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Impact of Infection on Transplantation Tolerance

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Summary

1.

Allograft tolerance is the ultimate goal of organ transplantation. Current strategies for tolerance induction mainly focus on inhibiting alloreactive T cells while promoting regulatory immune cells. Pathogenic infections may have direct impact on both effector and regulatory cell populations, therefore can alter host susceptibility to transplantation tolerance induction as well as impair the quality and stability of tolerance once induced. In this review, we will discuss existing data demonstrating the effect of infections on transplantation tolerance, with particular emphasis on the role of the stage of infection (acute, chronic, or latent) and the stage of tolerance (induction or maintenance) in this infection-tolerance interaction. While the deleterious effect of acute infection on tolerance is mainly driven by proinflammatory cytokines induced shortly after the infection, chronic infection may generate exhausted T cells that could in fact facilitate transplantation tolerance. In addition to pathogenic infections, commensal intestinal microbiota also has numerous significant immunomodulatory effects that can shape the host alloimmunity following transplantation. A comprehensive understanding of these mechanisms is crucial for the development of therapeutic strategies for robustly inducing and stably maintaining transplantation tolerance while preserving host anti-pathogen immunity in clinically relevant scenarios.

Keywords

infections; transplantation tolerance; donor negative vaccine; co-stimulation blockade; T cells; regulatory immune cells; intestinal microbiota

2. Introduction

Organ transplantation is an effective treatment for patients with end stage organ diseases and is a widely accepted clinical practice. However, to prevent allograft rejection, most of the

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transplant patients still rely on lifelong immunosuppressive medication, which may bring significant side effects to patients, such as drug toxicity, infection and cancer susceptibility¹. Transplant tolerance represents the state where the allograft is accepted by recipient in the absence of immunosuppressive treatment. Achieving a tolerogenic state will allow patients to avoid the deleterious side effects of immunosuppressive drugs. Transplant tolerance has been successfully achieved in animal models by various strategies². Based on different strategies, they can be classified into hematopoietic stem cell (HSC) transplant-based and non-HSC transplant-based tolerance induction strategies. HSC transplant often results in hematopoietic chimerism and central tolerance by deletion of allo-specific T cells in thymus and bone marrow; whereas non-HSC approaches usually results in peripheral tolerance and suppression, but not deletion, of allo-specific T cells³. Hematopoietic chimerism has been tested in clinical kidney transplantation with promising results⁴, while non-HSC transplant approaches remain in pre-clinical stages. However, the efficacy and stability of these strategies can be challenged by many factors, including pathogen infections and gut microbiota⁵. Infection as a major complication in clinical transplantation has been associated with suboptimal allograft outcome⁶. In experimental models, a detrimental impact of infection on transplant tolerance has also been demonstrated⁷. In this review, we will review current literature on the impact of infection on transplantation tolerance and the mechanistic basis of altering alloimmune T cell responses by infection. To be complete, we will also briefly discuss the influence of commensal gut microbiota on transplantation tolerance.

3. Impact of infection on alloreactive immune responses

Adaptive immune response against donor antigens is the major barrier to successful transplantation, and alloreactive T cell responses play a central role in mediating graft rejection⁸. Therefore, most efforts for inducing donor-specific tolerance have specifically focused on inhibiting alloreactive T cell immune responses. With an increasing knowledge of transplant-mediated adaptive immunity, a growing number of tolerance-inducing strategies have exhibited excellent long-term immunosuppression-free graft protection in preclinical models as well as in clinical trials². However, graft protection by these promising strategies seen in quiescent hosts may be challenged by inadvertent host infections, as a series of immune responses elicited in response to infection may alter the immune microenvironment in the body^{9,10} and have a significant impact on both tolerance induction and maintenance in transplantation¹¹. Therefore, understanding how alloreactive T cell immune responses may be modulated by exposure to pathogens and pro-inflammatory signals is critical for the development of therapeutic strategies that can induce and maintain potent transplantation tolerance in setting of inadvertent infections. In this section, we discuss how microbial infections may affect alloreactive T cell activation and regulation.

3.1 Effects of infection on alloreactive T cells

Full activation of alloreactive T cells requires three signals^{12,13}, all of which can be affected by infections (Figure 1). Signal 1 is the recognition and binding of alloantigenic peptide on antigen presenting cells (APCs) by the cognate TCR complex expressed on alloreactive T cells. The antigen binding component of the TCR complex specifically recognizes the proteolytically processed alloantigenic peptides presented by major histocompatibility

complexes (MHC) on APCs^{14–16}. However, it has long been known that certain pathogen-specific T cells also have the potential to recognize MHC molecules structurally similar to pathogen epitopes, hence the term “cross-reactive T cells” was coined¹⁷. Amir *et al* reported that a substantial proportion of virus-specific T cells, including those with specificities to the Epstein-Barr virus (EBV), cytomegalovirus (CMV), varicella zoster virus (VZV), and influenza virus, also respond to allogeneic stimulations¹⁸. More recently, van den Heuvel *et al* demonstrated that the polyclonal immune repertoire directed against CMV alone is associated with a memory response to six allogeneic human leukocyte antigen (HLA) molecules¹⁹. Reciprocally, a single HLA-specific memory T cell clone can also respond to multiple viral specificities. These data indicate a wide cross-reactivity between virus-specific and alloantigen-specific T cells. Existing studies have reported that cross-reactive virus-specific T cells indeed contributed to allograft rejection^{20,21}. In addition, bacteria-specific T cells such as those specific to *Leishmania major* infection also exhibit significant cross-reactivity to alloantigens and portend a negative impact on allograft survival²². These findings collectively suggest that recipients with prior pathogen exposures may already harbor a repertoire of allo-reactive memory T cells forming a pre-existing barrier to tolerance induction and maintenance.

