+$ T cells total).

Tissues Imaging

Allo-HSCT was performed with 10×10^6 WT T cell-depleted BM cells and 1×10^6 FACS-sorted $CD8^+$ or 1×10^6 FACS-sorted $CD4^+$ T cells (from B6-luc or *Itk*$^{-/-}$*luc* mice) and bioluminescence imaging of tissues was performed as previously described (21). Briefly, 5 min after injection with luciferin (10 μg/g body weight), selected tissues were prepared and imaged for 5 min. Imaging data were analyzed and quantified with Living Image Software (Xenogen) and Igor Pro (Wave Metrics, Lake Oswego, OR).

Cytokine Production, Cytotoxicity, and BrdU Incorporation Assays

On Day 7 post-transplantation, serum and single cell suspensions of spleens were obtained. Serum IL-33, IL-1α, IFN-γ, TNF-α and IL-17A content was determined by multiplex cytokine assays (Biolegend LEGENDplex). T cells were stimulated with anti-CD3/anti-CD28 for 4–6 h in the presence of brefeldin A (10 μM) and stained intracellularly for cytokines (IFN-γ and TNF-α). Control cells were stimulated with PMA and ionomycin in the presence of brefeldin A.

Proliferation Assays

For detection of BrdU, mice were given BrdU with an initial bolus of BrdU (2 mg per 200 μl intraperitoneally) and drinking water containing BrdU (1 mg/ml) for 2 days. BrDU incorporation was performed using BrDU kit (Invitrogen) according to the manufacturer's instructions.

Cytotoxicity Assays

For cytotoxicity assays, luciferase-expressing A20 cells were seeded in 96-well flat bottom plates at a concentration of $3x10^5$ cells/ml. D-firefly luciferin potassium salt (75 μg/ml; Caliper Hopkinton, MA) was added to each well and bioluminescence was measured with the IVIS 50 Imaging System. Subsequently, ex vivo effector cells (MACS-sorted or FACS-sorted $CD8^+$ T cells from bone marrow-transplanted mice) were added at 40:1, 10:1, and 5:1 effector-to-target (E:T) ratios and incubated at 37°C for 4 h. Bioluminescence in relative luciferase units (RLU) was then measured for 1 min. Cells treated with 1% Nonidet P-40 was used as a measure of maximal killing. Target cells incubated without effector cells were used to measure spontaneous death. Triplicate wells were averaged and percent lysis was calculated from the data using the following equation: % specific lysis = 100 × (spontaneous death RLU–test RLU)/(spontaneous death RLU – maximal killing RLU) (22).

Migration Assays

Lethally irradiated BALB/c mice were injected intravenously with 10×10^6 WT $_{TCD}$BM cells from B6.PL-*Thy1*^{a}/CyJ mice, along with FACS-sorted $CD8^+$ or $CD4^+$ T cells from B6.SJL and *Itk*$^{-/-}$ mice, mixed at a 1:1 (WT:*Itk*$^{-/-}$) ratio. Seven days post-transplantation, the mice were sacrificed and lymphocytes from the liver, small intestine, spleen, and skin-draining lymph nodes were isolated. Livers were perfused with PBS, dissociated, and filtered with a 70 μm filter. The small intestines were washed in media, shaken in strip buffer at 37°C for 30 min to remove the epithelial cells, and then washed, before digesting with collagenase D (100 mg/ml) and DNase (1 mg/ml) for 30 min in 37°C, and followed by filtering with a 70 μm filter. Lymphocytes from the liver and intestines were further enriched using a 40% Percoll gradient. The cells were analyzed for $H2K^b$, $CD45.1^+$ and $CD45.2^+$ $CD8^+$ T cells by flow cytometry, but we excluded any bone marrow-derived T cells ($Thy1.1^+$).

RNA Sequencing

T cells from WT C57Bl/6 or *Itk*$^{-/-}$ mice were MACS purified and FACS sorted, and 2×10^6 FACS sorted $CD8^+$ T cells were

transplanted into BALB/c mice, along with $_{TCD}$BM as described above. Seven days post transplantation, donor cells were purified from spleen. Samples were submitted to SUNY Upstate Medical University Sequencing core facility for RNA sequencing. We were unable to sort enough donor T cells from small intestine of the recipient mice that received *Itk*$^{-/-}$ T cells. Therefore, we generated RNA sequencing data from five groups: WT-Pre and *Itk*$^{-/-}$Pre cells prior to transplantation; WT-Spleen, and *Itk*$^{-/-}$ Spleen using cells isolated from 7 days post-transplantation. Copy numbers were further analyzed in Gene Spring for normalization, quality control, correlation, principal component analysis, and gene differential expression. The sequencing data is deposited in (https://www.ncbi.nlm.nih.gov/geo/).

Western Blotting

Cells were lysed in freshly prepared lysis buffer using RIPA buffer (Fisher Scientific) and Complete Protease Inhibitor Cocktail (Fisher Scientific) and centrifuged for 10 min at 4°C. Aliquots containing 70 µg of protein were separated on a 12–18% denaturing polyacrylamide gel and transferred to nitrocellulose membranes for immunoblot analysis using specific Abs.

qPCR Assay

To confirm the differences observed in RNA sequencing, pre- and post-transplanted donor T cells were FACS sorted from recipient mice on H2K^{b} markers, and total RNA was isolated from T cells using the RNeasy kit from Qiagen (Germantown, MD). cDNA was made from total RNA using a cDNA synthesis kit (Invitrogen). qRT-PCR assay was performed with a premade customized plate (Fisher Scientific, Hampton, NH).

Human Patient Samples

We also isolated plasma from GVHD patients and healthy donors and performed cytokine ELISAs on these plasma samples using multiplex ELISA kits (Biolegend, San Diego, CA). This work was done under approved IRB protocol 1522145-2.

Statistics

All numerical data are reported as means with standard deviation. Data were analyzed for significance with GraphPad Prism. Differences were determined using one-way or two-way ANOVA and Tukey's multiple comparisons tests, or with a student's t-test when necessary. P-values less than or equal to 0.05 are considered significant. All transplant experiments are done with N=5 mice per group, and repeated at least twice, according to power analyses. Mice are sex-matched, and age-matched as closely as possible.

RESULTS

Ablation of ITK Retains GVL effect but Avoids GVHD During Allo-HSCT

To determine whether TCR-mediated activation of ITK impacts GVHD pathogenesis after allo-HSCT, we examined the effects of ITK signaling on donor CD4^{+} and CD8^{+} T cells in an allo-transplant model, using C57Bl/6 mice (MHC haplotype b) as donors and BALB/c mice (MHC haplotype d) as recipients. To induce GVHD, we used MHC-mismatched donors and recipients, $_{TCD}$BM from B6.PL-*Thy1*^{a}/CyJ (Thy1.1) mice, and T cells from C57BL/6 (B6) WT or *Itk*$^{-/-}$ mice. Lethally irradiated BALB/c mice were injected intravenously with 10×10^{6} wild-type (WT) $_{TCD}$BM cells along with 2×10^{6} FACS-sorted donor T cells (1×10^{6} CD8^{+} and 1×10^{6} CD4^{+}), followed by intravenous challenge with 2×10^{5} luciferase-expressing B-ALL-*luc* blast cells as described (16). Recipient BALB/c mice were monitored for cancer cell growth using IVIS bioluminescence imaging for over 60 days >>(**Figure 1A**). While leukemia cell growth was observed in T cell-depleted BM-transplanted mice without T cells, leukemia cell growth was not seen in mice transplanted with T cells from either WT or *Itk*$^{-/-}$ mice. As expected, mice transplanted with WT T cells cleared the leukemia cells but suffered from GVHD. In contrast, mice transplanted with *Itk*$^{-/-}$ T cells cleared the leukemia cells and displayed minimal signs of GVHD. Most animals transplanted with *Itk*$^{-/-}$ T cells survived for more than 65 days post-allo-HSCT (**Figure 1B**), with significantly better survival and reduced clinical scores compared to those transplanted with WT T cells [scored based on weight, posture, activity, fur texture, and skin integrity as previously described (19) (**Figures 1C, D**)]. BALB/c mice transplanted with *Itk*$^{-/-}$ T cells showed only residual tumor cell growth (as measured by bioluminescence), showing that the donor cells maintained GVT functions similar to WT T cells (**Figure 1E**). Donor CD8^{+} T cells are more potent than CD4^{+} T cells in mediating GVL effects, but both CD4^{+} and CD8^{+} T cells mediate severe GVHD in mice and humans (23–25). To determine whether CD4^{+} T cell-intrinsic ITK signaling might be sufficient to induce GVHD, we repeated the same experiments using purified CD4^{+} T cells from either WT or *Itk*$^{-/-}$ mice in the MHC-mismatch mouse model of allo-HSCT (B6→BALB/c) (**Supplementary Figures 1A–C**). Recipients of WT CD4^{+} T cells exhibited worse survival compared to mice receiving $_{TCD}$BM cells alone (**Supplementary Figure 1A**). In contrast, recipients of $_{TCD}$BM mixed with *Itk*$^{-/-}$ CD4^{+} T cells had greatly reduced mortality and clinical scores (**Supplementary Figure 1B**), indicating that CD4^{+} T cell-intrinsic ITK signaling can contribute to the severity of GVHD. Our results indicate that ITK signaling is dispensable for anti-leukemia immunity, but required for GVHD.

T Cells Innate Memory Phenotype Is Not Sufficient for GVHD Effects, and the Regulatory Function of ITK in GVHD Is T Cell-Intrinsic

The innate memory phenotype (IMP: CD44^{hi}CD122^{hi} and Eomes^{hi}) (26) of *Itk*$^{-/-}$ CD8^{+} T cells arises in the thymus during development, as opposed to memory CD8^{+} T cells that are also CD44^{hi}, but largely arise in the periphery of WT mice in response to foreign antigens or due to homeostatic proliferation (27). We examined pre-transplanted CD8^{+} T cells for CD44^{hi}CD122^{hi} and Eomes^{hi} expression, and observed that

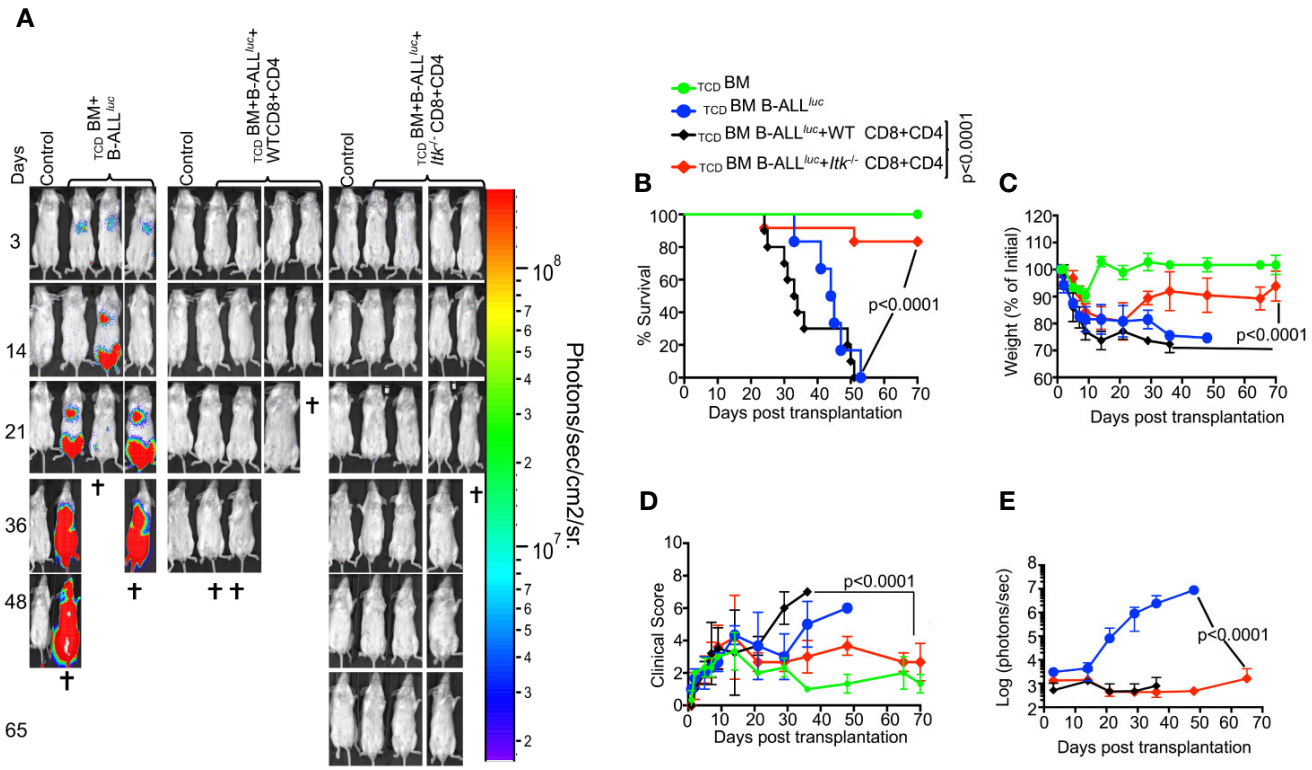

FIGURE 1 | Absence of ITK avoids GVHD while retaining GVL effects during allo-HSCT. 1 × 10^6 purified CD4+ and 1 × 10^6 CD8+ T cells (WT or *Itk*$^{-/-}$) were mixed at a 1:1 ratio, and transplanted along with 2 × 10^5 B-ALL-*luc* cells into irradiated BALB/c mice. Host BALB/c mice were imaged using the IVIS imaging system 3 times a week. Group 1 received 10 × 10^6 T cell depleted bone marrow only (labeled as $_{TCD}$BM). Group 2 received 10 × 10^6 $_{TCD}$BM along with 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM+B-ALL ^{luc}+), Group 3 was transplanted with 1 × 10^6 purified WT CD8$^+$ and 1 × 10^6 CD4$^+$ T cells (1:1 ratio) along with 2 × 10^5 B-ALL-*luc*+ cells 10 × 10^6 ($_{TCD}$BM+B-ALL ^{luc}+ WT CD8+CD4). Group 4 received 1 × 10^6 purified *Itk*$^{-/-}$ CD8$^+$ and 1 × 10^6 CD4$^+$ T cells (1:1 ratio) along with 2 × 10^5 B-ALL-*luc*+ cells 10 × 10^6 ($_{TCD}$BM+B-ALL^{luc}+ *Itk*$^{-/-}$ CD8+CD4). **(A)** Recipient BALB/c mice were imaged using IVIS 3 times a week. **(B)** The mice were monitored for survival, **(C)** changes in body weight, and **(D)** clinical score for 65 days post BMT. **(E)** Quantified luciferase bioluminescence of leukemia cell growth. Statistical analysis for survival and clinical score was performed using log-rank test and two-way ANOVA, respectively. For weight changes and clinical score, one representative of 2 independent experiments is shown (n = 3 mice/group for BM alone; n = 5 experimental mice/group for all three groups. Survival is a combination of 2 experiments. P values presented with each group. Two-way ANOVA and students t-test were used for statistical analysis. *Note: Controls are naïve mice used as negative control for bioluminescence (BLI).*

Itk$^{-/-}$ T cells expressed higher levels of CD44^{hi}CD122^{hi} and Eomes^{hi} compared to CD8$^+$ T cells from WT mice (**Figure 2A**). We sought to understand whether the emergence of IMP T cells is sufficient to separate GVHD from GVL. To test this, we generated WT IMP T cells using a mixed-bone marrow approach in which T cell-depleted BM from WT and *Itk*$^{-/-}$ mice were mixed at a 3:1 (WT: *Itk*$^{-/-}$) ratio (26). The irradiated congenic (B6) Thy1.1 hosts were reconstituted with this mixture of $_{TCD}$BM CD45.2$^+$ WT and CD45.1$^+$*Itk*$^{-/-}$ BM cells, along with a control group receiving mixed CD45.2$^+$ WT and CD45.1$^+$ WT BM cells (**Figure 2B**). WT BM-derived CD8$^+$ thymocytes that develop in such mixed BM chimera acquire an IMP phenotype due to their development in the same thymus as the *Itk*$^{-/-}$ T cells (26), which we also observed in our experiments (**Figure 2B**). Ten weeks after reconstitution of the T cell compartment, T cells derived from WT (CD45.2$^+$Thy1.1$^-$) and *Itk*$^{-/-}$ (CD45.1$^+$) donor cells were sorted from the bone marrow chimeras. These sorted T cells were transplanted into irradiated BALB/c mice along with $_{TCD}$BM in the allo-HSCT model as described above, and tested for their function in GVHD and GVL. Analysis of the BALB/c recipients of these different IMP CD8$^+$ T cells indicates that WT IMP cells were not able to separate GVL and GVHD (**Figures 2C–G**). Thus, the appearance of IMP is not sufficient to separate GVHD from GVL.

As previously discussed, *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ T cells exhibit attenuated TCR signaling and an IMP (26), as indicated by expression of high levels of CD44, CD122, and Eomes, specifically by CD8$^+$ T cells (**Figures 3A, B**). To examine whether these IMP T cells from *Itk*$^{-/-}$ mice mount GVL responses, we utilized the MHC-mismatch mouse model of allo-HSCT (WT, *Itk*$^{-/-}$→BALB/c, i.e., H2K^{b+}→H2K^{d+}). We then sorted H2K^{b+}donor T cells back from recipient mice and determined their cytotoxicity against B-ALL-*luc* cells. We found that these donor cells effectively killed primary leukemia cells *in vitro*, even in the absence of ITK (**Figure 3C**). Moreover, we observed significantly increased expression of perforin in CD8$^+$

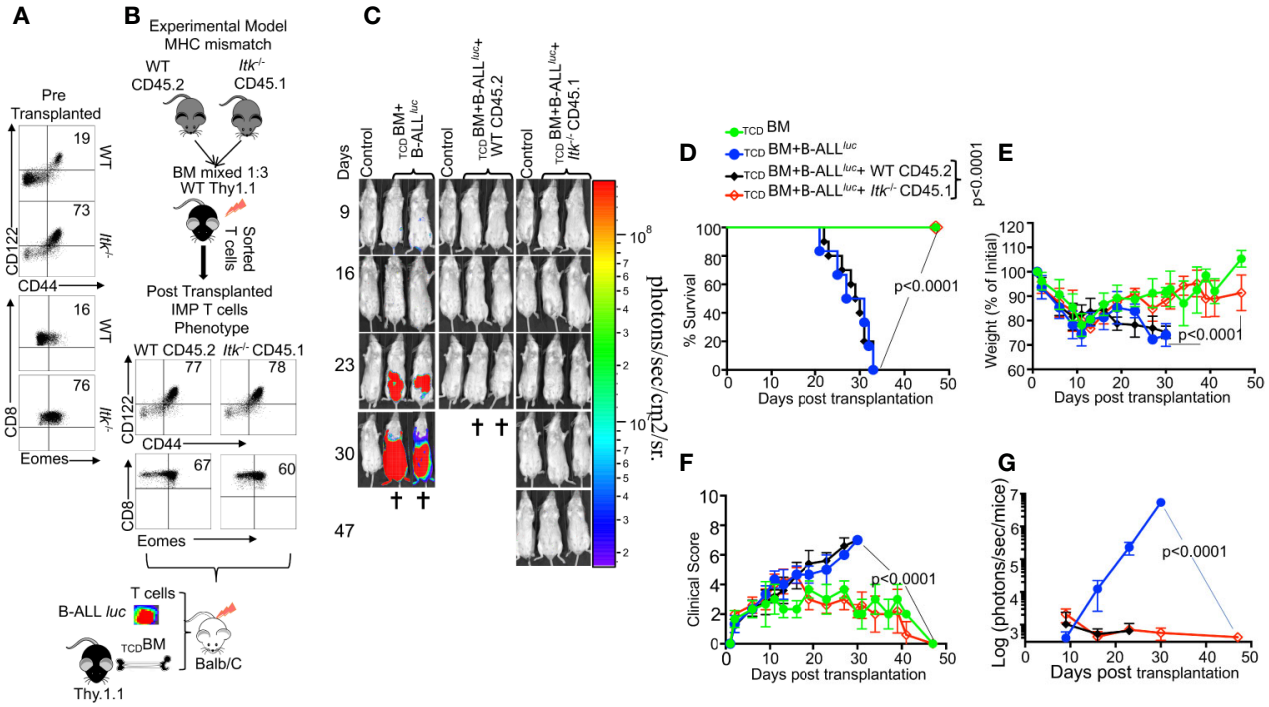

FIGURE 2 | The regulatory function of ITK in GVHD is T cell-intrinsic. **(A)** Purified WT and *Itk*$^{-/-}$ CD8^{+} T cells were examined for expression of CD44, CD122, and Eomes prior to transplantation. **(B)** Whole bone marrow cells isolated from C57Bl/6 WT (CD45.2) and *Itk*$^{-/-}$ (CD45.1) mice were mixed in 1:3 ration WT: *Itk* $^{-/-}$, and transplanted into irradiated Thy1.1 C57Bl/6 mice. 9-10 weeks later CD8^{+} T cells were sorted by CD45.2 and CD45.1 expression (donor T cells) and exclusion of Thy1.1 positive (host T cells). Isolated sorted T cells were examined for expression of CD44, CD122, and Eomes and transplanted into irradiated BALB/c mice. This experiment was repeated more than three times. **(C)** Irradiated BALB/c mice were divided in four different groups and transplanted with the sorted T cells described in **(B)** as follows: Group one was transplanted with 10 × 10^{6} $_{TCD}$BM alone ($_{TCD}$BM). Group two was transplanted with 10 × 10^{6} $_{TCD}$BM and 2 × 10^{5} B-ALL-*luc*, ($_{TCD}$BM+B-ALL^{luc}). Group three was transplanted with 10 × 10^{6} $_{TCD}$BM along with 1 × 10^{6} purified WT CD8^{+} T cells and 2 × 10^{5} B-ALL-*luc* ($_{TCD}$BM+B-ALL^{luc}+WT CD45.2). The fourth group was transplanted 10 × 10^{6} $_{TCD}$BM along with and 1 × 10^{6} purified *Itk*$^{-/-}$ CD8^{+} T cells and 2 × 10^{5} B-ALL-*luc* ($_{TCD}$BM+B-ALL^{luc}+*Itk*$^{-/-}$ CD45.1). These mice were monitored for leukemia cell growth using the IVIS system. **(D)** The mice were monitored for survival, **(E)** changes in body weight, and **(F)** clinical score for 47 days post BMT. For body weight changes and clinical score, one representative of 2 independent experiments is shown (n = 3 mice/group for BM alone; n = 5 experimental mice/group for all three groups). **(G)** Quantified luciferase bioluminescence of luciferase expressing B-ALL-*luc* cells. Statistical analysis for survival and clinical score was performed using log-rank test and two-way ANOVA, respectively. One representative experiment out of 2. Survival is a combination of 2 experiments, 3 mice per group of control $_{TCD}$BM, and 5 mice per group for all of the experimental groups. P value presented with each figure. *Note: Controls are naïve mice used as negative control for BLI.*

T cells from *Itk*$^{-/-}$ mice compared to T cells from WT mice, in the absence of activation (**Figure 3D**). Our findings demonstrate that CD8^{+} T cells from *Itk*$^{-/-}$ mice have enhanced activation, and exert cytotoxicity against primary leukemia cells.

IL-4 is known to upregulate Eomes in CD8^{+} T cells (26, 28), which we verified by comparing T cells from WT and *Itk/Il4ra* double KO (DKO) mice. Removing IL-4 signaling from the *Itk*$^{-/-}$ mice led to decreased expression of Eomes in *Itk*$^{-/-}$ T cells compared to T cells from *Itk*$^{-/-}$ and WT pre-transplanted (**Supplementary Figure 2A**). Next, we used the short-term allo-HSCT model, where T cells from WT or *Itk/Il4ra* DKO were transplanted into irradiated BALB/c mice. 7 days post transplantation, WT or *Itk/Il4ra* DKO donor T cells were then sorted back from the BALB/c recipient mice, and Eomes expression on these donor T cells was determined. We did not observe any differences between the donor WT or *Itk/Il4ra* T cells upon allo activation (**Supplementary Figures 2A–C**). Next, we tested the function of *Itk/Il4ra* DKO T cells in the long term allo-HSCT model, and observed that donor T cells from *Itk/Il4ra* DKO mice did not induce GVHD, and most of the animals survived compared to recipients of WT T cells (**Supplementary Figure 2D**). BALB/c transplanted with *Itk/Il4ra* donor T cells also had much less weight loss and significantly better clinical scores compared to BALB/c mice transplanted with WT donor T cells (**Supplementary Figures 2D–G**). Furthermore, *Itk/Il4ra* DKO donor T cells cleared leukemia cells without inducing GVHD. These data show that the IMP T cell phenotype may not be critical for GVHD, but modulating ITK does impact GVHD without affecting GVL.

To investigate the role of Eomes in clearing leukemia cells and in cytotoxic function, we crossed *Itk*$^{-/-}$ mice with *Eomes*$^{flox/flox}$ mice, and crossed these offspring with CD4cre mice, to delete Eomes specifically in T cells (28, 29) to generate (*Itk/Eomes* DKO). We performed similar allo-HSCT experiments as described above, and used WT or *Itk/Eomes* DKO T cells. Seven days post-transplant, donor T cells were sorted using

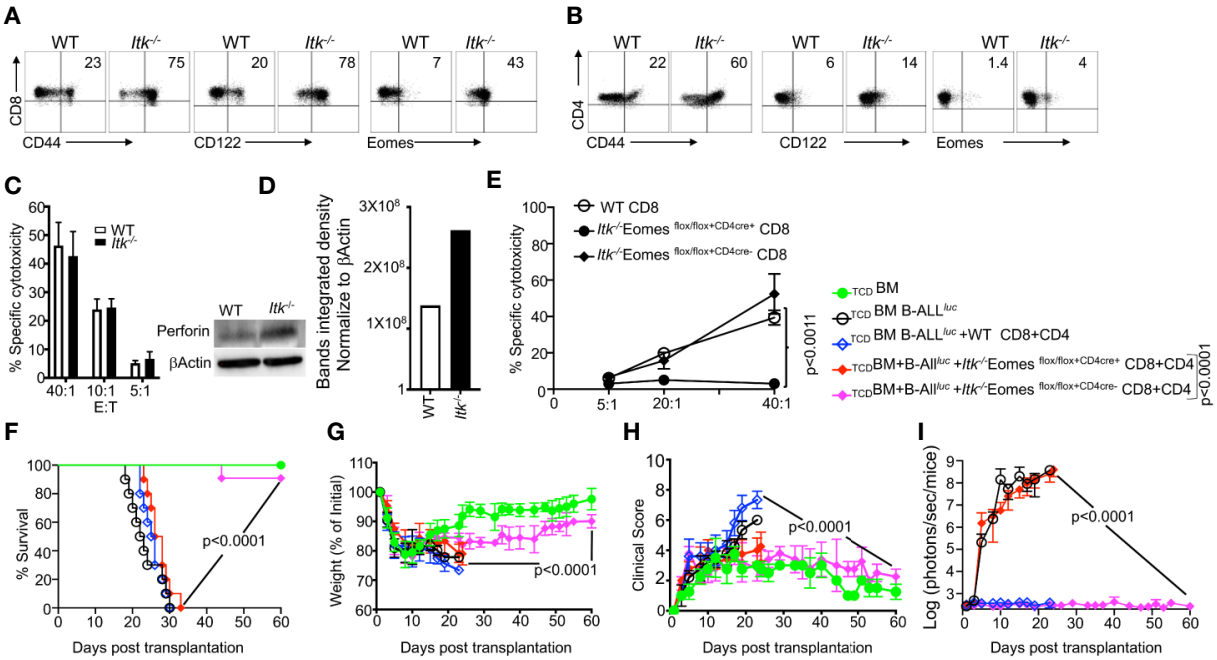

FIGURE 3 | IMP T cells are not sufficient for GVL effect. **(A, B)** Purified WT and *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ T cells were examined for expression of CD44, CD122, and Eomes by flow cytometry. **(C)** Purified WT or *Itk*$^{-/-}$ T cells were transplanted into irradiated BALB/c mice, at day 7 purified T cells were sorted using H2K^d, CD45.1 and CD45.2 expression. *Ex vivo* purified CD8$^+$T cells were used in cytotoxicity assay against primary leukemia target B-ALL*luc*+ cells at a 40:1, 20:1, or 5:1 ratio. **(D)** Purified T cells were examined for perforin by western blot. Quantitative analysis of perforin expression by western blot with data normalized against β–Actin. **(E)** Purified WT or *Itk/Eomes* DKO donor T cells were transplanted into irradiated BALB/c mice. On day 7 donor T cells were purified as described and used in an *ex vivo* cytotoxicity assay against B-ALL^{luc}-cells at 5:1, 20:1, and 40:1 ratio. **(F)** 1 × 10^6 purified WT and *Itk*$^{-/-}$ *Itk/Eomes* DKO CD8$^+$ T cells and 1 × 10^6 purified CD4$^+$ T total of 2 × 10^6 mixed CD4$^+$ and CD8$^+$ T cells were mixed and transplanted along with 2 × 10^5 B-ALL-*luc* cells and 10 × 10^6 $_{TCD}$BM into irradiated BALB/c mice. Host BALB/c mice were imaged using IVIS 3 times a week. Group one received 10 × 10^6 $_{TCD}$BM alone as ($_{TCD}$BM). Group two received 10 × 10^6 $_{TCD}$BM along with 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM+B-ALL^{luc}). Group three were transplanted with 10 × 10^6 $_{TCD}$BM and 1 × 10^6 purified WT CD8$^+$ T cells +1 × 10^6 CD4$^+$ T cells and 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM+B-ALL^{luc}+WT CD8+CD4). Group four received 10 × 10^6 T $_{TCD}$BM and 1 × 10^6 purified CD8$^+$ T cells +1 × 10^6 CD4$^+$ T cells from *Itk/Eomes* DKO along with 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM+B ALL^{luc}+ *Itk*$^{-/-}$ EomesFF+CD4cre CD8+CD4). Group five received 10 × 10^6 $_{TCD}$BM and 1 × 10^6 CD8$^+$ T cells +1 × 10^6 CD4$^+$ purified T cells *Itk/Eomes* DKO CD4$^+$ T cells along with 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM+B-ALL^{luc}+ *Itk*$^{-/-\ EomesFF+CD4cre-}$ CD8+CD4). **(F)** The mice were monitored for survival, **(G)** body weight changes, and **(H)** clinical score for 60 days post BMT. For weight changes and clinical score, one representative of 2 independent experiments is shown (n = 3 mice/group for BM alone; n = 5 experimental mice/group for all three group. The survival groups are a combination of all experiments. **(I)** Quantitated luciferase bioluminescence of tumor growth. Statistical analysis for survival and clinical score was performed using log- Two-way ANOVA were used for statistical analysis confirming by students *t* test, p values are presented. *Note: Controls are naïve mice used as negative control for bioluminescence (BLI).*

H2K^b expression, and *in vitro* cytotoxicity assays were performed at a 5:1, 20:1 and 40:1 ratio (effector: target). Our data show that *ex vivo* donor *Itk/Eomes* DKO were unable to kill cancer targets (**Figure 3E**). To examine the role of Eomes in the allo-HSCT model, BALB/c mice were lethally irradiated and injected intravenously with 10 × 10^6 WT $_{TCD}$BM cells along with FACS-sorted CD8$^+$ and CD4$^+$ T cells from donor mice (WT, *Itk*$^{-/-}$*Eomes* DKO). This was followed by intravenous challenge with 2 × 10^5 luciferase-expressing B-ALL-*luc* blast cells as described (17). Recipient animals transplanted with WT T cells cleared the tumor cells but had reduced survival and GVHD (**Figures 3F–I**). Recipient animals transplanted with *Itk*$^{-/-}$*Eomes* DKO T cells however, did not cleared the leukemia cells without showing signs of GVHD (**Figure 3I**). Notably, recipient animals transplanted with *Itk/Eomes* DKO T cells mice were unable to clear the tumor and all died from cancer burden. These data provided further evidence that Eomes is required for the GVL effect.

ITK Deficiency Results in Reduced Cytokine Production

It is known that the conditioning regimen for allo-HSCT elicits an increase in the production of inflammatory cytokines by donor T cells, and this is considered to be one of the hallmarks of GVHD pathogenesis (30). We obtained blood samples from GVHD patients and healthy donors and examined the levels of serum inflammatory cytokines such as IL-33, IL-1α, IFNγ, TNFα and IL-17A. We observed that patients with GVHD have significantly higher levels of serum proinflammatory cytokines compared to healthy controls (**Figure 4A**). Next, we assessed cytokine production by *Itk*$^{-/-}$ CD8 and CD4 T cells in our allo-HSCT model (B6→BALB/c), examining the levels of serum inflammatory cytokines such as IL-33, IL-1α, IFN-γ, TNF-α and IL-17A on day 7 post allotransplantation (**Figures 4B, C**). We found that serum IFN-γ and TNF-α were significantly reduced in recipients that received *Itk*$^{-/-}$ CD8$^+$ T or CD4$^+$ T

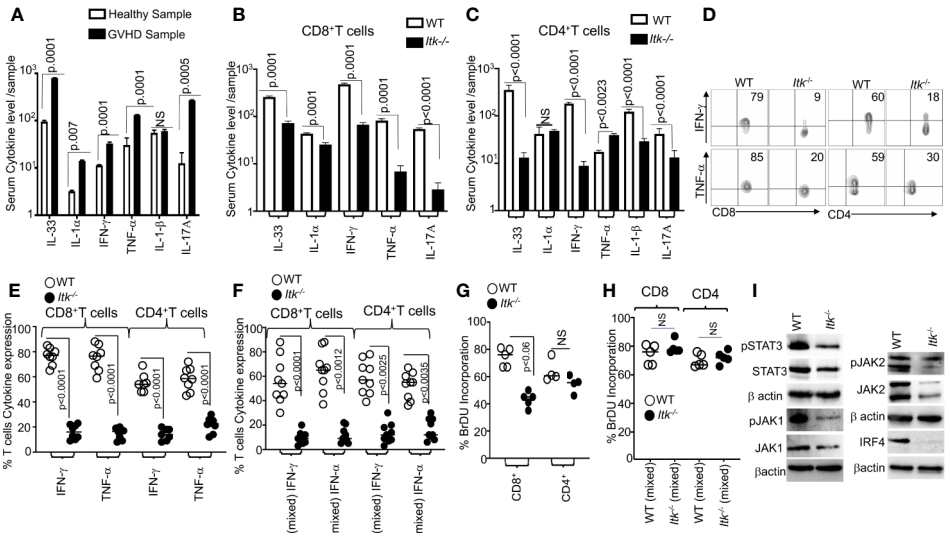

FIGURE 4 | ITK deficiency results in reduced cytokine production. **(A)** Serum from several GVHD patients was isolated and examined for inflammatory cytokine production (IL-33, IL1α, IFN-γ and TNF-α, IL1β and IL-17A) as determined by ELISA. **(B, C)** 1×10^6 purified WT or *Itk*$^{-/-}$ CD8$^+$ T or CD4$^+$ T cells were separately transplanted with into irradiated BALB/c mice. At day 7 post allo-HSCT, recipient BALB/c were euthanized and serum cytokines (IL-33, IL1α, IFN-γ, and TNF-α and IL-17A) were measured by ELISA. **(D)** Intracellular IFN-γ and TNF-α expression by donor CD8$^+$ and CD4$^+$ T cells after stimulation with anti-CD3/anti-CD28 as determined by flow cytometry. **(E, F)** Combined data from 3 independent experiments is shown for each experiment shown in figures. **(F)** Flow cytometry analysis of purified WT and *Itk*$^{-/-}$ T cells that were mixed at a 1:1 ratio for transplantation into irradiated BALB/c mice. At day 7 donor T cells were gated for expression of H-2K^b, CD45.1, and CD45.2 and intracellular expression of IFN-γ and TNF-α was analyzed by flow cytometry after stimulation with anti-CD3/anti-CD28. Combined data from four independent experiments is shown, and the p value for each experiment is shown. **(G)** Purified WT or *Itk*$^{-/-}$ donor CD8$^+$ and CD4$^+$ T cells were transplanted into irradiated BALB/c. At day 7 donor cells were analyzed for donor T cell proliferation by examining BrdU incorporation by flow cytometry. **(H)** Purified WT and *Itk*$^{-/-}$ donor T cells were mixed at a 1:1 WT: *Itk*$^{-/-}$ ratio and transplanted into irradiated BALB/c mice, at day 7 splenic donor T cells were gated for the expression of H-2K^b, CD45.1, and CD45.2 and analyzed for BrDU incorporation. **(I)** Purified WT and *Itk*$^{-/-}$T cells were stimulated with CD3 and CD28 overnight examined for the expression and phosphorylation of IRF4, JAK1/2 and STAT3 by western blot. For statistical analysis we used two-way ANOVA and student's *t* test, p values are presented.

cells compared to those that received WT CD8$^+$ or CD4$^+$ T cells (**Figure 4B, C**). Thus, we confirmed that the findings in our pre-clinical model correlated with what we found in human GVHD samples. We also isolated *Itk*$^{-/-}$ donor T cells from the secondary lymphoid organs of recipients using anti-H2K^b antibodies (expressed by donor C57Bl/6 cells). 7 days post allo-transplantation, cells were stimulated with anti-CD3/CD28 (**Figure 4D**), or PMA/ionomycin [to bypass the proximal TCR signaling defect (31) (**Supplementary Figure 3**)], in the presence of Brefeldin A, or left unstimulated for 6 h, followed by analysis of IFN-γ and TNF-α cytokine production. *Itk*$^{-/-}$ T cells were capable of producing IFN-γ and TNF-α at levels comparable to WT cells when both CD8$^+$ and CD4$^+$ T cell signaling was bypassed by re-stimulation with PMA and ionomycin (**Supplementary Figure 3**). However, the *Itk*$^{-/-}$ cells produced significantly less inflammatory cytokines when stimulated *via* TCR/CD28 than WT cells did (**Figures 4D, E**). Next, we determined whether the reduction of cytokine production by *Itk*$^{-/-}$ donor T cells was due to cell-intrinsic or -extrinsic factors. We mixed purified *Itk*$^{-/-}$ CD8$^+$ T and CD4 T cells with purified WT CD8$^+$ or CD4$^+$ T cells separately at a 1:1 ratio, and transplanted the mixed cells into irradiated BALB/c mice as described above. On day 7, donor T cells were isolated from recipient mice using H2K^{b+} and examined for IFN-γ and TNF-α expression as described above. We found that WT donor CD8$^+$ and CD4$^+$ T cells produced higher levels of inflammatory cytokines than *Itk*$^{-/-}$ donor CD8$^+$ and CD4$^+$ T cells, respectively, suggesting that the reduced cytokine production observed by *Itk*$^{-/-}$ donor T cells is T cell-intrinsic (**Figure 4F**).

We next examined donor CD4$^+$ and CD8$^+$ T cell proliferation using a BrdU incorporation assay. 7 days post allo-transplantation as described above, transplanted splenic CD4$^+$ and CD8$^+$ T cells were examined for proliferation by BrdU incorporation. *Itk*$^{-/-}$ donor CD8$^+$ showed statistically significantly reduced proliferation compared to WT donor CD8$^+$ T cells, although there was no difference in proliferation between WT and *Itk*$^{-/-}$ CD4$^+$ T cells (**Figure 4G**). To determine if the reduced proliferation of *Itk*$^{-/-}$ donor T cells was due to cell-intrinsic mechanisms, we mixed sort purified mixed *Itk*$^{-/-}$ and WT CD4$^+$ or *Itk*$^{-/-}$ and WT CD8$^+$ at a 1:1 ratio, followed by transplantation as described above. Interestingly, no difference was observed in BrdU incorporation in donor T cells from spleens of recipient mice between WT and *Itk*$^{-/-}$ donor CD4$^+$ and CD8$^+$ T cells in the mixed transplant models, indicating that the reduced proliferation of donor *Itk*$^{-/-}$ T cells proliferation was due to cell-extrinsic effects (**Figure 4H**). Thus, both cell intrinsic and extrinsic mechanisms regulate the behavior of *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ donor T cells.

The transcription factor IRF4 has been shown to play critical roles in maintaining TCR signaling, including TCR signal strength such as those regulated by ITK (32). The JAK/STAT signaling pathway is also critical for the response of T cells to cytokines (33, 34). To examine whether there was a difference in these signaling pathways between WT and *Itk*$^{-/-}$ donor T cells in the GVHD and GVL model, we examined expression of IRF4, JAK1, JAK2 and STAT3 by purified splenic T cells that had been stimulated overnight with CD3 and CD28 followed by lysis for analysis of protein. Our data showed that *Itk*$^{-/-}$ donor T cells expressed significantly less IRF4, JAK1, JAK2, and STAT3 as well as phosphorylated forms of JAK1, JAK2 and STAT3 (**Figure 4I** and **Supplementary Figures 4A–D**). Our data suggest that the lack of ITK affects the expression of IRF4, and thus the amount of cytokine signals the cells received. These data may explain the reduced cytokine production and proliferation in *Itk*$^{-/-}$ T cells observed above.

ITK Differentially Regulates Gene Expression in T Cells During GVHD

As an unbiased approach to further explore differences between WT and *Itk*$^{-/-}$ CD8$^+$ T cells, we employed RNA sequencing analysis to examine the differences in gene expression between WT and *Itk*$^{-/-}$ CD8$^+$ T cells following allo-HSCT. We sort-purified donor WT and *Itk*$^{-/-}$ CD8$^+$ T cells (using H-2K^b antigen expressed by donor T cells) before and 7 days after they were transferred into irradiated BALB/c recipients, and RNA sequences was done. Although WT and *Itk*$^{-/-}$ CD8$^+$ T cells are distinct prior to transplantation due to the enhanced IMP in the absence of ITK, WT and *Itk*$^{-/-}$ T cells which homed to the spleen post-transplantation are similar as revealed by the fact that they clustered within a close proximity in the Principal Component Analyses (PCA) (**Figure 5A**). We were unable to collect enough cells from the intestine of the *Itk*$^{-/-}$ T cell recipients, since they are deficient in homing to the intestine (see **Figures 6B–D**). To further determine the differentially expressed genes that are unique in WT CD8$^+$ T cells and associated with their ability to home to the GVHD target organs, we compared the lists of genes that were up- or down-regulated after the cells were transferred into the recipients and homed to different organs. Genes that are differentially expressed in WT T cells that were able to home to the GVHD target organ may reveal signals that are deficient due to the absence of ITK. We therefore extracted the list of genes that are up- or down-regulated in only WT T cells isolated from the gut of the recipient's post-transplantation (**Figures 5B, C** shows 20 up-regulated and 27 down-regulated genes). The differentially expressed genes between WT and *Itk*$^{-/-}$ donor T cells were enriched for transcripts encoding lymphocyte homing molecules such as adhesion molecules and chemokine signaling proteins, which might contribute to the defective homing capability of *Itk*$^{-/-}$ donor T cells (**Figure 5E**). The results of critical genes that were differentially expressed were confirmed by q-RT-PCR (**Figure 5D**). Using pathway enrichment analyses, our data also revealed a critical role for ITK in regulating genes involved in T cell cytokine/cytokine receptor interaction, cell adhesion, graft-versus-host disease, allograft rejection, and chemokine signaling pathways (**Figure 5E**). These data suggest that ITK regulates the expression of signature genes associated with the homing of the transplanted cells into the GVHD targeted organs, while it does not have an apparent effect on T cell homing in the spleen. This may, in part, explain the ability of *Itk*$^{-/-}$ T cells to maintain GVL effects while being unable to home to the GVHD target organs and participate in GVHD.

ITK Signaling Is Required for T Cell Migration to the GVHD Target Tissues

GVHD involves early migration of alloreactive T cells into the target organs, followed by T cell expansion and tissue destruction. Modulation of alloreactive T cell trafficking has been suggested to play a significant role in ameliorating experimental GVHD (35). Therefore, we examined the trafficking of donor T cells to GVHD target tissues as previously described (35). Irradiated BALB/c recipient mice were injected with CD8$^+$ and CD4$^+$ T cells from *Itk*$^{-/-}$ (CD45.2$^+$) and WT B6LY5(CD45.1$^+$) mice mixed at a 1:1 ratio (**Figure 6A**), and at 7 days post transplantation, recipient mice were examined for the presence of donor CD8$^+$ and CD4$^+$ T cells in the spleen, lymph nodes, liver and the small intestines. While the WT: *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ T cell ratio remained approximately 1:1 in the spleen and lymph nodes (**Figure 6B**), this ratio in the liver and small intestine was significantly elevated, suggesting that *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ T cells were defective in migration to and/or expansion in those tissues. Using histological staining for H&E, we also observed significant leukocyte infiltration into GVHD target organs – liver, skin, and small intestine (SI) (36) in WT T cell recipients but not in *Itk*$^{-/-}$ T cell recipients (**Figure 6C**). As an alternative approach, we tracked both CD8$^+$ and CD4$^+$ T cells in allo-BMT mice by using donor CD8$^+$ and CD4$^+$ T cells from WT and *Itk*$^{-/-}$ mice that also express luciferase, which could be monitored by bioluminescence (37). We observed that both CD8$^+$ and CD4$^+$ donor T cells from *Itk*$^{-/-}$ mice had significantly impaired residency in GVHD target organs, including the liver and small intestine (SI), compared to WT, despite no differences in spleen and lymph nodes (**Figure 6D**). Secondary lymphnodes (spleen and Lymph nodes) and GVHD target oragns small intestine (SI), and liver were quantified luciferase bioluminescence (**Supplementary Figures 5A, B**). In the mixed T cell transfer model, we had determined that *Itk*$^{-/-}$ T cell proliferation was comparable to that of WT cells; therefore, it is very likely that the reduced number of *Itk*$^{-/-}$ T cells in the liver and small intestine was due to impaired T cell trafficking. Pro-inflammatory conditioning treatment may promote T cell migration into GVHD target tissues (38, 39). Indeed, in the same mixed T cell transfer model, we found that chemokine and chemokine receptor expression (Aplnr, Cxcr5, Accr2, CCL12, CCL2, CCL5, Ccr9, Ackr4, and Cmtm4) was also significantly reduced in *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ T cells at day 7 post-transplantation (**Figure 6E**). These data suggest that *Itk*$^{-/-}$ CD8$^+$ T cells display attenuated chemokine receptor expression, which correlates with defective migration to GVHD target organs and reduced target organ pathology.