Signal 2 for T cell activation is the engagement of costimulatory molecules expressed on APCs with their corresponding ligands expressed on T cells. This signal is critical for driving T cell clonal expansion, survival, and differentiation²³. There exists a large group of co-signaling molecules, which can either promote (costimulatory) or inhibit (coinhibitory) T cell activation²⁴. These molecules together form a complex and kinetic network during the whole phase of adaptive T cell immune responses, and it is the constellation of all the costimulatory and coinhibitory signals that determines the fate of T cells^{23,25}. In the absence of costimulatory signals, T cells become inactivated following an antigen encounter, but remain alive in a hypo-responsive state called “anergy” for an extended period of time,^{26,27}. Naturally, this critical consequence of blocking costimulatory signals during T cell activation has generated tremendous enthusiasm towards targeting costimulatory signals for transplantation tolerance induction.

However, such a hypo-responsive state of T cells can be readily subverted by infections. IL-2 is a primary inflammatory cytokine in microbial infections²⁸ and is reported to reverse the hypo-responsiveness of anergic T cells. As Bendiksen *et al* reported, the ability of anergic T cells to proliferate and produce inflammatory cytokines in response to an antigen can be fully restored by receiving intermediate stimuli from the cognate antigens plus IL-2²⁹. The underlying mechanism of such anergy reversal was described by Myrianne *et al* and appears to implicate IL-2 receptor signaling through JAK3 and mTOR leading to the inhibition of expression of anergy-inducing genes³⁰. Another key interaction in driving anti-virus T cell immune responses is between OX40-OX40L^{31,32}, which has also been shown to rescue self- or tumor-reactive CD8 T cells from an anergic state³³. These data suggest that pathogen infections may result in reactivation of anergic T cells.

Interestingly, both costimulatory and coinhibitory signals play important roles in shaping the host T cell immune response to specific pathogens³⁴. For instance, in a chronic LCMV infection mouse model, Crawford *et al* have shown that both coinhibitory molecules

including PD-1, CTLA-4, 2B-4, LAG-3 and BTLA as well as costimulatory molecules such as OX40, ICOS and CD27 are increased on virus-specific T cells, though in a differential manner on CD4⁺ versus CD8⁺ T cells³⁵. Therefore, attention should also be paid to inhibitory signals upregulated on T cells during chronic infections. For instance, T cell exhaustion caused by chronic antigen stimulation is another hyporesponsive state of T cells which is characterized by decreased proliferative capacity and a loss of IL-2 production, followed by a reduced capacity to secrete tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ). These exhausted T cells are characterized by a significant upregulation of inhibitory receptor expression on their surface, among which the most well-characterized are PD-1 and CTLA-4^{36,37}. The current notion is that T cell exhaustion is likely to be beneficial for transplantation tolerance³⁸. For instance, in a human liver transplant study, Bohne *et al* reported that operational tolerance was associated with an expansion of exhausted PD1/CTLA4/2B4 positive HCV-specific circulating CD8⁺ T cells³⁹. Their findings suggest that persistent viral infections may exert immunoregulatory effects that could contribute to allograft tolerance. Taken together, immune responses elicited by infections may regulate both costimulatory and coinhibitory signals to form a complex co-signaling network, contributing to both transplantation rejection and tolerance.

In addition to signal 1 and signal 2, T cells also require a third signal (signal 3) for the optimal generation of effector and memory populations. In the absence of signal 3, antigen recognition and costimulation ligation propel T cells through only weak clonal expansion and proliferation, while fail to drive them to achieve strong effector functions or memory formation. Signal 3 is mainly provided by inflammatory cytokines such as IL-12 and type-I IFN^{40,41}. The critical role of IL-12 for CD8⁺ T cell effector function has been well elucidated. In a mouse Leishmania Infection model, Novais *et al* have recently shown that lesional CD8⁺ T cells fail to make effector cytokine IFN- γ because of a deficiency in IL-12, and consequently, the addition of IL-12 effectively increases their IFN- γ production in the leishmanial lesions⁴². In another mouse melanoma model, *ex vivo* IL-12-conditioning of mouse CD8⁺ T cells leads to a 10–100-fold increase in their persistence and anti-tumor efficacy upon adoptive transfer to lymphodepleted mice⁴³. Importance of IL-12 in alloreactive CD8⁺ T cell function has also been reported: in a heart transplant model, dendritic cells conditioned to produce IL-12 are needed to provide the “third signal” for effector CD8⁺ T cell differentiation and subversion of tolerance⁴⁴. Similar to IL-12, type-I IFN has also been shown to be necessary for virus-specific CD8⁺ T cell clonal expansion and memory formation. T cells deficient in cytokine receptors for type-I IFN show reduced clonal expansion and CD8 T cell memory formation during infection^{45–47}. While development of memory to vaccine is supported predominantly by IL-12, both IL-12 and type-I IFN contribute to memory formation in response to *Listeria*⁴⁸. The impact of signal 3 on T cell function is further supported by gene expressions induced by signal 3⁴⁹. Compared with stimulation with only signal 1 and signal 2, when IL-12 or type-I IFN is also present, T cells are reprogrammed to enhance the expression of many genes involved in effector functions, proliferation, costimulation, survival and trafficking⁴⁹. In summary, the inflammatory cytokines IL-12 and type-I IFN are critical for T cell effector function and memory formation. Therefore, their production by infectious pathogens may serve to

provide potent signal 3 to augment the effector function and memory formation of alloreactive T cells which in turn impact transplantation tolerance.

3.2 Effects of infection on regulatory immune cells

When fully activated in the presence of the aforementioned three signals, the robust adaptive T cell immune responses also require self-controlling mechanisms to prevent exaggerated inflammatory responses that will be undoubtedly deleterious to the body. Numerous regulatory immune cell populations exist to regulate the magnitude and duration of inflammatory immune responses^{50–53}. In the transplantation scenario, these regulatory immune cell populations may function to inhibit allogeneic T cell immune responses through multiple mechanisms, thus shifting graft rejection towards allograft tolerance⁵⁴. Such regulatory populations mainly include regulatory T cells (Tregs), regulatory B cells (Bregs), regulatory dendritic cells (DCs), regulatory macrophages, myeloid derived suppressive cells (MDSCs), and mesenchymal stromal cells⁵⁵. However, survival and function of all these regulatory cells can be modulated by their specific immune milieu^{50,56,57}, including pathogen infections (Figure 2). Therefore, understanding the underlying molecular mechanisms of how these regulatory cells modulate alloimmune responses in transplantation and the impact of infection on these populations will be highly informative for strategies for inducing and maintaining stable transplantation tolerance.

3.2.1 CD4⁺CD25⁺Foxp3⁺ regulatory T cells—The CD4⁺CD25⁺Foxp3⁺ Tregs are the most well-studied regulatory immune cell population in transplant immunobiology. Tregs comprise natural Tregs (nTregs) generated in the thymus and induced Tregs (iTregs) developed from Foxp3[−] T cells in the periphery during specific conditions, such as under the influence of TGF- β and/or mTOR inhibition⁵⁸. Both Treg populations are crucial to transplantation tolerance: while nTregs may be critical for the initiation of allo-specific tolerance, iTregs have been shown to participate in both tolerance induction and maintenance^{59,60}. Several mechanisms have been implicated in the immunosuppressive function of CD4⁺CD25⁺Foxp3⁺ Tregs. First, CTLA-4 expressed on the surface of Tregs can bind to costimulatory molecules CD80 and CD86 on APCs to inhibit their antigen presenting ability⁶¹. In addition, binding of CTLA-4 to CD80/CD86 can also enhance the production of indoleamine 2,3-dioxygenase (IDO) and its downstream inhibitory signal pathway⁶². Immunosuppressive cytokines, including IL-10, IL-35 and TGF- β , have also been implicated in the functional activity of Tregs^{61,63,64}. On the other hand, it has been shown that the survival and regulatory function of Tregs are largely inhibited by inflammatory signals⁶⁵. For instance, in the presence of inflammatory cytokines, such as IL-6, IL-21 or IL-1, both nTregs and iTregs can be re-programmed to down-regulate Foxp3 expression while up-regulating IL-17 production^{66,67}. Consistent with this notion, it has been observed that in inflamed tissues, both nTregs and iTregs exhibit transient or unstable Foxp3 expression, and acquire an activated memory phenotype and produce inflammatory cytokines⁶⁸. More importantly, IL-6 produced by DCs upon toll-like receptor (TLR) activation by microbial infections inhibits the immune suppressive function of Tregs in order to permit the activation of pathogen-specific adaptive immune responses⁶⁹. Collectively, these results indicate that inflammatory cytokines generated during infections may impair both the number and function of Tregs. Additionally, inflammatory cytokines can also

convert unstable Tregs to inflammatory phenotypes, ultimately disrupting transplantation tolerance mediated by Tregs.