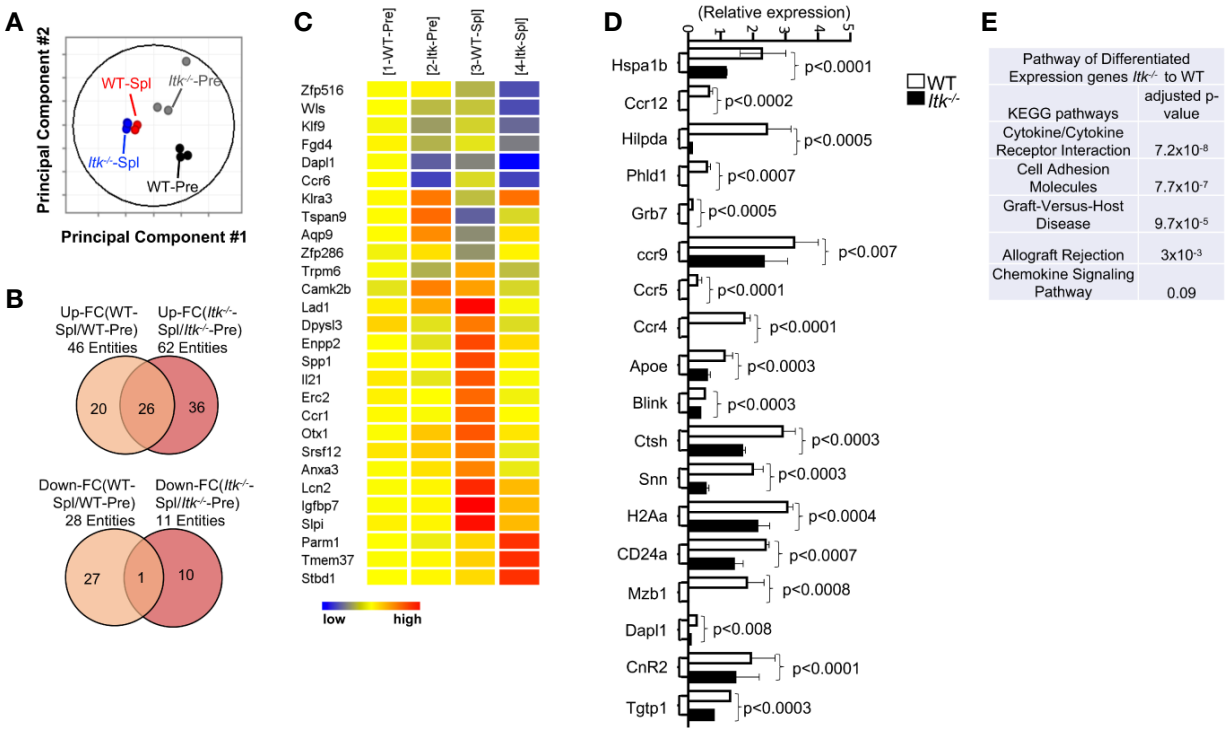

FIGURE 5 | ITK differentially regulates gene expression in T cells during GVHD. WT and *Itk*$^{-/-}$ CD8$^+$ T cells were FACS sorted then transplanted into irradiated BALB/c mice. At day 7 post-transplant, donor T cells were sort-isolated (based on expression of H-2K^b, CD3 and CD8) from host spleen. Sorted donor T cells were subjected to RNA sequencing. **(A)** Principal component analysis of genes with ≥2-fold change in any pairs of group combinations, with false discovery rate (FDR) ≤ 0.05. WT-Pre and *Itk*$^{-/-}$Pre denotes cells prior to transfer, and WT-Spl, and *Itk*$^{-/-}$Spl denotes cells isolated from the spleen (Spl) of the recipients post-transfer. **(B)** Venn diagram of genes that are ≥2-fold up- or down- regulated in the indicated comparisons, with FDR (*P*) ≤ 0.05. **(C)** Heat map of differentially expressed genes listed as (1) WT pre. (2) ITK-/- pre, (3) WT post spleen, and (4) ITK-/- post spleen. **(E)** Differentially expressed genes were enriched for pathway analysis comparing WT and *Itk*$^{-/-}$. **(D)** WT and *Itk*$^{-/-}$ CD8$^+$ T cells were FACS sorted then transplanted into irradiated BALB/c mice. At day 7 post-transplant, donor T cells were sort-isolated (based on expression of H-2K^b, CD3 and CD8) from host spleen and small intestine (Gut). Total RNA was isolated from sorted donor T cells were and qPCR was performed.

Given that *Itk*$^{-/-}$ T cells exhibit defective migration to target organs of GVHD, we predicted that although *Itk*$^{-/-}$ T cells can clear leukemia cells in the blood and secondary lymphoid organs, they would not be able to kill leukemia cells that reside in tissues. To test this possibility, lethally irradiated BALB/c mice were BM-transplanted together with FACS-sorted WT or *Itk*$^{-/-}$ CD8$^+$ T cells, and challenged with subcutaneously injected B-All *luc* cells. Although *Itk*$^{-/-}$ CD8$^+$ T cells did not cause GVHD, the subcutaneously injected leukemia cells were cleared only in mice transplanted with WT CD8$^+$ T cells, and not in those given *Itk*$^{-/-}$ CD8$^+$ T cells (**Figure 6F–J**). Together, these data suggest that the ITK signaling in T cells can separate GVHD from GVL effects, but only for leukemia cells that reside in the circulation and in secondary lymphoid organs (such as hematologic malignancies).

DISCUSSION

In this report, we demonstrate that the absence of the TCR-regulated kinase ITK significantly suppresses GVHD, while maintaining the GVL effect in models of allo-HSCT. Loss of ITK also altered expression of IRF4, and the JAK/STAT pathway components JAK1, JAK2, and STAT, which play critical roles in controlling cytokine expression (14, 39). Transcriptome analysis by RNA sequencing revealed that ITK signaling controls chemokine receptor expression during this process, which in turn affects the ability of donor T cells to migrate to GVHD target organs. Taken together, these data suggest that ITK could represent a potential target for the separation of GVHD and GVL responses after allo-HSCT.

The ability of *Itk*$^{-/-}$ T cells to induce GVL without causing GVHD indicates that the ITK signaling pathway is involved in the pathogenesis of GVHD. *Itk*$^{-/-}$ T cells develop into IMP cells (CD122$^+$ CD44^{hi} phenotype) in the thymus, and it is possible that such cells are responsible for the GVHD and GVL effects we observe. In experiments where WT T cells developed into IMPs, we found that they retained the capacity to induce both acute GVHD and GVL, suggesting a T cell-intrinsic function of ITK in promoting GVHD during allo-HSCT. Similarly, the cytotoxicity of *Itk*$^{-/-}$ CD8$^+$ T cells is not dependent on the IMP. While IMP cells express significantly higher Eomes compared to their WT non-IMP counterparts, we found that IMP CD8$^+$ T cells are not responsible for distinguishing GVHD and GVL. To our surprise,

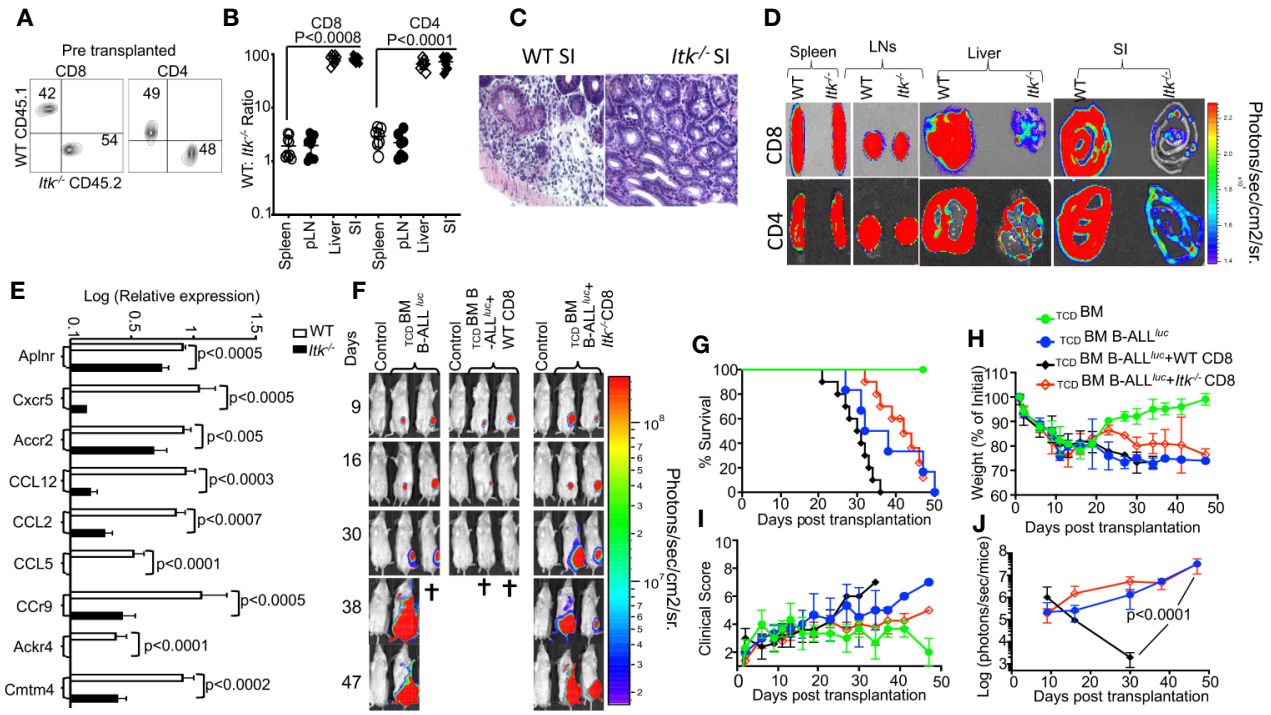

FIGURE 6 | ITK signaling is required for T cell migration to the GVHD target tissues. **(A)** Irradiated BALB/c mice were allo-HSCT-transplanted and injected with FACS-sorted WT and *Itk*$^{-/-}$ CD8$^+$ T and CD4$^+$ T cells mixed at a 1:1 ratio. FACS analysis of sorted T cells pre-transplant shown. **(B)** At day 7 post-BMT, the spleen, liver, and small intestine (SI) were examined for donor WT and *Itk*$^{-/-}$ T cells. The ratio of WT: *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ T cells in the organs was determined. **(C)** At day 7 post-allo-HSCT, small intestines were examined by H&E staining. **(D)** Irradiated BALB/c mice were BM-transplanted and injected with CD8$^+$ T CD4$^+$ T cells from luciferase-expressing WT or *Itk*$^{-/-}$ mice. **(E)** On Day 7 post-allo-HSCT, donor T cells were isolated and examined for the expression of Aplnr, Cxcr5, Accr2, CCL12, CCL2, CCL5, CCr9, Ackr4, and Cmtm4 using q-RTPCR. P values were calculated using 2-way ANOVA and Student's *t* test, p values are listed. **(F)** Irradiated BALB/c mice were transplanted with C57Bl/6-derived BM and FACS-sorted WT or *Itk*$^{-/-}$ 1 × 10^6 CD8$^+$ T cells, and challenged subcutaneously with 2 × 10^{5}luciferase-expressing B-All *luc* cells. Recipient animals were monitored for weight changes. Group one of recipient mice was transplanted with 10 × 10^6 $_{TCD}$ BM. The second group of recipient mice was transplanted with 10 × 10^6 $_{TCD}$BM and 2 × 10^5 primary B-ALL luc+ cells($_{TCD}$BM+B-ALL^{luc}). The third group of recipient mice was transplanted with 10 × 10^6 $_{TCD}$BM along with 1 × 10^6 T cell from WT mice along with 2 × 10^5 B-ALL-*luc*+ cells ($_{TCD}$BM+B-ALL^{luc}+WT CD8). The fourth group of recipient mice was transplanted with 10 × 10^6 $_{TCD}$BM and 1 × 10^6 T cell from *Itk*$^{-/-}$ mice along with 2 × 10^5 B-ALL-*luc*+ cells. ($_{TCD}$BM+B-ALL^{luc}+ *Itk*$^{-/-}$ CD8). Representative bioluminescence images of leukemia cell-bearing mice on days 9, 16, 30, 38, and 47 are shown. *Note: Controls are naïve mice used as negative control for* bioluminescence *(BLI).* **(G)** Animals were monitored for survival over 47 days, **(H)** for changes in weight loss, **(I)** and for clinical score. **(J)** Recipient mice were monitored for leukemia cell growth using the IVIS imaging system and quantified data is shown. For weight changes and leukemia cell growth, one representative of 2 independent experiments is shown (n = 3 mice/group for control, n = 5 mice for WT, and n = 5 mice for *Itk*$^{-/-}$). Survival groups were combined from both experiments. P values were calculated using two-way ANOVA and Student's *t* test, p values are listed.

we noted that *Itk*$^{-/-}$ CD8$^+$ T cells exhibit similar or higher *in vitro* cytotoxicity compared to WT CD8$^+$ T cells. This may be due to the higher levels of perforin expressed by *Itk*$^{-/-}$ T cells compared to WT T cells.

Our data also show that *Itk*$^{-/-}$ donor CD4$^+$ and CD8$^+$ T cells exhibit reduced expression of chemokine receptors compared to WT counterparts. Moreover, the migration of *Itk*$^{-/-}$ donor T cells to target organs was also severely defective, reflecting the reduced expression of key chemokine receptors. The defective migration of *Itk*$^{-/-}$ CD8$^+$ and CD4$^+$ T cells likely contributes to the attenuation of GVHD, since these T cells continue to display GVL effects against leukemia cells that were injected intravenously and reside in secondary lymphoid organs. In contrast, WT but not *Itk*$^{-/-}$ CD8$^+$ T cells were able to inhibit leukemia cell growth when the leukemia cells were injected subcutaneously. The compartmentalization of T cells to secondary lymphoid organs can be an effective strategy for preventing GVHD, while leaving GVL effects against hematologic malignancies intact. It is noteworthy that Ibrutinib, an inhibitor of BTK which can also inhibits ITK, is able to reduce chronic GVHD (12). In addition, previously published work showed that IFN-γR signaling constitutes a major mechanism for donor T cell migration to GVHD target organs (40, 41), and we observed that the lack of ITK affects production of IFN-γ. The retention of T cells to secondary lymphoid organs by FTY720-mediated inhibition of S1P1 also ameliorates GVHD while maintaining GVL effects (42, 43). Similarly, inhibition of T cell migration to GVHD target organs by targeting the chemokine receptors CCR2 or CCR5 protects against GVHD-induced pathology (44, 45), which at least with CCR2 deficiency was shown to preserve the GVL effect. Importantly, in a clinical study, CCR5 blockade by a small

molecule antagonist led to a reduction in GVHD with no significant difference in relapse rates, suggesting that blocking T cell migration to target tissues could reduce GVHD severity without compromising the beneficial GVL effect (45). In addition, the inhibition of CXCR3 ameliorates GVHD in allo-HSCT mice (46). Activated alloreactive $CD8^+$ T cells upregulate the expression of CX3CR1 and CXCR6 after allo-HSCT (47, 48), and these receptors are important for the homing of $CD8^+$ T cells to the liver and intestines. Thus, CXCR6 deficiency or blockade of the CXCR3 and CXCR6 ligands attenuates GVHD (47). Importantly, the GVL effect is still maintained under these conditions (49). Thus, blocking T cell migration by chemokine receptor blockade could be beneficial in the treatment of GVHD after allo-HSCT. Since activated $Itk^{-/-}$ T cells displayed significantly reduced expression of chemokine receptors, the compartmentalization of $CD8^+$ T cells to secondary lymphoid organs likely contributes to the preservation of GVL effects while severely attenuating GVHD (48).

Although suppression of TCR signaling can prevent GVHD, the complete suppression of T cell responses negates the beneficial GVL effect that is also provided by the same donor T cells after allo-HSCT (50). The fact that mice transplanted with $Itk^{-/-}$ T cells are able to mount GVL responses is an exciting feature. The preservation of the GVL response could have occurred for several reasons. First, the proliferation and cytotoxic activity of $Itk^{-/-}$ T cells are preserved compared to pro-inflammatory cytokine production. The manifestations and severity of GVHD are highly influenced by local cytokines, which then activate transcription factors and drive development toward a cytokine storm. In addition, proinflammatory cytokines exert direct effects on GVHD target tissues (51–53). Indeed, the presence of cytokine storm is considered one of the hallmarks of GVHD pathogenesis (54), and our data showed that cytokine production was significantly reduced in mice that received $Itk^{-/-}$ T cells. We also confirmed that cytokine production is T cell-intrinsic while proliferation is T cell-extrinsic. To explore the potential mechanism of this observed difference in cytokine and chemokine receptor expression between WT and $Itk^{-/-}$ donor $CD4^+$ and $CD8^+$ T cells, we analyzed key transcription factors and pathways that may be involved in these processes. We found significant differences in expression of the transcription factor IRF4 and the JAK/STAT signaling pathways, which regulate the expression of key molecules required for the maintenance of T cell effector function, cytokine production, and chemokine receptor upregulation. Since IRF4 has been shown to play critical roles in modulating TCR signal strength and T cell function (32), it is likely that reduction in the activation of IRF4 and of the JAK/STAT pathway contribute to reduced cytokine expression, thus alleviating the cytokine storm in GVHD (15). Our data show that the reduced proliferation seen in donor T cells from $Itk^{-/-}$ mice is cell-extrinsic. ITK deficiency has been shown previously to affect T cell proliferation (54) and cytokine production, but during allogenic activation, ITK-deficient T cells can still proliferate. This might be due to the redundant function of ITK and other Tec kinases (55). This finding is in line with our cytokine data, which show that $Itk^{-/-}$ T cells produce less cytokines, both in serum and on a per-cell basis. When transplanting either $CD4^+$ or $CD8^+$ T cells in a 1:1 ratio of WT:$Itk^{-/-}$ cells, we observed similar levels of proliferation for both WT and $Itk^{-/-}$ donor cells. Our data therefore provide further evidence that donor T cell proliferation is influenced by inflammatory conditions (56).

All together our data show that attenuating TCR signaling reduces donor T cell-mediated cytokine production, resulting in less severe GVHD. In addition, the inability of T cells to migrate to target organs may also affect this process, and thus explains the reduced ability of the $Itk^{-/-}$ donor T cells to induce GVHD.

DATA AVAILABILITY STATEMENT

The RNAseq data submission has been approved by NCBI GEO under the accession reference GSE161160. All data will be available to anyone.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB net 1140566-4. The ethics committee waived the requirement of written informed consent for participation. The animal study was reviewed and approved by SUNY Upstate Medical University and Cornell University.

AUTHOR CONTRIBUTIONS

MM, WH, AS, QY, SD, AB, and MK performed experiments. WT, YC, JP, and TG provided valuable reagents. RH assisted with data analysis, experimental design, scientific discussion, and manuscript editing. WH, QY, AA, AB, and MK designed experiments, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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SUPPLEMENTARY FIGURE 1 | *Itk*$^{-/-}$ CD4$^+$ T cells exhibit attenuated induction of GVHD compared to WT T cells. **(A)** 10 × 10^6 $_{TCD}$BM and 1 × 10^6 purified WT or *Itk*$^{-/-}$ CD4$^+$ T cells were transplanted into irradiated BALB/c mice. **(A)** The mice were monitored for survival, **(B)** changes in body weight, and (**C**) clinical score for 70 days post-BMT. For weight changes and clinical score, one representative of 2 independent experiments is shown (n = 3 mice/group for BM alone; n = 5 experimental mice/group for all three groups). The p values are presented. Two-way ANOVA and Student's *t* test were used for statistical analysis.

SUPPLEMENTARY FIGURE 2 | IMP T cells are not sufficient for GVHD effect. **(A-C)** *Itk/Il4ra* DKO and WT T cells were examined for Eomes expression pre- and post-transplantation. **(D)** 2 × 10^6 purified WT and *Itk/Il4ra* DKO CD8$^+$ T and 1 × 10^6 purified CD4$^+$ T cells were mixed and transplanted along with 2 × 10^5 primary B-ALL-*luc*+ cells into irradiated BALB/c mice. Recipient BALB/c mice were imaged using IVIS 3 times a week. Group one received 10 × 10^6 $_{TCD}$BM alone ($_{TCD}$BM). Group two received 10 × 10^6 $_{TCD}$BM along with 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM+B-ALL^{luc}). Group three was transplanted 10 × 10^6 $_{TCD}$BM with 2 × 10^6 purified (CD8$^+$ and CD4$^+$) from WT mice and 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM+B-ALL ^{luc} +WT CD8+CD4). Group four was transplanted 10 × 10^6 $_{TCD}$BM and 2 × 10^6 purified T cells (CD8$^+$ and CD4$^+$) from *Itk/Il4ra* DKO along with 2 × 10^5 B-ALL-*luc* cells ($_{TCD}$BM +B-ALL^{luc}+ *Itk/Il4ra* DKO CD8$^+$CD4). Recipient animals were monitored for survival, **(E)** changes in weight, and **(F)** clinical score. **(G)** Leukemia cell growth was monitored as in **Figure 1**, and quantitated bioluminescence is shown. One representative of 2 independent experiments is shown (n = 3 mice/group for BM alone; n = 5 experimental mice/group for all three groups. The survival groups were combinations of all experiments.

SUPPLEMENTARY FIGURE 3 | *Itk*$^{-/-}$ T cells are capable of cytokine production. Purified WT and *Itk*$^{-/-}$ T cells were transplanted into irradiated BALB/c mice. At day 7, donor T cells were gated for expression of H-2K^b, CD45.2, and CD45.1, and analyzed for intracellular expression of IFN-γ and TNF–α following *ex vivo* stimulation with PMA/ionomycin. Data from several experiments were combined and statistical analysis was performed using two-way ANOVA and Student's *t* test, with p values presented.

SUPPLEMENTARY FIGURE 4 | Quantitative analysis of JAK/STAT and IRF expression and phosphorylation. Quantitative analysis from western blots using Image Lab to normalize to β–Actin, data from 3 independent experiments. **(A)** Phospho and total STAT3. **(B)** Phospho and total JAK1. **(C)** Phospho and total JAK2. **(D)** Total IRF-4. For statistical analysis we used two-way ANOVA and student's *t* test, p values are presented.

SUPPLEMENTARY FIGURE 5 | Quantitative analysis of tissue BLI. For tissue imaging experiments, allo-HSCT was performed with 10 × 10^6 WT T cell-depleted BM cells and 1 × 10^6 FACS-sorted **(A)** CD8$^+$ T cells or **(B)** CD4$^+$ T cells (from B6-luc or *Itk*$^{-/-}$*luc* mice) and bioluminescence imaging of tissues was performed as previously described[20]. Briefly, 5 min after injection with luciferin (10 μg/g body weight), selected tissues were prepared and imaged for 1 min. Imaging data were analyzed and quantified with Living Image Software (Xenogen) and Igor Pro (Wave Metrics, Lake Oswego, OR).

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REVIEW
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Macrophages in Organ Transplantation

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Current immunosuppressive therapy has led to excellent short-term survival rates in organ transplantation. However, long-term graft survival rates are suboptimal, and a vast number of allografts are gradually lost in the clinic. An increasing number of animal and clinical studies have demonstrated that monocytes and macrophages play a pivotal role in graft rejection, as these mononuclear phagocytic cells recognize alloantigens and trigger an inflammatory cascade that activate the adaptive immune response. Moreover, recent studies suggest that monocytes acquire a feature of memory recall response that is associated with a potent immune response. This form of memory is called "trained immunity," and it is retained by mechanisms of epigenetic and metabolic changes in innate immune cells after exposure to particular ligands, which have a direct impact in allograft rejection. In this review article, we highlight the role of monocytes and macrophages in organ transplantation and summarize therapeutic approaches to promote tolerance through manipulation of monocytes and macrophages. These strategies may open new therapeutic opportunities to increase long-term transplant survival rates in the clinic.

Keywords: macrophages, immune tolerance, trained immunity, organ transplantation, nanotherapy

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INTRODUCTION

Organ transplantation is a life-saving strategy for thousands of patients with end-stage organ failure. Patients who find a compatible donor and receive a transplant are treated daily with multi-drug combinations designed to prevent rejection of the transplanted organ. Thanks to great progress in surgical techniques and immunosuppressive drugs, the percentage of short-term allograft rejection events has declined and 1-year allograft survival rates are above 90% (1). However long-term graft survival rates remain suboptimal (2, 3), arguing in favor of additional mechanisms of immune regulation associated with chronic allograft rejection that escape current immunosuppressive therapy.