3.2.2 Regulatory dendritic cells—DCs are a heterogeneous cell population that plays a pivotal role in linking the innate and adaptive immune responses. During an innate immune response, DCs can sense pathogen derived molecular patterns (PAMPs) via pattern-recognition receptors (PRRs), and activate downstream inflammatory immune responses. Conversely, during an adaptive immune response, DCs function as professional APCs for priming antigen-specific T cells, including those specific for alloantigens. However, the presence of an innate immune response may alter the phenotype and function of DCs by promoting their activation and maturation, which may in turn impact their antigen presenting ability during an adaptive immune response. In the steady state following transplantation, a special DC population has been described that functions to dampen allospecific T cell responses and facilitate transplantation tolerance^{70,71}. Consequently, these DCs are termed “tolerogenic DCs” (Tol-DCs). In general, Tol-DCs are characterized by an immature phenotype, with low expression of MHC-II and costimulatory molecules such as CD80 and CD86⁷². Their tolerogenic efficacy in transplantation and mechanisms of action have been well described in rodent and non-human primate (NHP) transplant models^{73–81}. First of all, downregulation of MHC-II and costimulatory molecules impair their antigen presenting ability, promoting hypo-responsiveness of cognate alloreactive T cells. Second, Tol-DCs can induce apoptosis of both naive and memory T cells through activation of Fas/FasL pathway and upregulation of IDO^{82–84}. Third, Tol-DCs promote induction and expansion of peripheral Tregs^{85,86}. Interestingly, Tregs in return prevent Tol-DC maturation through CTLA-4 and CD80/CD86 interaction as well as production of IL-10 and TGF- β , thereby forming a tolerogenic feedback loop between Tol-DCs and Tregs^{87,88}. This tolerogenic feedback loop is critical for maintaining the dominant role of Tol-DCs in peripheral tolerance.

The phenotype and immune regulatory function of Tol-DCs, however, may be greatly challenged by pathogen infections. Following activation of PRRs and downstream NF- κ B pathway, DCs undergo maturation with upregulation of MHC-II and costimulatory molecules and production of inflammatory cytokines. They then migrate to secondary lymphoid organs and trigger potent adaptive immune responses as a potent stimulator to T cells⁸⁹. In addition, type-I IFN produced during pathogen infections can also directly accelerate DC migration to lymphoid tissues and enhance their antigen presenting capacity⁹⁰. Loss of tolerogenic function of DCs breaks the Tol-DC-Tregs feedback loop, resulting in the impairment of Treg induction and expansion. Reciprocally, Treg induction and function can also be directly inhibited by infection via several mechanisms described above, further contributing to the disturbance to the Tol-DC-Treg feedback loop. In summary, like Tregs, the regulatory function of Tol-DCs on alloimmune responses during transplantation can also be threatened by pathogen infections.

3.2.3 Regulatory macrophages—Macrophages are a heterogeneous cell population with numerous subsets whose functions are determined by their tissue locations and microenvironment⁹¹. As a distinct subset of macrophages, regulatory macrophages (Mregs)

function to dampen inflammatory immune responses and prevent the immunopathology associated with prolonged classical macrophage activation ⁹². In transplantation, Mregs have been shown to suppress alloimmune responses and promote organ allograft survival. In a heterotopic mouse heart transplant model, donor strain Mreg infusion prior to transplantation significantly prolongs allograft survival via IFN- γ induced IDO activity. *In vitro* experiments also indicate that Mregs are capable of preferentially eliminating allogeneic T cells through direct phagocytosis ⁹³. Similarly, human Mregs derived from *in vitro* culture have also been shown to exert potent inhibitory effects on alloimmune responses in an IDO-dependent manner ⁹⁴. More recently, Hutchinson's group showed that human Mregs can convert allogeneic CD4⁺ T cells into IL-10-producing, TIGIT⁺Foxp3⁺ iTregs that exert bystander T cell suppression as well as inhibit DC maturation ⁹⁵. This ability is dependent on signals mediated by IDO, TGF- β , retinoic acid, Notch and the progesterone-associated endometrial protein. In a clinical study of living-donor kidney transplant recipients, preoperative administration of donor-derived Mregs resulted in an acute increase in circulating TIGIT⁺ Tregs ⁹⁵. Furthermore, human Mregs have also been shown to exert potent suppression of xenimmune responses via upregulation of IDO activity ⁹⁶. These novel findings indicate that Mregs may be a promising cell type for use as a cell-based tolerance therapy in solid organ transplant recipients. Direct evidence of effects of infections on Mregs in transplant settings are currently lacking. However, several studies support a role of pathogen infection in the development of Mregs. For example, a systemic IgG induction seen in Leishmania infection has been shown to engage the Fc receptor on macrophages and trigger downstream signaling including IL-10 production and development of Mregs ⁹⁷. Similar phenomena have also been observed in infections by the African trypanosomes ⁹⁸, Bacillus anthracis ⁹⁹, Coxiella burnetii ¹⁰⁰ and Dengue virus ¹⁰¹. Therefore, such infection-induced Mregs may have complicated consequences in transplantation, and their role in promoting graft survival as well as in pathogen controls and overall patient survival warrants further studies.

3.2.4 Myeloid derived suppressor cells (MDSCs)—MDSCs is yet another heterogeneous population of immature myeloid cells that have been shown to negatively regulate the adaptive immune response ^{102,103}. Based on different origins, MDSCs are divided into granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). In physiological conditions, immature myeloid cells (IMCs) tend to develop into DCs, macrophages, and neutrophils; whereas in pathological states such as tumors, stress, and infections, IMCs can differentiate into MDSCs to exert suppressive functions ^{54,102}. Several mechanisms of T cell suppression by MDSCs have been demonstrated. Arginase-1 produced by MDSCs can result in essential amino acid depletion and lymphocyte starvation ¹⁰⁴. In addition, inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate oxidase-2 expressed by MDSCs lead to oxidative stress through production of nitric oxide and reactive oxygen species (ROS) ¹⁰⁵. Lastly, MDSCs have also been shown to promote Treg expansion and to mediate their immunosuppressive capacity ¹⁰⁶. For transplantation tolerance, perioperative recipient infusion of MDSCs has been shown to prolong allograft survival in mouse islet transplantation ¹⁰⁷, corneal transplantation ¹⁰⁸ and skin transplantation ¹⁰⁹. In various infection models, the expansion and immunosuppressive function of MDSCs have been well-studied. On the one hand, both G-MDSCs and M-

MDSCs have been shown to expand post-infection which in turn inhibit subsequent T cell immune responses^{110,111}. On the other hand, we have recently shown that CMV infection in a murine model indeed impairs MDSC differentiation, thereby breaking transplantation tolerance mediated by MDSCs¹¹². Acute CMV infection is found to inhibit G-MDSC expansion and impair the suppressive function of M-MDSCs¹¹². In support of our findings, in an independent clinical study of lung transplant recipients, G-MDSCs are found to decrease in number with infection while expand in stable lung recipients¹¹³. Furthermore, patients with chronic lung allograft dysfunction also has a lower level of G-MDSCs in the periphery¹¹³. However, there are also conflicting data reporting significant and persistent increase of M-MDSCs after bacterial infection in renal transplant recipients which contributes to post-infection immunodeficiency in these patients¹¹⁴. Therefore, it is conceivable that the ultimate impact of infections on MDSCs in the setting of transplantation is determined by numerous variables including the type of pathogens and infections, immunosuppressive regimens and tolerance induction strategies used, and possibly the organ transplanted. While it is evident that infections do influence MDSC differentiation and function in transplantation settings, further studies are needed to delineate such influences in a more granulated manner in order to better utilize MDSCs for purposes of transplantation tolerance induction and maintenance.

4. A brief overview of peripheral tolerance induction strategies

Allograft tolerance has been a goal of the organ transplantation field for over sixty years. It was first reported in 1945 that red blood cells of one dizygotic twin cattle could survive long-term in the other dizygotic twin cattle without being rejected, indicating the possibility of host acceptance of allogeneic hematopoietic precursors¹¹⁵. The notion of inducing hematopoietic chimerism for establishing immune tolerance to solid organ transplantation has been since brought to test in numerous clinical trials, and has shown great efficacy in allograft protection^{116–118}. Hematopoietic chimerism is a form of central tolerance whose mechanisms of tolerance primarily involve clonal deletion of donor-reactive T cells and B cells in the thymus and bone marrow, respectively^{119,120}. However, this tolerance approach often requires aggressive recipient bone marrow conditioning regimens and further, exposes recipients to long-term risks for graft-versus-host disease¹²¹. Alternative to hematopoietic chimerism, several other approaches, including co-stimulatory signal blockage, donor negative vaccination and regulatory immune cell infusion, aim to establish peripheral tolerance^{118,122,123}. These approaches rely on mechanisms (discussed below) that are more susceptible to disturbances by concurrent infections, therefore will be the focus of this review.

4.1 Costimulatory signal blockade

As discussed earlier, full activation of alloreactive T cells requires costimulatory signals (signal 2). TCR signaling (Signal 1) in the absence of signal 2 renders T cells anergic, namely hyporesponsive to subsequent stimulations^{26,27}. Furthermore, costimulatory signals also play a crucial role in regulating the balance between Tregs and effector T cells (Teffs)¹²⁴. Based on this concept, a large number of studies have been conducted to specifically modulate co-stimulatory/inhibitory signals for purpose of inducing transplantation tolerance.

To date, allograft tolerance and long-term survival have been achieved by such strategies in both murine and non-human primate transplantation models. More importantly, some of these approaches have also shown promising results in clinical trials of human transplantation^{125–128}. Here, we will discuss strategies used to target B7-CD28/CTLA4 and CD40-CD154(CD40L) pathways.

4.1.1 B7-CD28/CTLA4 pathway—The B7(CD86/CD80)-CD28/CTLA4 pathway is a well characterized costimulatory signal pathway in transplant tolerance induction. CD28 is constitutively expressed on naïve as well as activated and memory T cells^{129,130}, whereas CTLA-4 is localized in intracellular vesicles in resting T cells and traffics to cell surface after TCR ligation and CD28 costimulation^{131,132}. After being ligated by B7 molecules, CD28 on T cells provides a costimulatory signal that promotes T cell survival, and triggers their proliferation and inflammatory cytokine production¹³³. On the contrary, upregulated by T cell activation, CTLA-4 on T cell surface competes with CD28 and binds to CD86/CD80 with a much greater affinity. Stimulation of CTLA-4 transmits an inhibitory signal to T cells^{134,135} leading to their inhibition and cell death^{136,137}. Furthermore, B7-CTLA4 interaction has also been shown to inhibit Th17 differentiation and suppress Th17-mediated autoimmunity¹³⁸. Both CTLA-4 and CD28 are critical for Treg function and survival^{139–143}. Tregs constitutively express CTLA-4, and its expression is further augmented by Treg activation. On the other hand, CD28 signaling is required for the maintenance of a stable pool of Tregs in the periphery by enhancing their survival and promoting their self-renewal^{139–143}.