To promote long-term organ transplant survival in the absence of chronic immunosuppressive therapy, transplant immunologists have historically focused on targeting the adaptive immune response. This is in response to early work on allograft rejection, which demonstrated that T cells are both necessary and sufficient for allograft rejection (4, 5). More recent work has focused on developing novel tolerogenic protocols that target the adaptive immune response using methods that include depletion of effector T cells (6), induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells (7) and blockade of co-stimulatory signals (8). The latter was achieved using monoclonal antibodies (mAb) or immunoglobulins (Ig) against cell surface molecules (CD4 (9); CD4 + DST (10); CD3 (11);

non-depleting CD3 (12); CD40L (13); CD40L + CD28 (14); LFA-1^{+} + ICAM-1 (15); CD2 (16); CD2 + CD3 (17); LFA3-Ig (18); CD80 and CD86 (19); CD40 (20); and CTLA4-Ig (21) (**Figure 1A**). While promising results have been obtained using these therapeutic approaches in experimental animal models, translation of these tolerance promoting methodologies that target innate immune cells in the clinic remain largely elusive (**Figure 1B**). Considering that consistent induction donor specific unresponsiveness remains a difficult task in the clinic, there is a major unmet need for the development of additional immune regulatory programs to improve long-term allograft survival in the clinical practice. Since innate immune cells participate in allograft recognition, developing therapeutic approaches that target myeloid cells in the clinic could open novel avenues to improve long-term transplantation outcomes.

It is widely accepted that allograft rejection is the result of a complex series of interactions between both the innate and the adaptive immune systems (22, 23). Recent advances in our understanding of the mechanisms that determine the outcome of the immune response to transplanted organs have highlighted the importance of the innate immune response (24). This ancient part of the immune system precedes cellular and humoral immunity and consequentially regulates the function of the adaptive immune response. The innate immune response initiates inflammatory signals as a defense mechanism against pathogens and tissue injury. Non-self-inflammatory stimuli induced by exogenous infectious agents are considered pathogen-associated molecular patterns (PAMPs), while tissue injury is recognized by self-derived damage-associated molecular patterns (DAMPs). Both PAMPs and DAMPs are recognized through pattern recognition receptors (PRRs), which include Toll-like receptors (TLR), NOD-like receptors (NLR) and C-type lectin receptors. PRRs are expressed on the cell surface and in the cytoplasm of innate immune cells, including macrophages, and mediate intracellular signaling cascades leading to transcriptional expression of inflammatory mediators (25).

Macrophages belong to the mononuclear phagocyte system and have a dual role in allograft transplantation, either triggering inflammatory response or inducing a tolerogenic environment (26). Local activation of macrophages through PRRs can lead to upregulation of major histocompatibility complex (MHC) and co-stimulatory molecules (signals 1 and 2), as well as the production of pro-inflammatory cytokines (signal 3) which result in T cell proliferation and differentiation (27, 28). More recently, it was demonstrated that macrophages adopt a long-term pro-inflammatory phenotype following an initial PRR stimulation of the C-type lectin receptor dectin-1, which results in a non-specific memory of the innate immune cells mediated by epigenetic reprogramming (29). This novel macrophage functional state has been termed trained immunity and is associated with pro-inflammatory cytokine production (TNFα and IL-6) after a second PRR stimulatory signal with TLR4 agonists (30). Understanding the immune biology of trained immunity has important implications for the design of novel therapeutic approaches. Preventing the accumulation of trained macrophages while promoting the development of regulatory macrophages represents an attractive, innovative approach to promote organ transplant acceptance. Herein, we highlight recent studies on the role of macrophages in organ transplantation and summarize the therapeutic potential of targeting macrophages for the induction of tolerance.

MACROPHAGE HETEROGENEITY AND PLASTICITY

Monocytes and macrophages are key elements of innate immunity and have crucial roles in host defense, inflammation and tissue

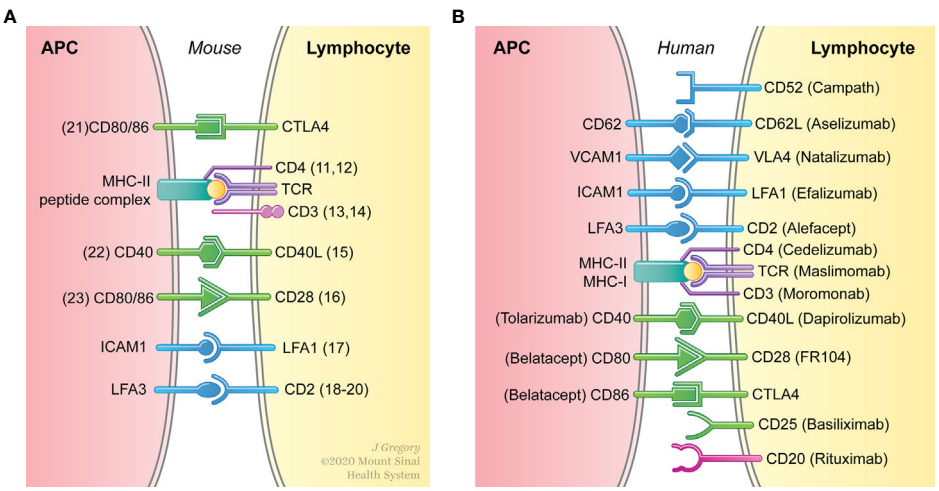

FIGURE 1 | Interfering with binding of the TCR to antigenic peptide complexed with MHC (signal 1) and engagement of co-stimulatory molecules (signal 2) prevents T cell activation. **(A)** Prolonged allograft survival and induction of tolerance has been achieved in experimental animal models buy targeting signals 1 and 2 in both T cells and antigen presenting cells (APC). **(B)** The clinical translation of therapeutic approaches that specifically target APC *in vivo* remain largely elusive.

homeostasis (31, 32). Monocytes originate from myeloid progenitor cells in the bone marrow and circulate in the blood for several days before entering the tissue and differentiating into macrophages (33, 34). Monocyte-derived macrophages also have key roles in clearing pathogens and cell debris, antigen presentation and initiating adaptive immune responses (35). To do so, macrophages acquire specialized functions according to the stimuli present in the environment. In relation to their activation, Mills et al. proposed two phenotypes: classical (M1) *versus* alternative (M2), in analogy to T helper cells Th1 and Th2 (36, 37). M1/M2 macrophages are functionally distinct with M1 macrophages shifted to nitric oxide (NO) and citrulline secretion, while M2 macrophages shifted toward production ornithine and polyamine secretion (36, 37). Consequentially, M1-derived NO inhibits T cell proliferation and exhibits a potent microbicidal activity, while M2-derived ornithine promotes cell proliferation and repair through polyamine and collagen synthesis (38–40). Over the past few years, this nomenclature has been a matter of debate due to the difficulty of including within M1 and M2 classification the multiple phenotypes adopted by macrophages. While *in vitro* activation of macrophages allowed us to better understand the developmental requirements of different macrophage subsets, *in vivo* studies are more complicated because the stimuli they encounter are multiple, complex and occur simultaneously (31, 41, 42).

Various stimuli control the expression of macrophage genes encoding cytokine receptors, cell activation markers and cell adhesion molecules **(Figure 2)**. Classic or M1 macrophage activation increases in response to PAMPs, DAMPs and pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor-necrosis factor (TNF) (43, 44). The environment favors the production of inflammatory chemokines by M1 macrophages, which induce lymphocyte recruitment. Among the chemokines produced by these M1 macrophages are CXCL9 and CXCL10, strongly associated with Th1 immune response (45, 46) and CXCL16, which maintain M1 polarization (47). Upon activation, M1 macrophages produce high levels of pro-inflammatory cytokines such as TNF, IL-1β, IL-6, IL-12 and IL-23, which may result in functional $CD4^+$ T lymphocyte polarization toward Th1 (48–51) or Th17 (52–54). In addition, M1 macrophages produce high levels of inducible nitric oxide synthase 2 (iNOS2) and reactive oxygen intermediates (ROI) that participate in removing bacteria, viruses and parasites. Phenotypically, costimulatory molecules such as CD40, CD80 and CD86, important in antigen presentation, are upregulated in M1 macrophages in conjunction with major histocompatibility complex class II (MHC-II) (55–57).

In contrast, M2-polarized macrophages, also known as alternatively activated macrophages, are important in tissue repair. The M2 phenotype contains different macrophage populations with separated functions, which can be polarized by several stimulatory factors. Based on the stimuli and transcriptional changes, Mantovani and Rőszer divided the M2 phenotype into M2a, M2b, M2c and M2d subtypes (58, 59). The mutual characteristics of these subtypes are high secretion of IL-10 and low IL-12 levels, in conjunction with the generation of arginase-1 (Arg-1). M2a macrophages are induced by IL-4 and IL-13, express high levels of mannose receptor (CD206) and

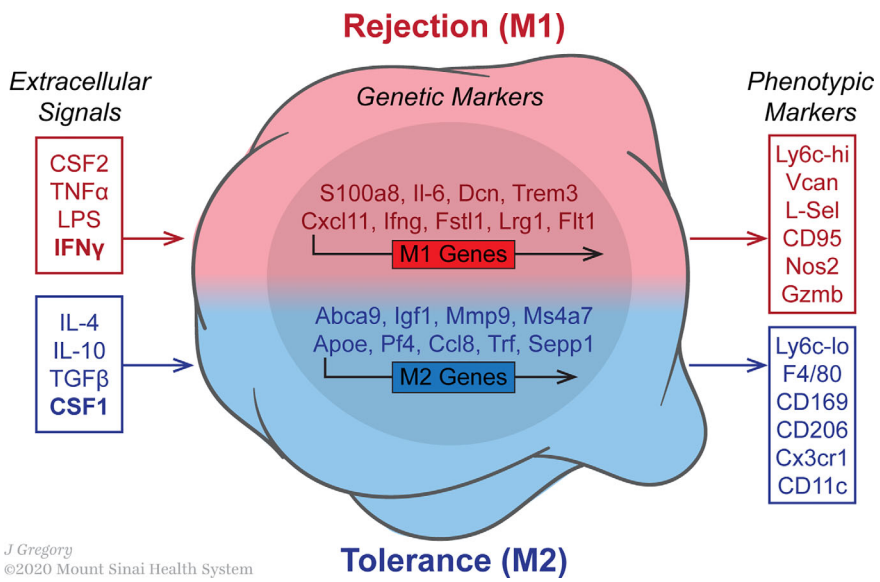

FIGURE 2 | Macrophages polarization in the transplanted allograft is influenced by various stimuli. The image indicates the expression of macrophage genes encoding cytokine receptors, cell activation markers and cell adhesion molecules. Data was acquired from published microarray obtained from graft-infiltrating macrophages on day 5 post-transplantation in either untreated rejecting or anti-CD40L mAb treated mice. The GEO accession number for the microarray data reported in this figure is GSE68648.

secrete pro-fibrotic factors, such as TGF-β, to contribute towards tissue repair (60–62). M2b macrophages have phenotypical and functional similarities with regulatory macrophages. They are activated by TLR or IL-1R agonists and produce both pro and anti-inflammatory cytokines, such as TNF-α, IL-1β, IL6 and IL-10 (41, 63). M2c macrophages, also known as inactivated macrophages, are induced by IL-10 and display anti-inflammatory functions. M2c secrete IL-10 and TGF-β (59, 64) and are efficient at phagocytosis and elimination of apoptotic cells (65). M2d macrophages have phenotypical and functional similarities with tumor-associated macrophages (TAMs). They are induced by A2 adenosine receptor (A2R) and IL-6 (66–68) and secrete IL-10, TGF-β and vascular endothelial growth factor (VEGF) to favor angiogenesis and cancer metastasis (68–70).

The need to update the M1/M2 classification has been evidenced in numerous studies addressing signaling pathways and genetic signatures associated with M1/M2 polarization (71–75). M1 and M2 share many genes implicated in cellular functions, such as phagocytosis, metabolism and cytokine production. IL-8, Tissue Factor and Leukocyte extravasation signaling pathways are shared among M1 and alternatively activated M2 (76, 77). On the other hand, recent works show specific signatures for M1 and M2 (78). For example, Jablonski et al. identified a new set of common and distinct M1 and M2 macrophage genes. They showed that CD38, Gpr18 and Fpr2 were M1-specific while c-Myc and Egr2 were M2-specific genes, proposing a new way to define both states of polarization based on their phenotypes: $CD38^{+}$ $Egr2^{-}$ (M1 macrophages) and $CD38^{-}$ $Egr2^{+}$ (M2 macrophages) (71). In addition, Buscher et al. demonstrated a strong gene-environment interaction in activated macrophages using a hybrid mouse diversity panel (HMDP). They showed different genetic signatures associated with lipopolysaccharide (LPS) responsiveness among a wide spectrum of macrophage phenotypes from several different inbred strains (72). Recently, Orecchioni et al. compared both transcriptomes obtained from Jablonski (*in vitro*) and Buscher (*in vivo*) to define differential signatures present in M1/M2 macrophages (79) and concluded that Fcγ receptor-mediated phagocytosis, MAPK signaling, MAPK, JAK1 and JAK3 signaling are upregulated in M1 upon LPS activation. These pathways control several inflammatory genes that allow the macrophages to exhibit their pro-inflammatory properties (80, 81). In contrast, the main pathways specifically expressed in M2 are adipogenesis, fatty acid synthesis and integrin signaling pathways, which are important for tissue infiltration, removal of necrotic tissue and initiation of tissue regeneration (82).

While bone marrow monocytes are mobilized early after transplantation and recipient monocyte-derived macrophages represent the majority of macrophages in the transplanted organ (83), it is important to acknowledge the immune regulatory role of tissue resident donor macrophages. Tissue-resident macrophages (TRMs) arise from fetal liver or yolk-sac progenitors and are phenotypically distinct from monocyte-derived macrophages in steady state conditions (84). While TRMs are primarily characterized by the expression of CD11b, F4/80, CD64, CD68 and MerTK and low levels of MHC-II on the cell surface in mice, monocyte-derived macrophages are characterized by CD11b, CD209, CD64 and MerTK expression on the cell surface (85). TRMs are functionally considered to be immunosuppressive because of their fundamental roles in maintaining homeostasis, inhibiting T cell activation and promoting the resolution of inflammation (75, 86). TRMs are divided into subpopulations according to their anatomical sites and functionality. For instance, Kupffer cells in liver (87, 88) or alveolar macrophages in lung (89) exhibit critical roles in generating CD4 regulatory T cells (Treg) and promoting tolerance. In the context of organ transplantation, Terry Strom and colleagues identified a subset of donor TRM that express high levels of the phosphatidylserine receptor TIM4 and CD169. The study demonstrated that this population of macrophages migrates to the draining lymph nodes following oxidative stress during ischemia-reperfusion injury (IRI) associated with transplantation and induces antigen-stimulated Treg. Interestingly, these M2-like TIM-4+CD169+ donor TRM were demonstrated to be immunoregulatory and to promote the engraftment in a murine cardiac allograft model (90). Contrary to this view, it has been suggested that ischemia/reperfusion primes innate immune cells for an excessive response to a subsequent inflammatory, which promotes organ injury. In the lung, alveolar macrophages under shock/resuscitation events increase their TLR4 expression in the cell surface due to oxidative stress (91). As a result, alveolar macrophages are primed and exhibit an exaggerated LPS response following a secondary stimulation. The source of the endotoxin is not clear, but it has been suggested that LPS may leak from the gut under ischemia/reperfusion conditions (92). This has major implications in lung transplantation as oxidative stress induced during IRI, coupled with an increase in the endotoxin levels in the donor organ is associated with increased neutrophil recruitment as well as physiological markers of allograft injury mediated by tissue resident alveolar macrophages through TLR4/MyD88 dependent pathways (93). Consequentially, presence of endotoxin in the lung predisposes the donor organ to the fatal syndrome of primary graft dysfunction (PGD) and compromises the survival of the allograft following lung transplantation. Overall, the data suggests that while TRMs present in the donor organs may favor immunoregulatory mechanisms that promote allograft engraftment (94), their suppressive activity may be reversed toward a pro-inflammatory functional state (95), compromising organ transplant survival.

Macrophages and Rejection

Macrophage accumulation has long been recognized as a feature of allograft rejection (96). The total number of graft infiltrating macrophages correlates with worse clinical outcomes (97, 98) and with acute allograft dysfunction in kidney transplant recipients (99). Early studies from Hancock and colleagues demonstrated that macrophages represent the majority of cells that infiltrate an allograft during severe rejection episodes (100). Using immunohistochemical approaches, their study reported that macrophages represent 60% of graft-infiltrating cells in severe rejection, 52% in mild rejection and 38% in moderate rejection (100). Looking at the patterns of graft-infiltrating cells during the first days after transplantation, various human studies

have shown that the initial accumulation of monocytic cells occurs in all grafts (rejecting and non-rejecting) (101) and that infiltration of kidney allografts by macrophages within the first week of transplantation is associated with worser clinical outcomes (102). Similarly, Schreiner et al. showed an initial accumulation of macrophages in the first 24–48 h after transplantation for both donor kidney allografts and isografts, with a marked increase in monocytes/macrophages being observed only in allografts 96 h after engraftment. As such, it is not surprising that depletion of macrophages has been used to attenuate graft injury and decrease inflammation in acute rejection models (103, 104). To this end, Jose et al. by depletion of macrophages with liposomal-clodronate in a renal transplant rat model showed the contribution of macrophages to tissue damage during acute rejection (105). In another study, Ma et al. demonstrated that the depletion of monocytes/macrophages with c-fms kinase inhibitor resulted in less renal allograft dysfunction and structural damage compared to the vehicle-treated rats (106). Data from our laboratory demonstrated early after transplantation that M1-like monocytic precursors leave the bone marrow and infiltrate heart allografts in transplanted mice (107). Importantly, while M1-like monocytes rapidly convert to M2-like regulatory macrophages in the allografts of transplant recipients under costimulatory blockade treatment with anti-CD40L mAb, untreated recipients maintain M1-like inflammatory macrophages in the rejecting allografts (108). Interestingly, depletion of recipient CD11b cells using CD11b-DTR mice as recipients, prevented the induction of tolerance. This suggests that initial events that regulate macrophage polarization (M1 to M2) rather than depletion may control the fate of the immune response, since depletion of macrophages may affect the protective role of wound healing and tissue remodeling macrophages that are required to restore homeostasis in the donor organ after the transplant surgical procedure.

Despite the significant progress in determining the roles of macrophages in acute graft rejection, the mechanisms by which macrophages mediate tissue injury are not completely understood. One of the suggested mechanisms by which macrophages mediate graft loss is through the production of nitric oxide contributing to the endothelial cell cytotoxicity and tubular injury (103). Acute rejection in heart transplant recipients was associated with severe fibrosis in 1-year biopsies, which was associated with higher $CD68^+CD163^+$ M2 macrophages compared to barely present $CD68^+CD80^+$ M1 macrophages in graft (109). Similarly, infiltrating macrophages in renal allograft 1-year after transplantation exhibited an M2 phenotype with $CD68^+$ $CD206^+$ dual staining (110). It has also been suggested that $CD16^+$ monocytes might be responsible for the development of acute allograft rejection after liver transplantation, which may be associated with inhibition of Treg cells (111). Furthermore, whole-genome transcriptome analysis of biopsy samples identified an inflammatory macrophage polarization–specific gene signature, which is upregulated during acute rejection (112). In fact, the degree of macrophage infiltration correlates with increased incidence of allograft rejection (34). Consistent with the increased of macrophage/monocytes infiltration, the level of monocyte colony stimulating factor (M-CSF), a key cytokine in monocyte recruitment, is elevated in the graft during clinical rejection (113). Moreover, activated monocytes are detectable in the circulation before the clinical symptoms of acute rejection occur (114).

Gradual replacement with recipient-derived macrophages over time leads to chronic rejection through mechanisms that involve cell death, fibrosis, smooth muscle proliferation and cytokine-mediated inflammation (115). Although inflammation is supposed to be short lived and self-limited, acute inflammation can sometimes shift toward a long-lived and self-perpetuating chronic inflammatory response (116). Chronic inflammation develops within months to years after organ transplantation and is the major cause of long-term graft loss (115). The main feature of chronic rejection is obliterative vasculopathy, often accompanied by parenchymal fibrosis which results in ischemia, cell death and progressive graft failure (115, 117). Chronic rejection is characterized by infiltrating T cells and macrophages, although other cellular compartments include natural killer cells, dendritic cells, B cells and plasma cells also play a role in chronic rejection (116). However, the high number of infiltrating macrophages in the allograft, as well as their potential to produce cytokine/growth factor suggests the crucial role of macrophages as end-effector cells in a final common pathway toward cardiac allograft vasculopathy (CAV) independent of T-cell or B-cell alloreactivity (118).

Accumulation of alternatively activated M2-type macrophages is the major macrophage population localized in areas of interstitial fibrosis in chronic kidney allograft injury and correlates with the severity of fibrosis and graft rejection (110, 119). M2 polarization is considered to be anti-inflammatory, immunoregulatory and important for tissue repair and regeneration. However, during chronic rejection, the pro-fibrotic function of M2-polarized macrophages promotes interstitial fibrosis and contributes to graft failure (120). Graft-infiltrating macrophages during chronic rejection are a heterogeneous population expressing markers that are associated with M1 inflammation but also with an M2 immunoregulatory phenotype. It is possible though that immunoregulatory M2 cells are derived from M1 cells in the graft, when the pro-inflammatory microenvironment subsides over time. The predominance of a certain macrophage polarization state in the graft might determine the clinical success of the transplantation. In human kidney transplant recipients, a higher M2 ratio is associated with chronic glomerular injury and poorer graft function (121). Despite the apparent predominant role of M2 macrophages in chronic graft rejection, M1 macrophages might critically contribute with the production of eicosanoids, proteases, ROS and NO (122). To prevent chronic rejection, Liu et al. investigated the effect of macrophage depletion for a certain amount of time in a rat allogenic heart transplant model (123). Their results suggested that macrophage depletion after heart transplantation could alleviate chronic rejection through M2 polarization of regenerated macrophages, as well as the alternation of expression levels of IFN-γ, TNF-α, MCP-1 and IL-10 (123). These approaches deplete macrophages and blocking monocyte recruitment by targeting CCR- and CXCR-mediated chemotaxis that reduce vasculopathy (118, 124, 125).

The granulocyte-macrophage colony-stimulating factor (GM-CSF) and the macrophage colony-stimulating factor (M-CSF) are some of the known factors that regulate differentiation, proliferation, and function of tissue macrophages and determine the outcome of the immune response (126). While GM-CSF induces a state in which macrophages are primed for M1, M-CSF induces M2 macrophage polarization (125, 127). In a recent study, our group elucidated the molecular mechanisms behind CSF-1-mediated macrophages polarization. Our results exhibited that graft-infiltrating neutrophils in tolerized recipient allografts secreted higher levels of M-CSF compared to neutrophils from untreated rejecting mice, suggesting a potential role of M-CSF producing neutrophils in mediating regulatory M2 macrophage accumulation in the transplanted allograft (128).