Exploiting the much higher affinity for B7 by CTLA-4 than by CD28, a fusion protein CTLA4-Ig has been generated for the purpose of competitively blocking the CD28-B7 costimulatory signaling pathway. *In vitro* mixed leukocyte reactions (MLR) have shown that CTLA4-Ig effectively inhibits allo-specific T cell activation¹⁴⁴. *In vivo* blockade of the CD28 pathway using CTLA4-Ig promotes tolerance of cardiac, renal and islet allografts in rodent models^{145–148}. More importantly, in an international phase III clinical trial^{127,128}, belatacept, a high avidity CTLA4-Ig, was shown to be associated with superior renal function and similar kidney graft survival in comparison to cyclosporine in kidney transplant recipients, although a higher rate of early acute rejection was also observed¹²⁸. However, several concerns warrant caution in using CTLA4-Ig. First, as we outlined above, Th17 immune response can be suppressed by CTLA4-B7 interaction. CTLA4-Ig has been shown to be relatively ineffective in controlling Th17 responses, suggesting that CTLA4-Ig can block CTLA4-B7 negative signaling when CD28 is absent¹³⁸. Second, CTLA4-Ig has been shown to accelerate rejection in a Treg-dependent mouse model of transplant¹⁴⁹ and to inhibit allo-specific Treg generation in humans¹⁵⁰. Third, there continues to be a belatacept shortage due to transitioning of the ongoing manufacturing process¹⁵¹. Due to these considerations, reagents more specifically inhibit CD28, while leaving CTLA-4 intact, have been generated and tested both in small and large animal models^{152,153}. Compared with belatacept, these selective CD28 blocking agents have been shown to preserve negative signaling through CTLA-4 and promote superior graft survival^{152,153}. FR104, a CD28-specific targeting antibody, has now entered a phase I study and may have potential future applications in clinical transplantation^{125,126,154}. In addition, another CD28 domain-specific

antibody, lulizumab, has also moved to a phase I/II clinical trial in kidney transplantation (clinicaltrials.gov).

4.1.2 CD40-CD154 pathway—The CD40-CD154 (CD40L) interaction is another well studied costimulatory signal pathway in transplantation. As a member of the TNFR superfamily, CD40 is expressed on the surface of multiple types of cells, including B cells, DCs, macrophages, fibroblasts, endothelial cells and tumor cells ¹⁵⁵. Expression of CD40 is constitutive at relatively low level on immature DCs, but can be significantly upregulated by several maturation signals including encounter with microbial products (e.g. Toll-like receptor ligands), pathogens (e.g. viruses and bacterial) or uptake of apoptotic cells ^{156–158}. CD154 is the ligand for CD40, and is expressed on activated T cells and subsets of NK cells, eosinophils and platelets ¹⁵⁹. Inhibiting CD40-CD40L interaction effectively reduces the expansion and differentiation of allo-specific T cells through preventing the maturation of alloantigen-presenting DCs and promoting the generation of tolerance-inducing plasmacytoid DCs ¹⁶⁰. CD154 blockage also promotes conversion of conventional CD4 T cells to Foxp3⁺ Treg cells, and increases accumulation of Tregs within the allografts and graft-draining lymph nodes ^{161,162}. Furthermore, inhibition of the CD40-CD154 interaction has also been shown to lead to upregulation of coinhibitory molecules on alloreactive T cells, including PD-1 and KLRG-1, and to impede B and T interactions necessary for the development of alloreactive B cells within the germinal center and to mitigate antibody-mediated rejection in mice ^{163,164}.

With these effects on alloimmune responses, not surprisingly blocking CD40-CD154 interaction has been shown to have a beneficial effect on allograft survival in numerous murine as well as in NHP transplant models ^{165–167}. Furthermore, blocking CD40-CD154 is also synergistic with other tolerance induction strategies. For example, blocking CD40-CD154 combined with CTLA4-Ig can lead to long-term graft survival in skin and cardiac allografts in mice ^{168,169}. In other studies, co-administration of CD40-CD154 blockade with donor-specific transfusion (DST) or donor bone marrow transplantation results in tolerance induction for skin, heart, and islet transplantation ¹⁷⁰. However, a significant incidence of thromboembolic complications of the anti-CD154 antibody was observed in NHP models, as this antibody also targets platelet CD154 ^{171–173}. Therefore, more contemporary approaches of targeting this pathway have focused on the development of CD40-blocking antibodies. ASKP1240 is a humanized anti-CD40 antibody which has been found to effectively prolong kidney, liver and islet allograft survival without thrombotic complications in NHPs ^{174–176}. A phase I clinical trial using ASKP1240 combined with standard immunosuppression has demonstrated long-term allograft survival in kidney transplant recipients without obvious side effects, including drug-related thromboembolic complications previously seen with the anti-CD154 antibody ¹⁷⁶. Encouragingly, several other anti-CD40 antibodies have also been developed and have demonstrated promising effect for transplantation tolerance induction ^{177,178}.