Manipulation of M1/M2 polarization represents another therapeutic approach to prevent allograft rejection. Xian Li and colleagues demonstrated that M1/M2 macrophage polarization is dependent on tumor-necrosis factor receptor-associated factor 6 (TRAF6) and mammalian target of rapamycin (mTOR), respectively (129). While mice deficient for TRAF6 in macrophages prevents accumulation of M1 macrophages in recipient mice that develop severe transplant vasculopathy, deletion of mTOR prevents accumulation of M2 macrophages in long-term allograft survival without histological indications of chronic rejection, emphasizing the role of M2-polarized macrophages in chronic allograft rejection (129). The Xian Li laboratory further investigated differences between M1 and M2 macrophages and identified the adenosine triphosphate (ATP)-gated ion channel (P2x7r) as a marker of M2 cells (130). Interestingly, blockade of P2x7r using oxidized ATP, prevented M2 polarization *in vitro* and graft-infiltration *in vivo*, leading to long-term heart allograft survival. This study demonstrated that pharmaceutical targeting of M2 graft-infiltrating macrophages during chronic rejection is a promising strategy to prolong graft survival. Consistent with this view, specific deletion of RhoA or inhibition ROCK kinases with a combination of Y27632, Fasudil and Azaindole inhibited vessel occlusion and tissue fibrosis, decreased M2 macrophage infiltration and abrogated chronic rejection of cardiac allografts (131, 132).

Besides their M1/M2 pro-inflammatory and immunoregulatory functions, it is also possible that macrophages contribute to graft rejection by additional mechanisms. Macrophages in biopsy specimens from patients with active chronic renal allograft rejection co-expressed the macrophage marker CD68 as well as the myofibroblast marker a-smooth muscle actin (a-SMA), suggesting that macrophages undergo a macrophage-to-myofibroblast transition leading to interstitial fibrosis and reduced graft function (133). Similarly, cells co-expressing macrophage and a-SMA markers were found in allografts in mice. These cells derived from recipient bone marrow cells, thus were infiltrating the graft and also co-expressed M2 marker CD206. Further mechanistic studies identified a crucial role for Smad3 in macrophage-to-myofibroblast transition (133).

One key feature of circulating monocytes is their ability to migrate to the inflamed tissue and to initiate the immune response against non-self antigens. Fadi Lakkis and colleagues reported that F4/80^{-}Ly6C^{+} neutrophils, F4/80^{int}Ly6C^{+} monocytes and F4/80^{hi}Ly6C^{-} macrophages rapidly infiltrate sites of inflammation and elicit an allospecific immune response. Remarkably, in contrast to the allogeneic non-self recognition by T cells that recognize MHC molecules, macrophages were shown to recognize non-MHC molecules (134). Using B6-OVA (H-2^{b}) and B6F1-OVA (H-2$^{b/d}$) donor heart grafts transplanted into B6 Rag$^{-/-}\gamma c^{-/-}$ (H-2^{b}) recipients, this group further demonstrated that only monocytes and DC from B6 Rag$^{-/-}\gamma c^{-/-}$ recipient mice receiving B6F1-OVA (but not B6-OVA) grafts, were able to promote acute cellular rejection upon transfer of OVA antigen-specific CD4^{+} OT-II cells. The Lakkis laboratory, went on to demonstrate that monocytes and macrophages detect the polymorphic molecule signal regulatory protein α (SIRPα) on donor cells to initiate the innate alloresponse (135). SIRPα is a regulatory immunoglobulin superfamily receptor that represents a key member of the "do-not-eat-me" signaling pathway that avoids the to avoid immune response by phagocytes. SIRPα is expressed by myeloid (136) and myeloid-derived suppressor cells (MDSC) that accumulate after organ transplantation and mediate allograft tolerance (137). Mechanistically, engagement of SIRPα with its ubiquitous ligand CD47 delivers inhibitory signals and suppresses the phagocytic function and inflammatory signaling of macrophages (138–140). In the context of organ transplantation, the Lakkis laboratory demonstrated that blocking SIRPα or CD47 with monoclonal antibodies induced graft dysfunction and rejection. Blocking of SIRPα-CD47 interaction results in MDSC differentiation into myeloid cells overexpressing MHC class II, CD86 costimulatory molecule and increased secretion of macrophage-recruiting chemokines leading to loss of tolerance (141). However, a donor allograft with a SIRPα molecule that is mismatched with CD47 leads causes monocytic cell activation and initiation of the immune response to the transplanted organ (135). More recently, the Lakkis laboratory also demonstrated that polymorphisms in the SIRPα gene were required to induce monocyte memory is against non-self MHC molecules. In this study, it was demonstrated that deleting the PIR-A in the recipient or blocking the paired immunoglobulin-like receptor-A (PIR-A) binding to donor MHC-I with a PIR-A3/Fc inhibits alloantigen specific memory of myeloid cells and promotes indefinite allograft survival in a murine kidney and heart transplant model (142). Overall, these studies provide compelling evidence demonstrating that monocytes initiate the immune response, determine the critical role of SIRPα polymorphic differences in the activation of graft reactive macrophages and that the immunological memory to innate myeloid cells can be potentially targeted to promote the induction of transplantation tolerance.

Macrophages and Tolerance

The participation of graft-infiltrating macrophages in the rapid, stereotypical inflammatory reactions that cause secondary tissue damage during ischemia-reperfusion injury (143) and acute episodes (144) has been long-recognized. However, we are also beginning to understand the vital role of suppressor macrophages in preventing rejection and re-establishment of tissue homeostasis after transplantation (145). Given their influence over transplant

outcome, manipulating the balance between graft-protective and graft-destructive macrophage activities represents an attractive therapeutic strategy (146). Various approaches to controlling macrophage responses have been proposed, including adoptive cell therapy with regulatory macrophages (Mregs). In previous work, it was shown that treatment with *ex vivo*-generated $CD11b^+$ $Ly6C^{-/low}$ $Ly6G^-$ $CD169^+$ Mregs could prolong fully-allogeneic heart graft survival in non-immunosuppressed mice (147). Mechanistically, Mregs can directly suppress T cell proliferation and survival through an iNOS-dependent pathway and the secretion of anti-inflammatory factors (148). More recently, Riquelme et al. demonstrated that Mregs induce $TIGIT^+FoxP3^+$ Tregs that produce IL-10 and non-specifically mediates bystander suppression of allo-stimulated CD4+ and CD8+ T cells (149). An equivalent population of human $CD11b^+CD115^+DC\text{-}SIGN^+$ Mregs arises from peripheral blood $CD14^+$ $CD16^-$ monocytes that are cultured with M-CSF for 6 days prior to stimulation with IFN-γ (150). During this period, a gradual down-regulation of CD14 is observed, which may recapitulate the physiological transition of human M1-like $CD14^+$ $CD16^-$ inflammatory monocytes into M2-like $CD14^{-/low}$ $CD16^+$ resident macrophages. Interestingly, presence of human Mregs correlates with an increase in $TIGIT^+FoxP3^+$ Treg in kidney transplant recipients (149), which is consistent with the preclinical experiments described above. In the clinical setting, Mregs are currently being investigated in humans in the *ONEmreg12* trial, a phase-I/II study to minimize maintenance immunosuppression in kidney transplant recipients (151). This and previous clinical studies suggest Mregs could be used as a cell-based tolerance-promoting therapy, and for this purpose a good manufacturing practice-compliant production process for manufacturing an Mreg-containing cell product, known as "Mreg_UKR," has been established (152).

Suppressive macrophages are also be generated in recipient mice treated with costimulatory blockade. Our laboratory demonstrated that anti-CD40L mAb favors accumulation of $CD11b^+CD115^+DC\text{-}SIGN^+$ expressing macrophages in the allograft, which promotes the expansion of Treg, while inhibited $CD8^+$ T cell accumulation (108). Mechanistically, DC-SIGN macrophages produce regulatory IL-10 and their *in vivo* accumulation is controlled by M-CSF, which is consistent with the Mreg development requirements, phenotype, and function as described by James Hutchinson laboratory above. Besides costimulatory blockade, nanoparticles have also been used to deliver immune regulatory agents to monocytes and macrophages *in vivo* (153). For example, delivery of mycophenolic acid (MPA) by means of PLGA nanoparticles (NP) results in a significant allograft survival prolongation compared to conventional MPA treatment in a murine model of skin transplantation. Mechanistically, Daniel Goldstein and colleagues demonstrated that uptake of NP-MPA by myeloid cells leads to upregulation of programmed death ligand-1 (PD-L1), which results in decreasing their potential to prime alloreactive T cells associated with prolonged allograft survival (154). More recently, our laboratory described a promising strategy to induce long-term allograft survival through *in vivo* targeting of macrophages with nanobiologics. Our laboratory used an effective *in vivo* platform to deliver an mTOR inhibitor (mTORi) and NF-kB inhibitor (TRAF6i) *via* high density lipoprotein nanobiologics (HDL) in a murine vascularized heart transplant model. The HDL-based nanobiologics preferentially targeted myeloid cells and promoted M2 regulatory macrophage polarization, which resulted in prevention alloreactive CD8 T cell-mediated immunity and expansion of Treg (155). As a result, we believe that nanobiologics-based delivery of immunotherapeutic agents has great potential in organ transplantation as they improve the pharmacokinetics, minimize the off-target effects, maximize its dosage at the site of action, and can be as used as controlled release systems in a spatiotemporal manner (156). Taken together, it has become evident that the *in vivo* manipulation of macrophages through the use of nanobiologics represents a promising strategy for long-term allograft survival.

Epigenetic Regulation of Macrophages and Innate Immune Memory

Macrophages are highly plastic cells that adopt M1 and M2 phenotypes through mechanisms ultimately resulting from integrating their preexisting history and surrounding environmental signals to enable a distinct transcriptional program. In addition, their distinct transcriptional program must enable their phenotype to be distinct from other myeloid cells. The transcriptional program that makes them distinct is controlled *via* various epigenetic processes, among which include DNA methylation, histone modification and expression of non-coding RNAs. These epigenetic modifications of the landscape lead to either compaction or opening of the chromatin, followed by the combination of DNA and DNA-binding proteins, which are associated to gene activation or repression. This is the basis of trained immunity, a new concept in the field, which postulates that innate immune cells can retain a memory of certain primary stimuli *via* epigenetic mechanisms, thus potentially priming them to initiate a stronger response upon a secondary stimulus.

The term "epigenetics" was first pioneered by C.H. Waddington, seeking to explain how phenotypes could be explained not solely by genetic inheritance (157). He later then proposed the concept of the "epigenetic landscape," which posited that as cells differentiate, they become restricted in their possible fates (158). This concept of the epigenetic landscape was further elaborated on by Thomas Jenuwein and David Allis with their proposal of a "nucleosome code," an extension of the "histone code" (159, 160). In their "nucleosome code" hypothesis, they propose that certain covalent modifications to the tails of histones in a region of DNA ultimately result in regional compaction or opening of chromatin. How closed or opened the chromatin in a particular region is then ultimately governs the ability of DNA-binding proteins and ultimately RNA Polymerase from binding to certain genes and subsequently transcribing. The histone modifications that encourage opening of the chromatin include H3K4me3, H3K9ac, and H3K27ac, weaken the grip tail of histone 3 (H3) to the DNA allowing other DNA-binding proteins to bind, while repressive histone modifications including H3K9me3, H3K27me3, and H3K36me3 enhance the grip of H3 to the DNA promote the opposite effect. How protected the DNA is

by chromatin opening or compaction, as a result of these histone modifications regionally, ultimately mediates the accessibility of RNA Polymerase to specific sites, thus governing gene activation or gene repression.

The link between an external stimulus to macrophages and modification of the epigenetic landscape, thus establishing the importance of the epigenome in macrophages, was first established in 1999, where LPS stimulation was shown to induce IL12 p40 production by the remodeling of nucleosomes positioned at its promoter (161). This process was later shown to be TLR-dependent *via* acetylation of residues on histone 3 and histone 4 typically associated with open chromatin. On a genome-wide level, TLR activation has been shown to induce a program where the "brakes" on inflammatory gene expression are withdrawn by removing repressive histone modifications. Specifically it was shown that the H3K27me3 demethylase JMJD3, is induced by LPS stimulation in macrophages, and thus promotes an inflammatory gene program (162). Conversely, histone modifications pertaining to gene activation, modifications that lessen the grip of nucleosomes on the DNA, are added on at specific loci upon LPS stimulation by various epigenetic writers including histone methyltransferase myeloid lymphoid leukemia (163). The fact that macrophages' epigenetic architecture is easily changeable upon external stimulation should not be surprising, given that large changes in histone methylation and acetylation patterns occur in the transition from monocytes to macrophages alone (29). In summary, these early studies made it clear that significant epigenetic changes were happening in macrophages.

Prior to stimulation to an exogenous substance, the epigenetic landscape of monocytes and macrophages must be properly established to develop their distinguished phenotype. This is done by the LDTFs (lineage-dependent transcription factor) PU.1 and the C/EBP family of transcription factors, which bind to macrophage-specific genes and enhancers and are critical for proper monocyte and macrophage development (164). These transcription factors are thought to prime these sites, including those of inflammatory genes, suggested by the fact that these loci are marked by the presence of PU.1, H3K4me1, and open chromatin. However, to keep the brakes on the expression of inflammatory genes, these same loci of inflammatory genes are decorated with repressive histone marks that promote chromatin compaction including H3K9me3, H3K27me3, and H4K20me3 and are bound by co-repressors (165–168). Only upon exogenous stimulation, these brakes are released by appropriate epigenetic erasers on the enhancers and promoters of inflammatory genes, and concurrently activating histone marks are added on by appropriate epigenetic writers.

Trained immunity is a relatively new compelling concept in immunology, whose foundation is primarily epigenetic based. It posits that innate immune cells can retain a memory after a primary stimulus and after a return to a resting phase enact a heightened response upon a secondary stimulus (169). The concept was first proposed in 2011 as a means to explain the phenomenon in vertebrates of protective effects of vaccinations or infections, including BCG vaccination and *C. albicans* infection, to unrelated stimuli in a manner independent of the adaptive immune system (170). Soon after, the mechanisms underlying these memory phenomena were soon determined to be based on epigenetic and metabolic reprogramming, with the two being intertwined (29, 171–173). Specifically, significant H3K4me3 deposition upon either BCG vaccination or ß-glucan stimulation was found at the gene promoters of inflammatory genes including TNF-α, IL-6 and glycolysis genes including hexokinase and phosphofructokinase, thus establishing a memory in macrophages. This process was shown to be was mTOR-dependent (172, 173) and preventing epigenetic changes through the use of mTOR inhibitors, inhibited the shift in metabolism toward glycolysis and the acquisition of H3K4me3 at key inflammatory gene promoters.

With regards to organ transplantation, Fadi Lakkis and colleagues described that monocytes are able to recall skin grafts exhibiting memory features normally attributed to adaptive immune cells. Using BALB/c $Rag^{-}/^{-}$ mice as recipients of BALB/c ($H\text{-}2^{d}$), allogeneic B6 ($H\text{-}2^{b}$) and "third-party" C3H ($H\text{-}2^{k}$) donor skin grafts rechallenged with B6 splenocytes 1 week after engraftment, the study demonstrated that monocytes were able to mount an inflammatory response 1 week after transplantation independently of the adaptive immune system (134). Interestingly, BALB/c recipients mounted an allo-dependent response to allogeneic B6, but also to "third-party" C3H (134). Although the third-party response was statistically lower than the allo-dependent response, the data suggests that monocytes are able to respond to non-specific recall stimuli, a feature of trained immunity. Challenging the view of non-specific responses mediated by macrophages, studies from Xian Li and colleagues reported that reconstituted $Rag^{-}/^{-}\gamma c^{-}/^{-}$ hosts with syngeneic B6 CD4+ T cells and donor BALB/c cells results in *in vivo* killing of donor BALB/c cells transferred 2 weeks after reconstitution but does not result in the rejection of "third-party" C3H cells (174). This argues in favor of further investigating epigenetic mechanisms of macrophage recall processes and the potential implication of SIRPα in these processes, as described above. Remarkably, this study demonstrated that macrophage-mediated rejection of recall responses can be prevented with CD40/CD40L costimulatory blockade during the first stimuli. This suggests that anti-CD40L mAb treatment may prevent the accumulation of memory-like macrophages in the donor allografts early after transplantation.

Inhibition of trained macrophages in the allograft can be achieved by targeting the mTOR pathway in myeloid cells *in vivo* (155). We recently demonstrated that vimentin promotes macrophage training *via* dectin-1 signaling, which results in increasing deposition of H3K4me3 at the promoter of TNF-alpha and IL-6 upon a secondary stimulation with HMGB1, another protein highly expressed in the donor allograft. The same trend in epigenetic changes occur *in vivo* using an experimental mouse model of heart transplantation. Interestingly, inhibition of trained immunity with mTORi-HDL nanobiologics promoted long-term allograft survival *via* Treg expansion and inhibition of cytotoxic T cells.

In addition to targeting trained immunity in organ transplantation *via* the administration of mTORi-HDL nanoparticles, there is the potential use of small molecules that inhibit epigenetic-related proteins including HDAC inhibitors (HDACi) and BET inhibitors (BETi). HDACi are thought to

primarily inhibit histone deacetylation, thus promoting gene expression at specific loci, while BETi inhibit the binding of BET proteins to acetylated regions of the genome, which normally promote gene expression at specific loci (175). However, reports specifically implicating their use in the context of transplant have been few. In regards to the use of BET inhibitors, a synthetic compound, I-BET, was developed that was shown to repress gene expression of LPS-inducible genes in bone marrow derived macrophages (BMDM) *ex-vivo* (176). The importance of BET proteins in aiding gene expression of inflammatory genes in macrophages was established through use of *brd2 lo* mice and silencing of BET proteins through siRNA studies (177). With regards to the use of an HDACi to prevent allograft refection, an inhibitor of HDAC6, KA1010, was shown to reduce allograft skin rejection through mechanisms that involved reduction in CD4 T cells with an increase in the Treg population (178). The effect of HDACi on macrophages on the other-hand is not clear and *in-vitro* experiments on BMDM treated trichostatin A (TSA), a class I and II HDACi, displayed a phenotype favoring progenitor-like myeloid cells rather than differentiated macrophages. These macrophages displayed a mixed M1/M2 phenotype according to cytokine and chemokine secretion analysis, suggesting that treatment with HDACi alone may not be a suggestable mode of therapeutic treatment (179). On the contrary, a study by Thangavel and colleagues demonstrated that combinatorial treatment of TSA with 5-Aza 2-deoxycytidine (Aza), a DNA methyl transferase (DNMT) inhibitor, was able to promote an M2 phenotype in macrophages and to reduce inflammation in an acute lung injury model (180). Overall, while drugs targeting epigenetic modifiers including HDACs, BET proteins and DNMTs do hold promise as therapeutic approaches that promote long-tern allograft survival in organ transplantation, it appears that successful use of these drugs to prevent graft rejection will require their use to be in combination with other drugs.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Organ transplantation is a life-saving strategy for terminal and irreversible organ failure. While the solid organ transplantation has achieved an excellent success in short-term graft survival rates, the long-term survival rates of organ transplants remain suboptimal. The pathophysiology of graft rejection is multifactorial and growing evidence suggests that macrophages are key mediators of acute and chronic graft loss, through the secretion of inflammatory mediators that activate the adaptive alloimmune response. Historically, accumulation of macrophages in the donor organ has been associated with transplant rejection (181, 182) as allogeneic antigen-primed macrophages mediate allograft rejection (183). However, not all macrophages are associated with graft loss. Different subpopulations of macrophages regulate the allograft immune response through protective mechanisms based on their phenotype and function. As a result, the identification of the *in vivo* signaling pathways that govern macrophage polarization and modulate their function may provide new therapeutic targets that promote allograft survival.

Therapeutic agents that regulate macrophage polarization that promote the accumulation of regulatory macrophages are potential candidates to promote long-term allograft survival in transplant recipients. In addition, identification of previously unrecognized pathways associated with chronic allograft rejection may offer new therapeutic avenues for intervention. Classically, the innate immune response has been defined as a non-specific rapid response, followed by a later-onset of antigen-specific adaptive immune cells. However, accumulating findings have challenged the fact that innate immune cells do not possess a memory, leading to the concept of innate immune memory and trained immunity. This concept postulates that stimulated innate immune cells are primed to recognize specific ligands and secrete specific cytokines more rapidly upon a second stimulus. This type of memory is retained by mechanisms of epigenetic and metabolic changes in innate immune cells exposed to particular ligands. As a result, therapeutic targeting of trained immunity represents a novel treatment paradigm to prevent allograft rejection. Thus, a comprehensive understanding of the immunobiology of different macrophage subsets is crucial to develop novel strategies that promote long-term allograft survival in transplant recipients and to translate macrophage-targeted therapeutic strategies in the clinic.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Untangling Local Pro-Inflammatory, Reparative, and Regulatory Damage-Associated Molecular-Patterns (DAMPs) Pathways to Improve Transplant Outcomes

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Detrimental inflammatory responses after solid organ transplantation are initiated when immune cells sense pathogen-associated molecular patterns (PAMPs) and certain damage-associated molecular patterns (DAMPs) released or exposed during transplant-associated processes, such as ischemia/reperfusion injury (IRI), surgical trauma, and recipient conditioning. These inflammatory responses initiate and propagate anti-alloantigen (AlloAg) responses and targeting DAMPs and PAMPs, or the signaling cascades they activate, reduce alloimmunity, and contribute to improved outcomes after allogeneic solid organ transplantation in experimental studies. However, DAMPs have also been implicated in initiating essential anti-inflammatory and reparative functions of specific immune cells, particularly Treg and macrophages. Interestingly, DAMP signaling is also involved in local and systemic homeostasis. Herein, we describe the emerging literature defining how poor outcomes after transplantation may result, not from just an over-abundance of DAMP-driven inflammation, but instead an inadequate presence of a subset of DAMPs or related molecules needed to repair tissue successfully or re-establish tissue homeostasis. Adverse outcomes may also arise when these homeostatic or reparative signals become dysregulated or hijacked by alloreactive immune cells in transplant niches. A complete understanding of the critical pathways controlling tissue repair and homeostasis, and how alloimmune responses or transplant-related processes disrupt these will lead to new immunotherapeutics that can prevent or reverse the tissue pathology leading to lost grafts due to chronic rejection.

Keywords: transplantation, ischemia–reperfusion injury, damage-associated molecular patterns, tissue repair and fibrosis, regulatory T cell, macrophage, alarmins, immunometabolism

INTRODUCTION

Tissue injury negatively impacts outcomes after the transplantation (Tx) of cells, tissues, or organs. In solid organ transplantation (SOTx) and vascularized composite allograft (VCA) transplantation ischemia reperfusion injury (IRI), surgical manipulations, donor trauma, and brain death initiate the release of self-derived molecules containing damage-associated molecular patterns (DAMPs) that alert immune cells to the damage (1, 2). Released DAMPs will act on resident donor and graft-infiltrating immune cells to shape local and systemic immune functions that determine SOTx short and long-term outcomes. Some DAMPs are not released from necrotic cells, but instead are exposed on stressed or dying cell membranes (3). This review briefly discusses recent advances in understanding how DAMPs contribute to inflammation that stimulates alloimmunity, but highly detailed information can be found in numerous excellent reviews (1, 2, 4–6). In this review, we also elaborate on emerging concepts in Tx that are developing from an evolving understanding of the potential beneficial function of DAMPs in tissue repair and systemic homeostasis. We will also discuss examples of how these reparative or homeostatic DAMP pathways may become dysregulated or re-appropriated throughout the graft by anti-donor immune responses to also contribute to chronic rejection (CR).

CURRENT UNDERSTANDING OF DAMPS AS DRIVERS OF ALLOIMMUNE RESPONSES AND POOR OUTCOMES AFTER SOTX

Numerous DAMPs released during cellular stress, tissue injury, or *via* inflammatory cell death pathways such as ferroptosis, necroptosis, pyroptosis, have been identified (6, 7). How these DAMPs initiate sterile inflammation and contribute to anti-AlloAg immune responses has been reviewed recently (1, 2, 4, 5). Well-characterized pro-inflammatory DAMPs active in Tx include nuclear materials, such as high-mobility group box 1 (HMGB1), interleukin (IL)-1α, cytoplasmic components, including ATP, heat shock proteins (HSPs), and s100 proteins, mitochondrial (mt) contents like mtDNA or mt transcriptional factor A, as well as extracellular matrix (ECM) components, including hyaluronan, fibronectin, and heparan sulfate have been assessed in Tx models (**Table 1**). Oxidative injury-induced neo-antigens and typically sequestered cytoplasmic proteins such as HSPs and the ER chaperone calreticulin can be exposed on or incorporated in the cell membrane.

Many defined DAMPs are recognized by conserved pattern recognition receptors (PRRs) that also recognize non-self materials containing pathogen-associated molecular patterns (PAMPs) to generate protective immune responses (**Table 1**).

TABLE 1 | Inflammatory DAMPs and their impact on SOTx outcomes.

Family	Molecule	Receptors	Role in Tx- related inflammation/immunity/outcomes	References
Inflammatory DAMPs				
	Histones	TLR2, TLR4, and TLR9	Causes TLR- and inflammasome-dependent generation of inflammatory response by innate cells	(6, 8, 9)
	HMGB1	TLR2, TLR4, RAGE, and TIM3	Promotes the production of pro-inflammatory cytokines and chemokines by innate immune cells Induces metabolic reprogramming supporting the pro-inflammatory functions of myeloid APC Promotion of AR and CR in experimental models Implicated in poor outcomes after clinical transplantation	(2, 10–15)
	IL1α	IL-1R	Promotes the production of pro-inflammatory cytokines and chemokines by innate immune cells	(16)
	ATP	P2Y2 and P2X7	Attraction and activation of innate cells Promotes inflammasome activity Causes the release pro-inflammatory cytokines supporting rejection Promotes IL-1β and IL18 secretion and initiates inflammatory cell death Stimulates alloimmunity	(6, 17–20)
	Vimentin	Dectin-1	Induces metabolic reprogramming supporting the pro-inflammatory functions of myeloid APC Induces macrophage TNF α and IL-6 production	(6, 10)
	Hyaluronan (HA)	TLR2 and TLR4	Low molecular weight breakdown products stimulate macrophages pro-inflammatory cytokine production Supports of alloimmunity	(21–23)
	S100s	TLR2, TLR4, RAGE	Potent immunostimulatory activity, monocytes and neutrophils recruitment	(6, 24)
	Mitochondrial DNA (mDNA)	TLR9	Macrophages and neutrophils activation Promotes inflammasome activity Causes the release pro-inflammatory cytokines supporting rejection Promotes IL-1β and IL18 secretion and initiates inflammatory cell death	(6, 25, 26)

This overlap has made it difficult to clearly define the role of specific DAMPs versus bacterial product contamination in early studies (6). Nevertheless, studies with antibodies targeting specific DAMPs, as well as the generation and use of DAMP KO mice, have established that self-derived materials influence SOTx outcomes in experimental transplant models (5). Other PRR-type receptors able to detect DAMPs include NOD-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptor (RLRs), C-type lectin receptors, and intracellular DNA sensors, such as cyclic GMP-AMP synthase (cGAS), and absent in melanoma 2 (AIM2) (6). Non-PRR receptors such as the receptor for advanced glycation end products (RAGE) as well as G-protein-coupled receptors like formyl peptide receptors (FPRs) and P2Y receptors, detecting extracellular nucleotides like ATP have been revealed to be important in sterile inflammation and supporting alloimmune responses leading to acute rejection (AR) and CR (1, 2). Exposed HSPs and calreticulin displayed on the cell surface can be recognized by CD91 and aid engulfment and presentation of alloAg by antigen presenting cells APC (27, 28). Natural antibody responses to displayed oxidative-induced neo-antigens can also trigger APC activation *via* the complement cascade (3, 4). DAMPs stimulation of pro-inflammatory immune response leading to increased alloimmunity is well appreciated to contribute significantly to both AR and CR in experimental models (10–12) and this important subject has been the focus of several recent and thorough reviews (1, 2, 4, 5). Clinically, DAMPs are implicated in AR of liver grafts (13) as well as CR of cardiac transplants (14). HMGB1 is induced by IRI in cadaveric kidney transplants, but absent from living donor grafts that have better outcomes (15). Similarly, recipients with a mutation in TLR4 that decreases the affinity for HMGB1 exhibit better early graft function (15).

The concept that the presence of DAMPs leads to poor early and late Tx outcomes is supported by the clinical observation that shorter IRI times result in reduced risk of AR and CR after SOTx. The finding that HLA mismatch, recipients of living, unrelated donor kidneys have significantly better long-term outcomes relative to those receiving HLA-matched cadaveric kidney grafts subjected to longer periods of ischemia support this premise (29). Furthermore, each hour of cold ischemia increases the odds of AR (30), early graft failure, and mortality after kidney Tx (31). Similar negative associations with outcomes with increased ischemia times have been made for cardiac (32, 33) and liver Tx (34, 35).

DIRECTLY TARGETING DAMP SIGNALING TO IMPROVE OUTCOMES

Clinical observations supporting DAMPs as a dominant initiator of IRI and contributor to alloimmunity and rejection have compelled efforts to antagonize them after SOTx. As outlined in **Table 1**, many DAMPs initiate function by activating TLRs and the downstream adapter MyD88. Pre-clinical studies using MyD88 or TLR deficient mice have identified both as effective targets to limit IRI, inflammation, and improve transplant outcomes (36). These early rodent studies utilizing TLR and MyD88 deficient mice have led to the development of numerous biologics targeting TLRs and MyD88, which have shown promise in promoting tolerance or limiting rejection (37–42). For example, newer agents like Eritoran, which is a synthetic analog of the lipid A portion of lipopolysaccharide (LPS) that can antagonize LPS binding (43, 44), or the 2-aminothiazole-derived MyD88 inhibitor TJ-M2010-5, are potent inhibitors of DC activation and promoted long-term heart and skin graft survival in rodents (37).

Nevertheless, successful translation of agents targeting these pathways remains to be realized. NI-0101 is a humanized, anti-TLR4 antibody that interferes with TLR4 dimerization and provides sustained blocking of LPS-induced cytokine production in healthy volunteers (45, 46). It, however, failed recently to alter disease in a Phase II, randomized, placebo-controlled, double-blind, international, multicenter study of individuals with moderate to severe rheumatoid arthritis (47). OPN-305 (Tomaralimab, Opsona Therapeutics) is a humanized, IgG4 monoclonal antibody against TLR2 under recent phase II investigation to prevent delayed graft function after kidney Tx (48). Both OPN-305 and OPN-201, its murine monoclonal parent antibody, have shown a potent ability to antagonize TLR2 signaling that is activated by HMGB1 and several HSPs, and limit IRI in rodents and swine (49, 50). An earlier Phase I study established that OPN-305 infusions were well tolerated and consistently inhibited heat-killed *listeria monocytogenes*-mediated IL-6 secretion by patients' peripheral blood cells for periods up to 90 days (51). While these studies would suggest promise, the future of OPN-305 is unclear. There have not been any published reports from the Phase II trial, and as of 2019, Opsona was liquidated after the search for a development partner or buyer for its leading drug therapy was fruitless (52). Given the importance of TLRs and MyD88 to the initiation of detrimental pro-inflammatory response to after IRI and demonstrated in pre-clinical Tx studies, it can be expected that efforts to identify clinical candidates that effectively target this pathway and limit early inflammation after Tx will persist. As trials of these agents move forward, it will be very interesting to observe if the benefits that potent TLR signaling pathway inhibitors have against DAMP-driven IRI and tissue damage and the generation of alloimmune responses can outweigh the expected blunting of effective anti-pathogen immunity (53). If this class of drugs only produces the generalized immunosuppression similar to that observed with non-specific TCR signaling inhibition with drugs like Tacrolimus, their impact will be limited. However, if potent drugs blocking TLR signaling can be delivered for only for a short window in or around Tx surgery for highly effective prevention of the general innate inflammation initiating AlloAg-specific T cell responses DAMP-activated antigen presenting cells (APC), this class of drugs blocking tissue damage-mediated inflammation could be transformative.

TARGETING IMMUNOMETABOLISM TO LIMIT THE PRO-INFLAMMATORY ACTIVITY OF DAMPS

Ischemia resulting from organ procurement not only causes cellular stress and cell death that releases DAMPs, but it will also cause graft hypoxia that will program graft-resident, donor immune cells and infiltrating recipient immune cells for an inflammatory response to these DAMPs. Myeloid cells in a hypoxic environment upregulate hypoxia-induced factor-1 alpha (HIF-1α), which is critical to coordinate a local pro-inflammatory response (54). HIF-1α dimerizes with HIF-1β and translocates to the nucleus to modulate transcription of genes with promoters containing HIF response elements (HREs), with many of the induced gene products supporting the recruitment, retention, and function of pro-inflammatory macrophages. The expression of HIF-1α is essential to myeloid cell transition to glycolysis during pro-inflammatory immune responses. Early studies by Cramer et al. demonstrated that HIF-1α deletion using a lysozyme 2 (*Lys2*, or L*ysM*)-driven Cre recombinase resulted in monocytes and macrophages that were defective in glycolysis and, as a result, impaired their capacity for motility, invasiveness, and phagocytic ability (55).

The binding of TLR agonists by myeloid APC also shifts the cellular metabolism of myeloid cells towards glycolysis, which will supply ATP to support their inflammatory functions in oxygen sparse environments, but also generate nucleotides, lipids, and reactive oxygen species (ROS) used for anti-pathogen effector functions (56–59). Such metabolic changes originate from TLR ligation-mediated inhibition of mitochondrial oxidative phosphorylation (OXPHOS) and associated remodeling of the tricarboxylic acid (TCA) cycle. A pivotal determinant of myeloid cells' metabolic reprogramming during the generation of a pro-inflammatory subset is the *de novo* expression of inducible nitric oxide synthase (iNOS). Expression of iNOS generates large quantities of nitric oxide (NO) that inhibits mitochondrial respiration through the stable nitrosation of Complex I of the electron transport chain (ETC), as well as reversible inhibition Complex IV and isocitrate dehydrogenase (60). Induced changes in the TCA cycle result in the generation of metabolic intermediates that are determinants of the macrophage inflammatory phenotype due to their enforced reliance on glycolytic metabolism and preventing macrophages' repolarization away from a pro-inflammatory macrophage subset (61). The O'Neill group established that TLR4 ligation limits glutamine-dependent anaplerosis, or the replenishment of TCA cycle intermediates, to cause elevated levels of succinate to reach levels causing HIF-1α stabilization resulting in augment production of IL-1β (62). Interestingly, hypoxia alone was a weak inducer of *Nos2* mRNA in myeloid APC but synergized with TLR3, TLR4, and TLR9 agonists to prevent HIF-1α-dependent upregulation of *Nos2* mRNA and iNOS protein (63). The stimulation of TLRs by DAMPs like HMGB1, S100 proteins, mRNA, and mtDNA released during or after IRI in the hypoxic graft is a dominant driver of the pro-inflammatory responses that lead to early graft injury and failure, as well as stimulation of alloimmune responses that cause acute and chronic SOTx rejection.

To date, TLRs and their pathways have proven challenging to antagonize early post-SOTx to limit early inflammation, but HIF-1α would seem like an attractive downstream target that could suppress myeloid cell pro-inflammatory activity by limiting glycolytic metabolism. However, limited pre-clinical studies indicated that non-specific targeting of HIF-1α might be detrimental due to important graft tissue protections provided by HIF-1α in stromal and parenchymal tissues. In an orthotopic tracheal Tx model, adenovirus-mediated HIF-1α gene transfer to the graft promoted repair of mouse airway allograft microvasculature and attenuated CR (64). This effect was due to the HIF-1α-dependent recruitment of recipient pro-angiogenic cells that contributed to repairing damaged airway microvasculature. HIF-1α delivery before Tx increased graft perfusion to decreased fibrosis and improve graft survival (64). Other studies have demonstrated the importance of protective signaling of HIF-1α in the stroma and parenchyma after heart and kidney IRI before transplant (65, 66). The deletion of HIF-1α in macrophages causes the decreased production of vascular endothelial growth factor (VEGF), which is an important stimulus for initiating repair and vascularization of tissues damaged by IRI. While the early activity of the HIF-1α-VEGF is vital for the initiation of tissue repair, the sustained activation of the HIF-1α-VEGF pathway may later contribute to CR (67). HIF-1α is a critical factor that shapes the immune response after IRI and Tx; effectively targeting it will require both myeloid cell-specific delivery and understanding whether the graft's current conditions will require a HIF-1α antagonist or agonist.

Recently, Ochando and colleagues completed exciting studies where they used myeloid cell-targeting nanoparticles to deliver an inhibitor of mammalian target of rapamycin (mTOR), which blocked HMGB1-induced glycolytic reprogramming (10). When mTOR-targeting nanoparticles were combined with antagonism of the CD40-TRAF6 axis, the result was long-term, fully MHC-mismatch cardiac allograft acceptance (10). The power of mTOR in stimulating macrophage pro-inflammatory functions was also demonstrated when the Medzhitov group established that IL-10 regulates macrophage pro-inflammatory responses by limiting glycolysis through the induction of a potent mTOR inhibitor, DDIT4 (68). IL-10 has long been understood to generate reparative and regulatory myeloid cells that support transplant tolerance, yet the mechanism(s) by which IL-10 mediates such potent impacts on myeloid cells has remained poorly understood. These studies established that myeloid cells stimulated with IL-10 resists the typical metabolic reprogramming induced by TLR4 ligation and, instead, maintained mitochondrial integrity and function to support OXPHOS. IL-10 also limits activation of the inflammasome by ATP in TLR4-stimulated macrophages. These recent investigations provide compelling evidence that myeloid cell-specific inhibition of mTOR and glycolysis will be an effective way to antagonist multiple DAMP pro-inflammatory pathways early after Tx.

ATP, while the energy currency of immunometabolism, has also emerged as a highly influential TLR-independent DAMP released by damaged, dying, and activated cells (6), particularly after Tx. Recent basic discovery and clinical studies have elucidated how immune cells release ATP as an inflammatory signal in response to allogeneic Tx and signal in a feedback mechanism *via* P2X7 to promote the release pro-inflammatory cytokines supporting rejection (17). The pro-inflammatory cytokine IL-1β plays a crucial role in early immune responses to tissue injury and pathogens, but even when induced by pro-inflammatory stimuli it is generated in an inactive pro-form that requires processing by caspase-1 to an active form. Activation of caspase-1 relies on the inflammasome, a multi-protein complex containing members of the nucleotide-binding domain- and leucine-rich repeat-containing receptor (NLR) family. The best-characterized inflammasome is NLRP3, whose activity is induced by a wide range of diverse stimuli, including PAMPs, numerous DAMPs, such as ATP and mtDNA, ROS, and even particulate matter (69). Activating the NLRP3 inflammasome and subsequent secretion of IL-1β requires two signals (69). First, a NFκβ activating signal, often a TLR-agonist, that induces and increases the expression of pro-IL-1β and NLRP3. A second signal, like the activation of the P2X7 receptor by extracellular ATP, or TLR detection of released mtROS or mtDNA, will cause inflammasome formation. The inflammasome activates associated caspase-1 that mediates the processing of pro-IL-1β or the closely related IL-1 family member, IL-18, into their mature and active cytokine form. Targeting P2X7 with an irreversible antagonist for 14 days after fully mismatched murine heart transplant promoted long-term cardiac transplant survival (18). Additional recent studies convincingly revealed that extracellular ATP is an early DAMP released by the transplant. ATP acts in a feed-forward loop to sustain high extracellular ATP levels in the graft by causing infiltrating recipient myeloid cells to release ATP locally. These high levels of ATP are crucial for augmented *Nlrp2, Casp1*, and *Il1b* expression in the graft, as well as, the secretion of IL-18 that contributes to type 1 alloimmune responses (17). While the survival benefits provided by inhibiting P2X7 in a rigorous skin transplant model was modest (17), the emerging importance of ATP and P2X7 after Tx suggest that targeting this pathway may be highly effective when combined with low doses of immunosuppression, or act synergistically with TLR antagonists to limit the activating signals feeding inflammasome/caspase-1 activation.

NATURAL PRO-INFLAMMATORY DAMP REGULATORS

As discussed above, the bulk of past Tx-related studies have sought to identify how to blunt DAMP pathways that initiate early inflammation after IRI responses with the prediction that this would lead to reduced alloimmune response or even aid tolerance induction. This approach has shown promise, particularly in experimental animal studies, where DAMP targeting with antibodies to DAMPs or their receptors reduces alloimmunity and limits AR. DAMP targeting also limited later development of CR-associated graft vasculopathy and fibrosis. Recently, another promising approach has been the identification of natural/endogenous pro-inflammatory DAMP regulators (**Table 2**) that the body utilizes to regulate pro-inflammatory DAMPs and harness them to limit alloimmunity or improve transplant outcomes. Past studies by Liu and colleagues demonstrated that the sialic-acid–binding immunoglobulin-like lectins (Siglecs)-CD24 signaling pathway suppresses inflammation triggered by DAMPs to protect against pathological inflammatory responses arising from cell death and necrosis (70). Importantly, they revealed that the Siglec-CD24 pathway only regulated DAMP signaling, while leaving the protective immune response to pathogen-derived PAMPs unabated (70). CD24 associates with DAMPs, particularly HMGB1, to negatively regulate their stimulatory activity by binding and presenting them to Siglecs that then downregulate immune responses *via* intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs) domains (71). Active CD24-DAMPs-Siglec axis limits the inflammatory signaling in myeloid antigen, especially DC, to blunt their pro-inflammatory functions, particularly the secretion of TNF-α, IL-1β, and IL-6 (71). Harnessing this pathway has been particularly promising as a means to limit alloimmunity (72, 110) and assessment of the CD24-DAMPs-Siglec axis as a way to limit AR and CR after SOTx may also be worthy of further focused investigation.

IMMUNE CELLS INVOLVED IN A REGULATED, IMMUNE-MEDIATED TISSUE REPAIR PROCESS

Tissue-Resident and Type 2 Cytokine-Activated Monocyte-Derived Macrophages

Tissues and organs of the body have resident populations of macrophages seeded during the embryonic or early postnatal period from early hematopoietic progenitors from the yolk sac and fetal liver, as well as a small subset originating later from circulating monocytes (111). In homeostatic mouse and human hearts, myeloid cells can be fractionated by their expression of the C-C chemokine receptor 2 (CCR2). At homeostasis, both rodent and human heart contain predominantly fetal-derived $CCR2^{-}$ $MHCII^{lo}$ macrophages, and small subsets of monocyte-derived $CCR2^{+}$ $MHCII^{hi}$ macrophages, as well as $CCR2^{+}$ $MHCII^{lo}$ monocytes (112). However, after the ischemic injury of organs and tissues, infiltrating monocytes and monocyte-derived $CCR2^{+}$ macrophages rapidly dominate damaged tissues where they initiate the pro-inflammatory response discussed above. Studies of the $CCR2^{-}$ population have demonstrated the importance of this subset to limit adverse remodeling, but the propensity to be lost at sites of IRI (90). Studies of IRI in commonly transplanted organs, such as the heart, lung, liver, and kidney, have provided evidence for the existence of certain DAMPs that not only initiate inflammation but also, or instead, initiate and sustain tissue injury resolution responses and repair

TABLE 2 | Regulatory or reparative DAMPs and related molecules in Tx.

Family	Molecule	Receptors	Role in Tx- related inflammation/immunity/outcomes	References
Regulatory or reparative DAMPs				
	CD24	Siglec	Associates with DAMPS to negatively regulate their stimulatory activity Protect against pathological inflammatory responses arising from cell death and necrosis Limits T cell alloimmunity	(70–73)
	IL-33	ST2	Promotes the systemic expansion of $ST2^+$ Treg able to limit alloimmunity Promotes the secretion of Areg and other growth factors act on tissues and stem cells to support repair Induces TCR-independent Treg secretion of IL-13 and Areg that to control local inflammation and the generation of reparative type macrophages Directly promotes the generation of reparative macrophage phenotype through a metabolic reprogramming that augments OXHPOS and FA uptake	(74–82)
	Heat Shock Proteins (HSPs)	CD91, TLR2, TLR4, SREC1, and FEEL1	Supports debris clearance and wound repair Protect organs from IRI Extend graft survival Induce IL-10 secretion by T cells Support polarization of macrophages towards regulatory and reparative subsets	(24, 83–89)
	Hyaluronan	Lyve1	High weight forms support the survival and localization of macrophage subsets that productively remodel ECM to support vasculature function after injury Contribute to tissue integrity and functional immunological niches	(23, 90–94)
Specialized pro-resolving mediators (SPMs) and related molecules				
	Annexin A1	FPR2/ALX	Polarization of macrophage towards a pro-reparative subset Prolong allografts survival with sub-therapeutic immunosuppression Protect organs after IRI	(95–99)
	Maresins, Lipoxins, and Resolvins	GPR32 and ALX/FPR2 receptors	Limiting neutrophil infiltration and induction of neutrophil apoptosis Directly limiting adaptive immune responses Organ-protective and regenerative actions after IRI Stimulate macrophage transition toward reparative subsets Enhance Treg functions Prolongation of allograft survival	(100–109)

by infiltrating immune cells. The study of mucosal injury and repair suggests a similar evolution where homeostatic resident myeloid cells are rapidly outnumbered by infiltrating monocytes and monocyte-derived macrophages (113). Through these models (114, 115), we now have a framework paradigm of an effective inflammation resolution and repair processes after tissue insult due to ischemic stimuli, and we are beginning to understand the DAMP-influenced processes and pathways directing immune cell-mediated response after injury (**Figure 1**). Findings in these injury models will not be confounded by the unique immunological situation found in SOTx where adaptive and innate immune cells will respond to non-self, allogeneic graft components. In injury models, a highly regulated, immune-mediated tissue repair process that is shaped by DAMPs after injury has emerged. This process consists of a pro-inflammatory phase, a resolution phase, and a repair phase (**Figure 1B**). How anti-AlloAg responses impact the typical signaling induced by DAMPs during the cellular responses leading to the early inflammatory phase and resolution and reparative phase after ischemic injury remains poorly understood. These questions are beginning to be addressed in recent rodent Tx studies described below, as well as speculated on in the later sections of our review.