4.2 Donor negative vaccination

Donor negative vaccination refers to a strategy of controlled exposure of donor antigens to the recipient immune system prior to transplantation to induce a donor-specific immune

tolerance state. In this case, assurance of an immune tolerance response in the recipients is absolutely critical, as an inadvertent immune activation may result in highly detrimental allo-specific memory cells and consequent allo-sensitization of the recipients^{179,180}. One potential form of donor negative vaccine is apoptotic donor cells whose non-inflammatory interaction with the recipient immune system mimics that by the billions of self-apoptotic cells cleared in our body on a daily basis¹⁸¹. There are several *in vitro* approaches of inducing apoptosis in donor cells, including γ irradiation, UV-B irradiation, and chemical treatments such as with ethylene carbodiimide (ECDI) or lytic anti-Fas monoclonal antibody¹⁸². Importantly, for tolerance induction, the donor cells infused must be at an early stage of apoptosis, as late stage apoptotic cells or further secondary necrotic cells engage receptors for damage-associated molecular patterns (DAMPs) and deliver inflammatory, instead of immunosuppressive signals¹⁸¹.

Infusions of UV-B or γ irradiated donor cells have been shown to provide significant protection to allografts in rodent bone marrow¹⁸³, heart^{184,185} as well as aorta¹⁸⁶ and islet transplantation models¹⁸⁷ without any additional immunosuppressive treatment. Our lab has experimented with donor splenocytes treated with 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide (ECDI-SPs) as a highly effective modality for tolerance induction in murine models of allogeneic and xenogeneic islet transplantation^{188,189}, allogeneic heart¹⁹⁰ and kidney transplantation (Luo et al, unpublished data), as well as in non-human primate allogeneic islet transplantation¹⁹¹. Graft protection in these studies correlates with a remarkable inhibition of allo-specific T cell immune response^{184–186}. Specifically, donor ECDI-SPs differentially target T cells with indirect versus direct allo-specificities. Following infusions of donor ECDI-SPs, T cells with indirect allo-specificity undergo a robust initial proliferation followed by rapid clonal depletion, whereas T cells with direct allo-specificity undergo limited proliferation and become anergized¹⁹². Supporting our findings, by using UV-B irradiation-induced donor apoptotic cells, another group independently showed that host DCs phagocytosing such cells subsequently present donor antigens in a manner that induce clonal deletion of donor reactive T cells with indirect allo-specificity^{185,186}.

Suppression of allo-specific T cells by donor ECDI-SP infusions is mediated by host DCs and macrophages in the spleen at the first point of encounter between the host and the infused apoptotic donor cells. Following uptake of apoptotic cells in the spleen, splenic CD11c⁺ DCs up-regulate co-inhibitory molecules PD-L1 and PD-L2, and subsequently mediate an abortive expansion followed by depletion of allo-reactive T cells^{188,192}. In addition, efferocytosis (non-inflammatory phagocytosis) of donor ECDI-SP by the efferocytic receptors on splenic macrophages also play a critical role in this process. One such efferocytic receptor is the Myeloid Epithelial Reproductive Tyrosine Kinase (MERTK)¹⁹³. MERTK is a member of the TAM (Tyro 3, AXL, MERTK) receptor tyrosine kinase (RTK) family. Its expression is thought to be primarily on macrophages where it mediates anti-inflammatory phagocytosis¹⁹⁴. MERTK expression is significantly up-regulated on splenic CD11b⁺F4/80⁺CD64⁺ macrophages following their engulfment of allogeneic ECDI-SPs¹⁹⁵. In addition, its phosphorylation is also significantly enhanced by co-culturing and phagocytosing allogeneic ECDI-SPs in an *in vitro* system¹⁹⁵, leading to activation of its downstream anti-inflammatory signaling. In addition to alterations of surface molecules, phagocytosis of apoptotic cells by APCs also creates a local immunosuppressive milieu by

upregulating the expression of anti-inflammatory factors such as IL-10, TGF- β , IDO, and at the same time downregulating the production of inflammatory cytokines such as IL-1 β , IL-6, IL-12, IL-23, TNF- α ^{183,185,187,190,196}. To further elucidate the obligatory role of DCs and macrophages in tolerance induction by donor ECDI-SPs, we depleted CD11c⁺ DCs in recipients by treatment with diphtheria toxin in CD11c-DTR mice or used MERTK knockout mice as recipients ^{192,195}. Supporting our hypothesis, depletion of DCs completely abolishes graft protection provided by donor ECDI-SP infusions ¹⁹². Likewise, macrophages with MERTK deficiency exhibit an exaggerated inflammatory response upon interaction with allogeneic ECDI-SPs, and consequently MERTK^{-/-} recipients fail to be tolerized by donor ECDI-SP infusions ¹⁹⁵. Consistent with this notion, Shi and colleagues similarly show that blocking phagocytosis *in vivo* abolishes the beneficial effect of apoptotic cells on heart allograft survival ¹⁸⁴. Therefore, collective evidence indicate that DC and macrophage phagocytic functions are crucial for mediating the tolerogenic signals triggered by preemptive donor apoptotic cell infusions.