As discussed above, myeloid cells are primary sensors of early damage. Yet, how infiltrating monocytes and tissue-resident macrophages respond to early hypoxia and DAMPs released due to ischemia is quite distinct. Infiltrating monocytes will differentiate into macrophages activated by pro-inflammatory cytokines (TNFα, IL-1β, IFNγ, IL-6) and DAMPs (HMGBI, ATP, Genomic DNA/Histones, IL-1α) into highly pro-inflammatory cells that, with neutrophils, dominate the pro-inflammatory phase after IRI. After ischemic injury due to myocardial infarction (MI), however, the resident $CCR2^-$ macrophage subset, while able to proliferate in non-damaged tissues, is lost due to anoxia and nutrient depletion (90). Thus, the ischemic areas are rapidly dominated by responding neutrophils and infiltrating $Ly6C^{hi}$ $CCR2^+$ monocytes, and $F4\text{-}80^+$ $Ly6C^{hi}$ $CCR2^+$ MHCII pro-inflammatory macrophages. The pro-inflammatory state of differentiation and functional activity of these macrophages will be enhanced by DAMPs like HMGB1 and Vimentin and local type-1 cytokines, particularly IL-1β, IFNγ, and TNFα. As described by Braza et al., HMGB1 and Vimentin promoted a pro-inflammatory training of cardiac graft-infiltrating macrophages that secreted increased TNFα and IL-6 (10). These type-1 cytokine-activated macrophages approximate the well-characterized "M1" macrophages generated *in vitro* by exposing macrophages to LPS and IFNγ. They use their high phagocytic capacity, robust production of NO, and pro-inflammatory cytokines to mediate the removal of

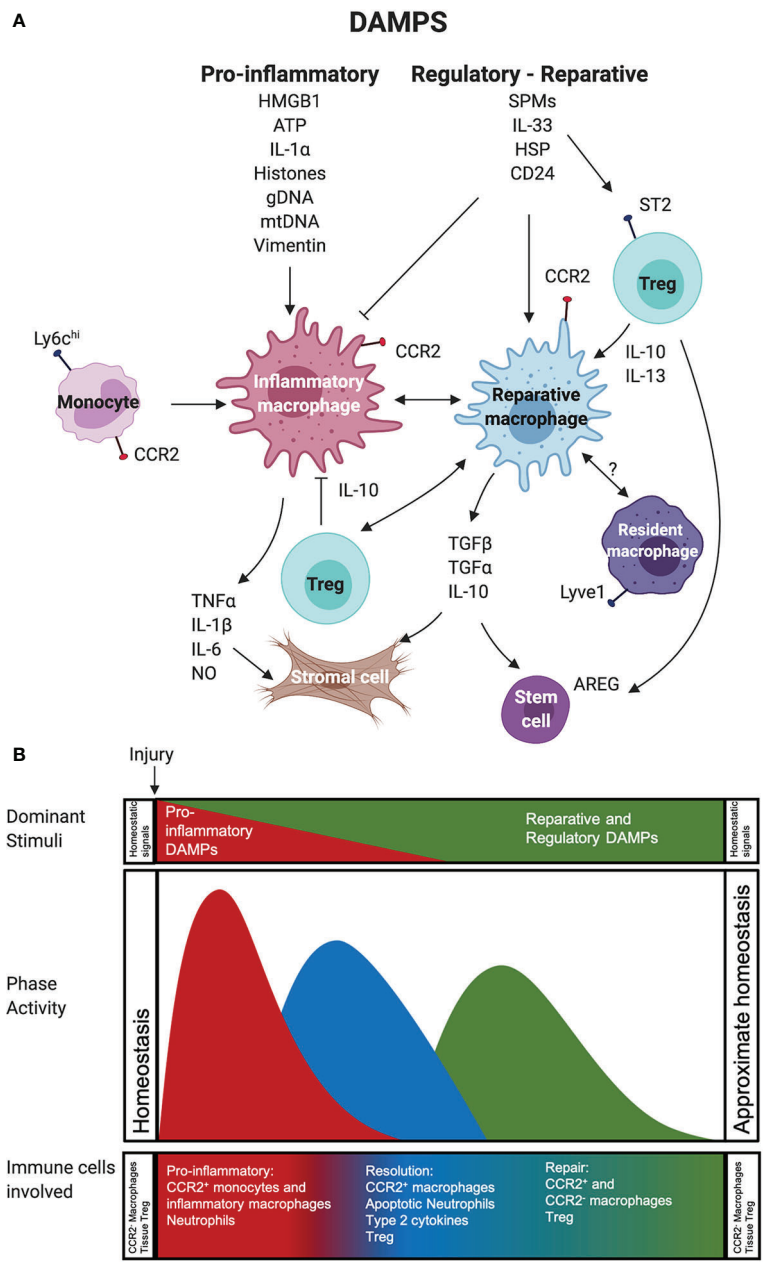

FIGURE 1 | Impact of pro-inflammatory versus reparative and regulatory DAMPs on immune cells during inflammation, resolution, and repair phases after tissue injury. **(A)** Recruited CCR2$^+$ monocytes and the macrophages derived from them participate in a highly regulated, immune-mediated tissue repair process shaped by *1.* Pro-inflammatory and *2.* Reparative and Regulatory DAMPs. These also act on Treg and potentially resident macrophages to support the survival and function of these immune cells. **(B)** This process can be divided into three overlapping phases, including a: *1.* pro-inflammatory phase (red), *2.* resolution phase (blue), and *3.* repair phase (green). In the first phase, pro-inflammatory DAMPs act on monocytes and macrophages to generate or support the function of highly phagocytic, inflammatory macrophages that use robust production of NO and pro-inflammatory cytokines to mediate the removal of any pathogens and damaged necrotic tissue. The transition to the resolution phase involves efferocytosis, or the phagocytosis of apoptotic cells, by macrophages receiving input from reparative and regulatory DAMPs and type 2 cytokines. These can both block the impact of pro-inflammatory stimuli on macrophages and contribute to the generation of Treg that support local immune suppression. The final phase involves little pro-inflammatory DAMP activity. It is dominated by reparative and regulatory DAMPs macrophage metabolism enabling the function of reparative and regulatory macrophages, such as secretion of cytokines, effector molecules, and growth factors that mediate responses in stromal, parenchymal cells, and stem cells to facilitate tissue repair. Regulatory and reparative DAMPs also act on Treg, which support the generation of reparative and regulatory macrophages and contribute growth factors to the repair environment. Reparative and Regulatory DAMP most likely also act on resident macrophages that are important for injury resolution and re-establishment and maintenance of tissue homeostasis. Abbreviations used: Areg, Amphiregulin; ATP, Adenosine Triphosphate; CCR2, CC Motif Receptor 2; DAMP, damage-associated molecular pattern; gDNA, Genomic DNA; HSP, Heat Shock Protein; HMGB1, High-mobility group box 1; IL, Interleukin; Ly6C, Lymphocyte antigen 6 complex, locus C1; mDNA, Mitochondrial DNA; NO, Nitric Oxide; SPM, Specialized pro-resolving mediators;TGF, Transforming growth factor; Treg, Regulatory T cell.

any pathogens and any damaged necrotic tissue. These macrophages will also have high levels of HIF-1α, facilitating their glycolytic metabolism and the release of pro-inflammatory cytokines and chemokines that attract and activate additional infiltrating neutrophils and monocytes. The result is the collateral damage of healthy tissue from the induction of this response; thus, the benefit of limiting the pro-inflammatory response after Tx is apparent.

Nevertheless, this pro-inflammatory process involving macrophages is essential to address pathogens and dead cells and crucial to the initiation of the resolution phase and subsequent repair phase (**Figure 1B**) of the wound healing responses (57, 59, 116). Indeed, if macrophages are depleted early after IRI injury, the overall inflammatory response is greatly diminished, yet this results in ineffective clearing of necrotic cells from the damaged site and leads to inefficient repair and regeneration (117). Thus, one lesson from these studies for the transplant community is not to seek the total absence of an inflammatory macrophage response after Tx, but instead encourage a restrained early response that is brief and limited in scope to not cause overwhelming tissue damage that leads to early graft failure or persisting tissue injury. The success of reagents like co-stimulatory blockade in tolerance induction may be due, in part, from their ability to limit the antigen-presentation function of myeloid, but not ablate myeloid cell injury resolution functions. A vital transition in local macrophages next occurs in ischemic areas where they assume a phenotype associated with immune regulation and wound-healing. This transition is orchestrated by macrophage efferocytosis, or the phagocytosis of apoptotic cells, especially neutrophils, in the absence of pro-inflammatory stimuli and the presence of the type-2 cytokines, IL-4 or IL-13 (118).

A wealth of knowledge regarding type-2 cytokine activated macrophages has been generated through the *ex vivo* study of macrophages treatment with IL-4. IL-4 augments fatty acid (FA) uptake and oxidative phosphorylation (OXPHOS) to supports macrophage regulatory and reparative functions (57, 61). The importance of FA uptake and β-oxidation in regulatory and reparative macrophage polarization has been controversial (119). Yet, the disruption of FA uptake through inhibitors or loss of the FA translocase CD36 in mice and humans limits the generation and function of immunosuppressive and regulatory myeloid cells (120, 121). Inhibition of this pathway blocks the IL-4-induced expression of crucial genes, including CD206, CD301, and RELMα that are functional phenotypic markers of reparative and regulatory macrophage (61). Macrophages programmed towards repair through efferocytosis and IL-4 secrete the anti-inflammatory cytokine IL-10. IL-10 will act in the local environment to support macrophage OXPHOS and preserve their respiratory capacity by facilitating the removal of dysfunctional mitochondria *via* mitophagy (68). Upregulation of peroxisome proliferator activated receptor γ (PPARγ) and PPARγ coactivator 1B is important for FA oxidation and mitochondrial biogenesis in IL-4-exposed macrophages (122, 123). In addition to IL-10, type 2 activated macrophages secrete TGF-β and express programmed cell death ligands to suppress local immune responses (116). Arginase 1 (Arg1) is also induced to generate ornithine from L-arginine to support tissue repair (124), but also generates metabolites that dampen T cell responses, including those of alloreactive T cells (125, 126). IL-4-activated macrophages aid injury resolution through the production of several growth factors, including platelet-derived growth factor, transforming growth factorβ1 (TGF-β1), insulin-like growth factor 1 (IGF-1), and VEGFα to promote cellular proliferation, blood vessel development, and attract and differentiate tissue fibroblasts into myofibroblasts (116). The capacity of reparative macrophages to control myofibroblasts that modulate the local ECM to initiate wound contraction and closure and direct re-vascularization makes them critical to the restoration of injured tissues and organs as close to a homeostatic state as possible (121).

The ability of type 2-activated macrophages to both repair tissue and suppress local T cell responses has made them an attractive target population in SOTx to support tolerance induction, as well as limit or potentially even reverse CR (127). Further research is necessary to understand what endogenous local molecules initiate or support monocytes' transition to reparative and regulatory macrophages at the end of the injury's pro-inflammatory phase. The signals that direct their reparative response to resolve damage and restore homeostasis or functionality to damaged tissues and organs will be important targets to define for the generation of new biologics for use in SOTx. With a clear picture of the stimuli that control both the initiation, magnitude, and length of the inflammatory and repair and resolution phases after injury comes the capacity to control the process through regulated delivery of agents directing the appropriate pathways at the correct time. The elucidation of these macrophage-mediated pathways in innovative transplant models will be vital. These studies should also explain how the typical process and pathways leading to effective repair in tissues and organs are impeded, augmented, or dysregulated by a persistent local immune response to AlloAg.

Lyve1^{hi} Macrophages

While infiltrating monocyte-derived macrophages are the prevailing effectors during the immune response to ischemic injury, other immune cells have been identified that also contribute significantly to effective healing and remodeling after tissue damage. A recent paper has described a Lyve1^{hi} MHCII^{lo} CX3CR1^{+} CCR2^{-} interstitial macrophage subset in the vasculature adventitia of the lung, heart, fat, and dermis (128). Depletion of this subset before bleomycin-induced lung injury or isoproterenol-induced cardiac hypertrophy augmented fibrotic disease in both models (128). Comparison of the arterial Lyve1^{+} vs. Lyve1^{-} macrophages revealed that the Lyve1^{+} subset was enriched for genes involved in homeostasis and ECM remodeling, and their deletion result in ECM abnormalities causing lost vascular wall integrity and impeded blood flow (91). Further mechanistic investigations revealed that Lyve1^{+} macrophages bind to smooth muscle cells (SMC) *via* ECM interactions and shape artery tone and function by regulating ECM collagen deposition (91). As mentioned briefly above,

studies by Dick et al. have shown that a similar population of fetal-derived, TIMD4$^+$ Lyve1$^+$ MHC^{lo} CCR2$^-$ cardiac resident cells are lost at sites of IRI. These cells are rapidly replaced by CCR2$^+$ monocyte-derived cells, some of which can take on a CCR2$^-$ resident phenotype, but lack expression of *Lyve1* or *Timd4* (90). The TIMD4$^+$ Lyve1$^+$ resident macrophage subset also repopulates after loss through proliferation in the peri-infarct area and their depletion post-MI resulted in poor cardiac function (90). Data generated using the depletion of resident CCR2$^-$ macrophages before syngeneic cardiac Tx established that the therapeutic benefit they provide after IRI is due, in at least part, to their capacity to inhibit CCR2$^+$ monocyte recruitment (92). It is not entirely clear if monocyte-derived Lyve1$^+$ CCR2$^-$ subsets are as effective as the fetal-derived subset they replace over time. There is, however, accumulating evidence that Lyve1$^+$ CCR2$^-$ macrophages are essential for the healing after cardiac IRI and contribute to local homeostasis by directing the infiltration of other immune cells through modulation of local ECM.

Tregs in Tissue Repair

CD25^{hi} forkhead box P3 (Foxp3)$^+$ regulatory T cells (Tregs) are an essential endogenous population of CD4$^+$ T cells that act as potent immunosuppressive cells to control autoreactive immune responses and limit tumor immunity. Tregs use multiple mechanisms for their immunosuppressive functions that limit the size and quality of other T cell responses. These mechanisms include the production of anti-inflammatory cytokines, such as IL-10, IL-35, and TGFβ, that act directly on T cells to suppress their expansion and effector functions, as well as promote their exhaustion and deletion. Tregs ample expression of CD25 allows them to sequester IL-2 from immunological microenvironments. The importance of their suppressive capacity was first made evident in the study of mice and humans with Foxp3$^+$ mutations that caused aggressive and lethal systemic autoimmunity (129, 130). Ongoing clinical trials are attempting to harness the potent immunosuppressive capacity of Tregs as cell therapy and reduce autoimmune pathology, or ideally, restore lost tolerance in patients with Crohn's Disease, Type 1 diabetes, and lupus (131). Based on rodent pre-clinical Tx studies' successes where administered polyclonal or AlloAg-specific Tregs support Tx tolerance induction, more than 15 clinical studies have been recently completed or underway in SOTx.

In addition to preventing tissue injury by limiting collateral damage mediated by an unrestrained immune response, Tregs also secrete factors that support the proliferation and survival of stem cells. Tregs secrete amphiregulin (Areg), a bi-functional growth factor that supports stem cell proliferation and differentiation through actions on the epidermal growth factor receptor (EGFR) (132). Related studies have identified Tregs secretion of keratinocyte growth factor as an import signal for alveolar epithelial proliferation and regenerative alveologenesis (133). In addition to the ability of Tregs to shape the function of monocytes and myeloid APC through secreted molecules like IL-10 and TGFβ, they also express indoleamine 2,3-dioxygenase that catalyzes the degradation of tryptophan to limit the function of CD8$^+$ T effector cells (134). Tregs also have the capacity, at least *in vitro*, to direct the polarization of monocytes towards macrophage populations exhibiting features of those exhibiting reparative and regulatory functions *in vivo*. When both mouse or human monocytes are cultured with Treg, they upregulate their expression of CD206 and Arginase 1, both functional phenotypic markers of reparative and regulatory macrophages, due to Tregs secretion of IL-10 and IL-13 (135). Tregs are also essential to limit the damage and support function after ischemic injuries to the heart and brain (136, 137). Thus, in addition to their canonical role in suppressing detrimental immune responses and maintaining immune homeostasis, Tregs participate in the repair of tissue damage.

REGULATORY AND REPARATIVE DAMPS AND SPECIALIZED PRO-RESOLVING MEDIATORS (SPMS) IN TISSUE INJURY RESOLUTION

In the above sections, we outline the importance of several macrophage subsets, with input from Treg, needed to complete a highly regulated resolution and repair process that is relatively universal across organs and tissues. While not the focus of this review, it should be mentioned that other immune cells, particularly dendritic cells, various T helper subsets, and innate lymphocytes, also play essential roles in the repair process initiated by DAMPs (3, 138). It is also hopefully more clear how pro-inflammatory DAMPs, like HMGB1, ATP, and mDNA, are important initiators of, or at a minimum - crucial contributor to the early pro-inflammatory phase of tissue injury. Nevertheless, other endogenous signals, like CD24, that quell local inflammation or initiate the resolution and repair phases after IRI or other Tx-relevant injuries remain poorly understood. Previously suggested pro-inflammatory DAMPs, particularly IL-33, HSPs, and HA, however, support the expansion of reparative cells. These DAMPs also drive the function of immune cells during the resolution and reparative programs induced in various injury models (**Table 2**). Other biomolecules, such as Annexin A1 (AnxA1) and specialized pro-resolving mediators (SPMs), including resolvins and maresins, also act as powerful endogenous signals that support immune-mediated inflammation resolution and the return to local homeostasis (**Table 2**).

When considered from a more general perspective, there are several common characteristics of regulatory DAMPs and SPMs that standout. First, these molecules are typically sequestered or shielded from recognition by the immune system until they are released after injury. Second, both groups limit local infiltration by inflammatory leukocytes and instead orchestrate the differentiation and function of immune cells that restore local homeostasis through inflammation resolution and repair. Third, most contribute to the differentiation of reparative- or regulatory-type macrophages *via* induced signaling and metabolic programming towards OXPHOS and FA uptake. Fourth, the capacity of regulatory DAMPs and SPMs to

directly stimulate Treg expansion and function, or support Treg expansion indirectly through actions on myeloid APC and macrophages is common. Finally, many of these molecules may be released at very low concentrations during normal cell turnover to sustain the immune cells maintaining homeostasis. The molecules and immune cells, like Treg and macrophages, restoring local and systemic homeostasis, may overlap considerably with cells and systems that typically maintain it. We discuss below the limited, but growing, literature describing a potential role for regulatory DAMPs and SPMs and their target cells in influencing alloimmunity and Tx outcomes.

Specialized Pro-Resolving Mediators (SPMs)

SPMs are a superfamily of lipid molecules that are generated locally after injury and target G coupled receptors (GPRs) in order to stop excessive neutrophil infiltration, counter pro-inflammatory signals, enhance efferocytosis, and the clearance of dead cells by macrophages (139, 140). SPMs are generated from essential polyunsaturated FA in enzymatic reactions completed by both leukocytes, platelets, and parenchymal and stromal cells into several related groups of immunoresolvins, including lipoxins, E, and D series resolvins, protectins, and maresins (100, 139, 140). Lipoxin is secreted by neutrophils and macrophages after being synthesized from arachidonic acid. It acts on cells expressing the G protein-coupled lipoxin A4 (ALX)/formyl peptide receptor (FPR2) or GPR 32, the aryl hydrocarbon receptor, estrogen receptor, as well as the cysteinyl leukotriene receptor (141). Important actions of lipoxin on innate immune cells after injury include limiting neutrophil infiltration and the induction of neutrophil apoptosis. Lipoxin also supports injury and inflammation resolution by delaying the apoptosis of macrophages completing efferocytosis and local debridement (142). Limited studies suggest that lipoxin may have the ability to directly regulate B-cell antibody production and proliferation, as well as limit T cell effector functions (101, 102). While lipoxin impacts on innate and adaptive immune cells would be expected to improve Tx outcomes, to date, however, the influence of lipoxin on alloimmunity and Tx outcomes has been poorly explored. A limited assessment in clinical lung Tx samples revealed the presence of lipoxin in these samples, and the delivery of a stable lipoxin analog provided subtle improvements in mouse heart and kidney Tx models (103). This initial testing was completed in MHC-fully mismatched models; thus, experimentation in less aggressive combinations is warranted to understand better if lipoxin can improve CR by limiting IRI, reducing alloimmunity, or initiating repair responses.

E-series resolvins are generated primarily by neutrophils from the exudate omega-3 FA eicosapentaenoic acid (EPA), where D-series resolvins are made by neutrophils from docosahexaenoic acid (DHA), which also serves for the starting blocks of maresins, which is synthesized from DHA by macrophages (100). Like lipoxin, both resolvins limit neutrophil infiltration and promote their apoptosis. Some resolvins have potent organ-protective and regenerative actions that would be highly relevant in surgery-induced IRI. SPM-stimulated macrophage transition toward those reflective of IL-4-activated macrophages, which, as discussed, are characterized by high levels of FA uptake and are the primary source of maresins (104). These lipid mediators have also been shown to induce macrophage production of IL-10 while reducing dendritic cell production of IL-12 (100, 105). SPMs also have potent anti-IRI activities demonstrated for kidney, liver, and lung mediated by limiting TLR4/MAPK/NF-κB pathway activity and activating the Nrf2 pathway to limit oxidative stress (106–108). Other intriguing studies have suggested that D-series resolvins and maresin can act on T cell GPR32 and ALX/FPR2 receptors to limit human and mouse pro-inflammatory cytokine production, while simultaneously enhancing Treg function. It has also been described how a decrease in resolvins and maresins are observed in obese subjects or individuals suffering from autoimmunity or systemic inflammatory diseases. These findings suggest the importance of these molecules in systemic homeostasis (143). Based on these effects, the role of SPMs in the early and late immunobiology of Tx deserves investigation.

Annexin A1 (AnxA1)

AnxA1 is a phospholipid-binding protein sequestered in the cytoplasm of neutrophils, monocytes, and macrophages and released upon their activation (144). The production of AnxA1 is highly responsive to glucocorticoids, with endogenous and delivered glucocorticoids increasing both AnxA1 expression and secretion (144). The anti-inflammatory and pro-resolving effects of AnxA1 are mediated through binding to FPR2/ALX, which limits neutrophil transmigration tissue infiltration and induces neutrophil apoptosis. AnxA1 acts on macrophage FPR2/ALX receptors to activate AMPK, which is a potent regulator of mTOR (95). This results in the polarization of macrophage towards a pro-reparative subset. These data indicate that AnxA1 acts as a natural factor that can regulate the pro-inflammatory DAMP metabolic reprogramming described by Braza et al. Thus, AnxA1 may act like the pro-tolerogenic signals generated when nanoparticles containing the mTOR inhibitor rapamycin were used targeted to graft macrophages after heart transplant (10). Delivery of an AnxA1 mimetic could prolong BALB/c skin grafts on B6 recipients, but only when given with sub-therapeutic cyclosporine A (96). Targeting FPR2/ALX with AnxA1, like lipoxin above, provides a protective, but not robustly immunosuppressant or protective effect after Tx. Given the importance of limiting early graft injury and rapidly transitioning from a local pro-inflammatory state to one of injury resolution and tissue repair, it is easy to envision how reagents targeting this pathway could be combined into immunosuppressive protocols to improve outcomes by limiting the pro-inflammatory phase and accelerating pro-inflammation resolution after IRI. Tx researchers have spent most of our energy looking for reagents that are potent immunosuppressants or able to induce tolerance. AnxA1 may be able to contribute here through actions on Dectin-1 (145). However, it is advisable that we also harness the wealth of past evidence that AnxA1 or its derivatives are useful when used to target FPR2/ALX to limit MI-mediated pathology and acute kidney injury (97–99). These data

would support an investigation into using these reagents to limit early graft failure or IRI under cover of immunosuppression.

Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) are highly conserved proteins grouped according to their molecular weights (e.g., Hsp 27, Hsp40). They are upregulated in response to stress conditions that result in damaged proteins, such as extreme heat, hypoxia, oxidative stress, inflammation, injury, or infections. They are essential for intracellular functions involving the initiation of protein folding, repair, refolding of misfolded peptides, and aiding in the degradation of irreparable proteins. However, upon necrotic cell death or cellular stress, HSPs are released and were initially characterized as pro-inflammatory DAMPs that acted on TLR4. However, several pre-clinical studies have demonstrated various extracellular HSPs that, when overexpressed or delivered, can protect organs from IRI (83, 84) and extend graft survival (85, 86). When delivered, various HSPs are potent inducers of T cell IL-10 production and support the polarization of macrophages towards regulatory and reparative subsets (87, 88). These findings and their implications in Tx have been recently expertly-reviewed (89) and thoroughly describes the literature supporting consideration of HSPs as a regulatory DAMP in Tx.

Hyaluronan (HA)

HA, also known as hyaluronic acid, is an important ECM component synthesized by HA synthase at the plasma membrane of predominantly mesenchymal cells. Here it associates with different HA-binding proteins to form pericellular and extracellular matrices that are important for creating space and a matrix allowing cellular migration and localization. HA is also generated as part of the tissue injury and repair response, where it directs and regulates the infiltration and function of fibroblasts, blood vessels, and immune cells (93). HA is connected to the early inflammatory responses after injury, as degraded HA induces signaling *via* TLR4 and TLR2 on macrophages to drive pro-inflammatory cytokine production (21). HA has a long history in Tx, as Goldstein and colleagues showed convincingly that HA fragments could induce DC maturation and initiate alloimmunity (22). That HA can induce alloimmunity is of clinical relevance as the bronchial lavage fluid of lung transplant patients undergoing AR displays significantly higher HA levels than those with no rejection (146). Additional studies established that HA was prominent in areas of intraluminal small airway fibrosis in lung transplants bronchiolitis obliterans, as was the message for HA synthase (147). Increased local and circulating levels of HA have also been noted in rodent skin and cardiac transplant models (22, 148). Yet, the impact of HA on alloimmunity is not clear and potentially double-edged, as different molecular weight HA products seem to produce pro-inflammatory or regulatory impacts depending on the transplanted organ. The accumulation of lower molecular weight HA stimulated lung inflammation after lung injury and was shown to contribute to lung transplant rejection, while high-molecular-weight HA attenuated allograft inflammation and contributed to lung epithelium integrity (21–23, 147). In contrast, low molecular weight HA delivery prolonged renal and cardiac allograft survival (149, 150). These contrasting findings may reflect different biological functions of HA between different organs and or distinct roles of HA in the different physiological processes happening, i.e., AR, CR, or tissue repair.

One aspect that should be discussed further that may account for these varied responses in transplanted organs is the emerging importance of intact, high molecular weight HA to the generation of functional immunological and repair niches. HA interactions are critical to hematopoietic and tissue-forming stem cell migration, function, and survival (93). HA stem-supporting characteristics also aid the function and survival of cancer cells, and HA in the ECM of the tumor environment supports tumor-associated macrophages' polarization and survival (151, 152). Thus, while the DAMP activity of HA is an important consideration, an equally important function of HA in the transplant microenvironment may be its role in providing the localizing, supporting structure to the hematopoietic and structural cells that are being tuned by DAMPs and other signals in the environment to shape any alloimmune response or resolve a local injury. HA contributions to "rejection" niches are implied by the observation that delivery of low molecular weight fragments antagonize cardiac graft infiltration by effector cells using the HA binding receptor CD44. This interaction can also be targeted effectively with anti-CD44 antibodies (153).

Nevertheless, several recent studies have revealed the importance of HA niches and protective HA-binding myeloid cells in them after IRI. As introduced above, Dick et al. described the importance of fetal-derived, self-renewing Lyve1^{+} MHCII^{lo} CCR2^{-} macrophage subset in productive repair after myocardial infarction (MI) (90). Lyve1 is the receptor for HA, and the expression of this receptor by CCR2^{-} macrophages appears to target them to HA dense areas, particularly the adventitial layer of arteries. Deletion of these macrophages post-IRI resulted in the dysregulated repair after myocardial infarction (90). Related studies also used a different system to completed targeted deletion of Lyve1^{+} macrophages and established that an important function this subset was to modulate the ECM in these arterial niches and prevent arterial stiffness at homeostasis (91). This function required Lyve1-HA-interaction-induced production of matrix metalloproteinase 9 (91). Further mechanistic investigations revealed that Lyve1^{+} macrophages bind to smooth muscle cells (SMC) *via* interactions with HA and shape artery tone and function by regulating ECM collagen deposition (91). HA's importance for healing after IRI was also demonstrated in mice with inducible deletion of HA synthase 2 (HAS2). HAS2 deletion before IRI resulted in a severely impaired hemodynamic function associated with a loss of cardiac macrophages, but not monocytes (94). The authors accounted poor function to increased apoptosis of macrophages in the absence of HA stimulation (94). The loss of HA also resulted in decreased myofibroblast in the infarct site, and *in vitro* studies outlined an intricate network were HA-positive fibroblasts and Lyve1^{+} macrophages communicate to generate functional ECM after IR. These observations mesh with syngeneic cardiac Tx studies completed by Kreisel and Lavine,

where they demonstrated that CCR2$^-$ macrophages inhibit monocyte recruitment, where the CCR2$^+$ macrophage subset promoted monocyte recruitment *via* MyD88-dependent mechanism and the release of monocyte chemoattractant proteins (92). These recent studies shed light on how critical local niches shaped by ECM components, including HA, will be important to outcomes after Tx.

Interleukin-33 (IL-33)

IL-33 is a member of the IL-1 superfamily sequestered in the nucleus due to a nuclear localization domain and chromatin binding motif (154, 155). IL-33 released during necrotic cell death and cellular stress is functional, but its activity is negatively regulated by caspases, oxidation, and chromatin occupancy. Several proteases can increase the activity of full-length IL-33 by cleaving off the nuclear localization domain and chromatin binding motif (154, 156). We have also recently demonstrated that bio-active IL-33 is present in vesicles bound to the ECM of stromal cells where it is protected from proteolytic modification (157).

IL-33 was originally identified and described as inflammatory DAMP that drives type 2-cytokine-mediated inflammation when it is released after tissue damage and stimulates immune cells *via* the IL-33 receptor IL-1R-like-1(IL1RL1), more commonly referred to as Stimulation-2 (ST2) (158). Numerous immune cells express varying levels of ST2. These include basophils, mast cells, eosinophils, group 2 innate lymphoid cells (ILC2s) (154), CD8$^+$ (159, 160), and CD4$^+$ T cells (161), particularly Th2 cells and Treg (74–77, 158, 162), B cells (163), macrophages (78, 157, 163), and DC subsets (162, 164, 165). IL-33 acts on these cells to support type 2 responses dominated by the cytokines IL-5 and IL-13. IL-33 induction of type 2 cytokines aides parasite clearance and drives allergic responses, lung inflammation, and fibrotic skin diseases. There is a close link between type 2 cytokines and tissue repair, and IL-33 has emerged as a crucial mediator of the repair process. Much of the known repair activity of IL-33 involves its capacity to target ST2$^+$ Tregs, a predominantly peripheral tissue-resident subset, and induce their expansion and production of IL-10, IL-13, and Areg (166). Seminal studies by the Rudensky group established an essential role for Tregs in the resolution of epithelial injury after virally-induced lung injury due to their secretion of Areg. Interestingly, it was Tregs recognition of IL-18 or IL-33, not TCR signaling, that led to this reparative action (74). IL-33 also induces TCR-independent Treg secretion of IL-13 that is critical to control local inflammation and after chemical or viral lung injury (77). Treg secreted IL-13 generates Arginase 1$^+$ macrophages implicated in tissue repair and homeostasis (77). ILC2 secrete IL-13 in response to IL-33 to promote lung regeneration by stimulating macrophage support of type 2 alveolar epithelial stem cell proliferation (167). There is a prominent role for IL-33 in regulating metabolic homeostasis, and disruption of the Treg-ILC2-Macrophage axis contributes to increased inflammation and obesity (168–170). Fibro/adipogenic progenitor cells in the skeletal muscle express IL-33 and sustains skeletal muscle Tregs that are important for muscle regeneration after injury through secretion of Areg that supports muscle satellite cells (75, 76) and potentially limits the local generation of inflammatory Ly6C^{hi} macrophages (75).

Numerous studies have suggested the potential to harness the emerging regulatory and reparative properties of IL-33 in Tx. Administration of IL-33 post-heart Tx expands ST2$^+$ Treg to prolong allograft survival across MHC barriers in rodent heart transplant models (79, 158). Skin graft acceptance could also be aided through IL-33-induced expansion of regulatory myeloid cells and Treg (80, 81). It was not until recently that we also revealed an essential regulatory function for graft-derived IL-33 that involved the direct targeting of infiltrating recipient monocytes and macrophages (78). We used heart transplants lacking IL-33 or recipients with ST2-deficient macrophages to clarify that a critical function of endogenous IL-33 was to promote the generation of reparative macrophage phenotype through a metabolic reprogramming augmenting OXHPOS and FA uptake. Thus, IL-33 is unique relative to DAMPs like HMGB1 that drives glycolysis and epigenetic modifications enabling inflammatory cytokine production (10). IL-33 instead blocks iNOS expression and, like IL-4, IL-10, and IL-13, increases mitochondrial function and FA uptake (61, 78). In total, it is safe to describe IL-33 as a regulatory DAMP in Tx, and it will be necessary to use tissue-specific disruption of IL-33 and immune cell-specific deletion of ST2 to help us further understand how IL-33 coordinates responses to IRI and alloinjury after SOTx.

UNDERSTANDING WHERE PRO-INFLAMMATORY AND REGULATORY AND REPARATIVE DAMP SIGNALS GET TANGLED AND LEAD TO POOR OUTCOMES AFTER TX

As outline above, the process of injury recognition, inflammation initiation and resolution, and then tissue repair after IRI is complex in both signals and cells involved. It is also subject to pathology when not perfectly orchestrated, or a phase in the process is amplified or incomplete. It is easy to appreciate how an augmented inflammatory response due to an extended ischemia period releasing prodigious amounts of pro-inflammatory DAMPs across an entire organ can lead to early graft dysfunction and failure. It is clear how this would also lead to AR due to widespread activation of resident DC and other APC presenting AlloAg, which then travel to the secondary lymphoid organs to stimulate an alloimmune response. The inflamed tissues would also be an ideal environment for the generation of inflammatory APC as infiltrating recipient monocyte differentiate into pro-inflammatory macrophages and DC that support local alloresponses that drive rejection (171). The ongoing efforts discussed above to block the early inflammation mediated by pro-inflammatory DAMPs, if found therapeutic, should have an impact here. However, despite the availability of potent immunosuppressants available to target adaptive immune

responses and the shortening ischemia times common in current clinical transplant medicine, CR remains a persistent problem. The development of CR in immunosuppressed individuals suggests that other factors beyond pro-inflammatory DAMPs may need to be considered. In this remaining section, we briefly postulate how unique aspects of SOTx may interfere with appropriate resolution or re-establish tissue homeostasis after Tx to lead to CR.

Alloimmunity Prevents Effective Resolution and Repair

Transplanted organs represent a unique immunological situation where non-self, allogeneic signals will impact the typical immune responses working toward resolving early ischemic injury and any damage caused by allorecognition. The reaction to AlloAg by the adaptive immune systems, as well as NK cells, has been long recognized, yet how innate alloimmune responses influence acute and chronic tissue injury resolution and repair responses remains unclear. Precise mouse studies have now established that graft infiltrating monocytes, in addition to detecting DAMPs, will recognize allogenic molecules, such as the polymorphic signal regulatory protein α (SIRPα). The binding of allogeneic SIRPα to the nonpolymorphic CD47 causes monocytes to mature into monoDCs expressing IL-12 and stimulating T cell proliferation and IFNγ production in the graft (172, 173). Murine monocytes and macrophages can also recognize and acquire memory specific to MHC-I antigens *via* paired immunoglobulin-like receptors-A (PIR-A) (174). As outlined in **Figure 1**, these infiltrating monocytes are the main coordinators of local DAMP and cytokine signals needed to initiate and then resolve tissue injury. It is easy to speculate how alloreactive macrophage will increase local IL-12 and IFNγ to prolong the pro-inflammatory phase or prevent transition to resolution and repair (**Figure 2A**). Nevertheless, these changes may augment counter-responses to increase damage and regulatory and reparative DAMPs (**Figure 2A**). Thus, CR may instead result from an overzealous or persistent resolution response mediated by reparative Treg and macrophages that sustain a response to an unresolved allogeneic injury.

Replacement of Donor CCR2$^-$ Macrophages by a Recipient CCR2$^+$ Pro-Inflammatory Subsets

The importance of fetal-derived, Lyve1$^+$ CCR2$^-$ resident macrophages cells to control pro-inflammatory CCR2$^+$ monocytes' infiltration and mediate ECM remodeling to allow inflammation resolution and productive tissue repair after cardiac IRI was laid out above. Comparable populations of self-replicating, fetal-derived macrophages are noted around the vasculature of the lung, fat, dermis (128), as well as in the kidney (175). The CCR2$^-$ macrophage subset appears critical for local control of inflammation and remodeling after injury, but susceptible to loss due to conditions typical of IRI and CR, including hypoxia and the loss of an HA-rich ECM. Once lost, this subset is rapidly replaced by a circulating CCR2$^+$ monocyte-derived subset, which lacks the capacity of the CCR2$^-$ subset for

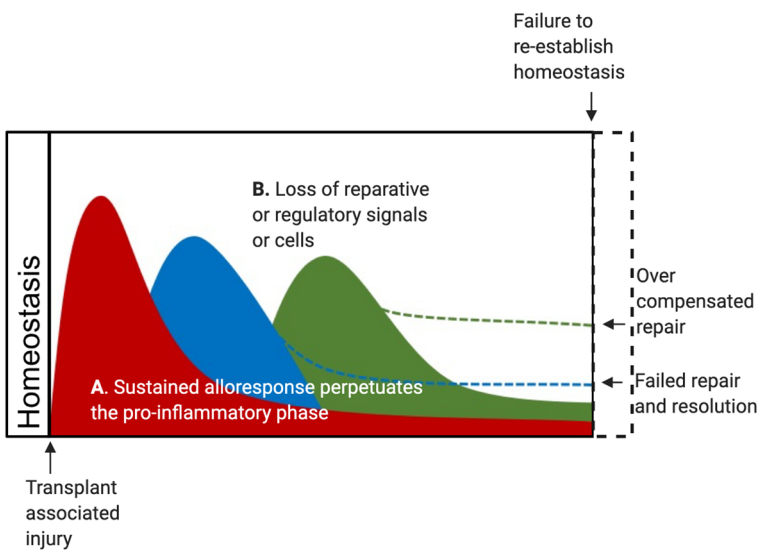

FIGURE 2 | DAMP may contribute to CR after Tx in several scenarios. **(A)** The alloreactive response of innate and adaptive cells may sustain the pro-inflammatory phase and lead to a failure to fully transition through the resolution phase and complete the repair phase. In this scenario, CR represents a failure to resolve and repair, leading to residual graft damage and failure to restore tissue homeostasis. Alternatively, these changes may cause an overzealous counter-responses initiated by reparative Treg and reparative macrophages in responses to regulatory and reparative DAMPs released by an unresolved allogeneic wound. **(B)** Alternatively, lost or depleted reparative and regulatory cells due to ischemia or alloimmune responses may lead to a failed resolution phase leading to residual graft damage. This scenario may also arise from a depletion of local reparative and regulatory DAMPs over time. In this case, CR would represent a failure to restore tissue homeostasis due to persisting graft damage.

limiting fibrosis (90–92). Similar findings in human transplant samples were observed when endomyocardial biopsies from sex-mismatched heart transplant recipients were assessed for the presence of donor tissue-resident CCR2$^-$ macrophages (112). These studies confirmed that the CCR2$^-$ subset was almost exclusively donor-derived. Parallel transcriptomic profiling of CCR2$^-$ and CCR2$^+$ macrophages isolated from failing human hearts were consistent with the conclusion that these two populations were distinct cell types. The CCR2$^+$ subset appeared monocyte-derived and expressed inflammatory mediators, including IL-1β, components of the inflammasome, and genes involved in adverse cardiac remodeling. The CCR2$^-$ subset instead expressed increased Lyve1, growth factors, and ECM genes implicated in tissue remodeling. The CCR2$^+$ macrophage subset was more abundant than the CCR2$^-$ subset in heart failure samples from those with worse left ventricular systolic dysfunction and adverse remodeling (112). This seminal study provides the initial confirmation of a potentially beneficial donor CCR2$^-$ macrophage population in transplanted organs.

Further studies can build on this work to define if shorter ischemia times, *ex vivo* normothermic perfusion, or specific immunosuppression protocols can prevent or slow the loss of donor CCR2$^-$ macrophages and the subsequent replacement by CCR2$^+$ recipient macrophages after SOTx. These examinations or related pre-clinical studies will provide an understanding if AR, CR, IRI, or recipient alloimmunity causes the CCR2$^-$ subset to drop below a significant reparative threshold after Tx. It would be expected that this would lead to increased inflammation and a sustained loss of homeostasis (**Figure 2B**). Such a scenario would account for increased alloimmunity and fibrosis, that culminates CR pathology after SOTx (**Figure 2B**). An important additional question to answer if local reparative DAMPs support the survival or local proliferation of the CCR2$^-$ subset during homeostasis or after IRI.

Dysregulated Local Niches in the Tx Microenvironment

Immunological niches typically provide a hospitable place that concentrates the signals needed to nurture the immune and stromal cells need to maintain an effective local immune response or local homeostasis. An organized immunological microenvironment, or niche, controls local immune responses during tumor development, is necessary for regulating immune cell functions in the secondary lymphoid organs, and fundamental to the production of blood cells in the bone marrow (176–178). We briefly discussed how HA-rich niches in the adventitia are critical for the homeostatic maintenance of vessel function and how these can be disrupted through the loss of specific cells like CCR2$^-$ macrophages or local environmental signals like HA. The role of immunological niches in transplant outcomes is currently entirely speculative but is an exciting concept. However, based on what is known about both IRI and the alloimmune response after Tx, we would expect that "homeostatic," "acute rejecting," and "injured/reparative," "dysregulated/fibrotic/CR" areas all would be observed, and often co-exist, throughout the lifespan of a transplant. Work in this space by the Halloran group revealed using unsupervised principal component analysis and archetypal analysis on microarray assessment of HTx endomyocardial biopsies (EMB) identified that samples that abnormal EMB did not associate cleanly with rejection and instead expressed transcripts indicating a tissue injury response (179, 180). These samples were enriched for transcripts for DAMPs, as well as macrophages. The injury-related scores were also high at early times post-transplant and routinely diminished over time. The decreasing rejection scores suggest that repair and resolutions of global IRI injury to the graft is indeed typical unless interrupted by a local alloimmune response that is not effectively inhibited by immunosuppression.

IL-33 has been implicated in adventitial vascular niches, where the IL-33 deletion causes an inadequate local immune response to pathogens (164). Ablation of IL-33 from white adipose niche caused immune dysregulation in these niches resulting in immune dysfunction and obesity associated with increased pro-inflammatory myeloid cells (181). A high-fat diet also reduces IL-33 expression in the white adipose niches to produce similar outcomes. As discussed above, we found that the upregulation of IL-33 during clinical and experimental HTx rejection decreased CR due to this regulatory DAMP's potent capacity to limit the generation of pro-inflammatory macrophages from monocytes infiltrating the grafts (78). Nevertheless, it is yet to be determined how IL-33 or other regulatory DAMPs are maintained in SOTx regions of the graft with acute or sustained alloimmunity. Limited evidence from EMB suggests that grafts maintaining IL-33 display less CR (78). It is known that IL-33 decreases with age in the muscle leading to inadequate regenerative responses associated with decreased Treg and increased inflammatory macrophages. How the expression of these DAMPs are modulated in fibrotic areas to instruct local CCR2$^-$ and CCR2$^+$ macrophages will be an essential question to answer. A lost local repair response may become further augmented when niches become depleted of reparative, or regulatory DAMPs or the niche ECM becomes unsupportive of cells needed for repair and instead overtook by alloreactive T cells that are stimulated by pro-inflammatorymacrophages. As vessels become occluded due to damage or CR, the niche will become hypoxic and may further drive macrophages towards pro-inflammatory subsets supporting rejection. Conversely, sustained hypoxic environments in areas of the graft could instead favor the generation of regulatory macrophages due to the induction and modulation of local DAMPs. Both HMGB1 and IL-33 functionality is impacted by their redox state. While oxidation of IL-33 into a disulfide-bonded form negatively regulates its function (182), oxidized HMGB1 induces the expression of proinflammatory cytokines and chemokines by macrophages through its binding to MD-2 and TLR4 (183). Conversely, reduced HMGB1 associates with the chemokine CXCL12 and binds the CXCR4 receptor to recruit circulating leukocytes and stem cells to the site of damage and promote tissue regeneration (184, 185). Reduced HMGB1 in hypoxic tumor sites is suggested to generate regulatory and reparative macrophages that shape an immunosuppressive tumor microenvironment (186). HSPs would

also be induced in hypoxic areas or by cell stress associated with ischemia, rejection, and fibrosis. Important future studies will be needed to establish how reduced, oxidized, or induced DAMPs function in hypoxic versus normoxic regions of solid organ transplants to dictate short and long-term outcomes.

CONCLUSIONS

The original concept that DAMPs function after Tx as endogenous PAMPs in one-way paths that can be blocked to prevent early inflammation while regulatory and repair signals proceed unabated is dated. Our current understanding, generated from limited Tx data and studies of organ IRI models, is that the transcription factors and metabolic processes activated by pro-inflammatory DAMPs triggering inflammation after IRI and tissue injury are an essential part of a dynamic process that needs to function to trigger resolution and allow damaged tissues to return to homeostasis. It is also clear that regulatory and reparative DAMPs, closely related SPMs, are also significant players in shaping the ideal size and duration of the inflammatory phase after tissue injury. Regulatory and reparative DAMPs are also active mediators of subsequent resolution and repair phases.

As tools such as scRNAseq and spatial transcriptomics become more widely applied in Tx, it will become more apparent how these different subsets of DAMPs contribute to immune cell networks during effective responses to IRI and how these are altered by local alloimmune responses by innate and adaptive cells. Value-added histology approaches utilizing mapped total RNA analysis (i.e., 10x Genomics Visium technology) or multiplexing immunofluorescent tags detecting RNA messages or specific proteins (i.e., Nanostring GeoMx technology) will be incredibly helpful to add to our understanding of the DAMP-driven immunology and physiology existing throughout the graft. Chronological graft assessment will define how sustained generation or depletion of regulatory or reparative signals triggering inflammation, resolution, and repair are modulated in graft AR and CR in crucial spaces, such as the vasculature. Applying advanced bioinformatics techniques such as artificial intelligence and machine learning can be used to investigate immune cell/DAMP interactions to help establish how these interactions shape the active signaling networks at each step after injury, inflammation, resolution, and repair of the graft (187). Using these types of analyses with precise mouse models allows for temporal control of the local DAMPs or AlloAg, which will allow us to untangle AlloAg input into pro-inflammatory, resolution, and repair pathways after SOTx.

This knowledge will provide the transplant community with a framework for developing precision-medicine approaches where biologicals direct immune processes in the graft effectively. This family of drugs will be delivered to modulate dominant networks active in the graft instead of typical efforts to target individual immune populations or cytokines. Given the emerging evidence that DAMPs are important mediators of both early inflammation, injury resolution and repair there is significant therapeutic potential in manipulating the expression or delivery of DAMPs during the course SOTx. We have shown that the delivery of regulatory biomolecules, such as IL-33, using a hydrogel immediately post transplantation could improve outcomes by reducing the generation of inflammatory macrophages in HTx early after transplantation (78). Exploration into the *ex vivo* manipulation of organs prior to transplantation as a means to minimize inflammation and induce the expression of regulatory DAMPs is warranted. With the more recent development of normothermic *ex vivo* organ perfusion storage to mitigate IRI, there is a window of opportunity to biologically modify the donor organ either through the delivery of biomolecules, including regulatory DAMPs, encased in biovesicles or synthetic nanoparticles or potentially through gene therapy (188, 189). Another potential therapy to investigate is hypoxic pre-conditioning before transplantation in order to induce the expression of HSPs and other DAMPs that are regulatory in their reduced form. Future studies will be needed to establish the best timing and mechanism of therapeutic delivery of regulatory DAMPs following solid organ transplantation to limit AR and CR.

AUTHOR CONTRIBUTIONS

HT and GD together generated the text and figures. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: HT is a listed inventor on patent application PCT/US2019/030547 ("MATRIX BOUND VESICLES (MBVS) CONTAINING IL-33 AND THEIR USE").

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