Regulatory immune cell populations such as CD4⁺ Tregs and MDSCs have also been shown to be critical in mediating transplantation tolerance by apoptotic donor cells ^{188,192}. In mouse islet transplantation models, we demonstrate a significantly higher number of Tregs in the spleen, draining lymph nodes, as well as the islet allograft in recipients treated with donor ECDI-SPs compared with untreated recipients ^{188,192}. Depletion of Tregs completely abolishes tolerance induction by ECDI-SPs ¹⁸⁸. Similar observations of Treg expansion and accumulation in transplanted grafts in response to infusions of donor apoptotic cells have also been reported by others ^{185–187}. In addition to Tregs, in mouse heart allografts there is also an impressive accumulation of CD11b⁺IDO⁺ cells following infusions of allogeneic ECDI-SPs which correlates with a significant prolongation of heart allograft survival ¹⁹⁰. Further investigation reveals that the CD11b⁺ population induced by ECDI-SPs phenotypically resembles monocytic-like (CD11b⁺Ly6C^{high}) and granulocytic-like (CD11b⁺Gr1^{high}) MDSCs. These cells suppress T cell proliferation *in vitro* and traffic to cardiac allografts *in vivo* where they exert an inhibitory effect on local T cell expansion ¹⁹⁷. Recipients treated with an anti-GR1 antibody to deplete both Ly6C^{high} and Gr1^{high} cells promptly rejected their cardiac allograft ¹⁹⁷, indicating the obligatory role of MDSCs in mediating tolerance induced by allogeneic ECDI-SPs. More recently, we demonstrate that the expansion of MDSCs in response to allogeneic ECDI-SP infusions is dependent on MERTK, and that the immunosuppressive capacity of MDSCs is compromised by an inflammatory milieu seen in MERTK^{-/-} mice in response to allogeneic ECDI-SP infusions ¹⁹⁵. These findings underscore the role of MERTK in mediating the tolerogenic effect of ECDI-SPs via promoting the expansion and function of immune regulatory cells such as Tregs and MDSCs.

4.3 Regulatory immune cell infusions

Regulatory immune cells can facilitate allograft tolerance by inhibiting allo-specific T cells⁵⁴. Accordingly, adoptive transfer of regulatory immune cells in transplant recipients may promote transplantation tolerance. CD4⁺ Treg adoptive transfer has shown promising efficacy in allogeneic mouse skin, cardiac and islet transplantation models ^{198–203}. Notably, alloantigen-specific Tregs are more potent than polyclonal Tregs in suppressing

alloimmunity^{200,201}. A pilot clinical study in living donor liver transplant recipients demonstrates that infusion of *ex vivo* generated cells enriched for regulatory T cells is safe and possibly effective for minimization of immunosuppressive drugs and induction of operational tolerance²⁰⁴. In addition to expansion by donor cell stimulation, alloantigen-specific Treg can also be generated through transfection of viral vectors encoding chimeric antigen receptors (CARs)²⁰⁵. CARs are synthetic fusion proteins consisting of a single-chain variable fragment (scFv, a binding moiety of monoclonal antibody), an extracellular hinge, a transmembrane region, and an intracellular signaling domain. Transfected CARs in nTregs are capable of redirecting the specificity of nTregs toward the desired antigens without altering their regulatory phenotype or epigenetic stability²⁰⁶. HLA-A2, a MHC class I molecule highly prevalent among human donors, has been used as a target antigen to generate CAR-Tregs for suppressing transplant immunity²⁰⁷. Infusion of HLA-A2-specific CAR Tregs (A2-CAR-Tregs) has been shown to completely prevent rejection of allogeneic skin graft in immune reconstituted humanized mice in the absence of any immunosuppression²⁰². In their study, compared with polyclonal nTregs, A2-CAR Tregs exhibited a superior control of allo-specific immune responses *in vitro* and in humanized mouse models²⁰². In a mouse xenogeneic GVHD model in which human PBMCs were engrafted into immunodeficient NOD.SCID. $\gamma c^{-/-}$ (NSG) mice, human A2-CAR-Tregs, compared with polyclonal nTregs, were again superior at preventing xenogeneic GVHD caused by HLA-A2⁺ T cells²⁰⁸. Furthermore, Antonio *et al* developed a new type of CAR termed mAbCAR expressing a FITC-targeted CAR on Tregs that could be activated in a flexible way by various mAbs covalently conjugated to FITC²⁰⁹. Based on this approach, donor-specific H-2D^d-mAbCAR Tregs have been shown to significantly prolong survival of allogeneic islets expressing the MHC I antigen H-2D^d²⁰⁹. Together, these findings indicate that CAR Tregs may have a great therapeutic potential in transplantation.

Infusion of tolerogenic DCs (Tol-DCs) has been widely experimented in animal models of allogeneic transplantation. A systematic review of thirteen mouse and rat studies concludes that Tol-DCs infusion is effective in prolonging MHC-mismatched islet allograft survival and further synergizes with immunosuppressive or costimulation blockade in promoting graft tolerance²¹⁰. Most recently, Ezzelarab reports in a nonhuman primate allogeneic kidney transplantation model that pre-transplant Tol-DC infusion induces and maintains donor-reactive CD4⁺CTLA4^{high} T cells with a regulatory phenotype following transplantation, even in the presence of CD28 co-stimulation blockade²¹¹. Likewise, Mreg infusion has also been exploited in both animal models and clinical trials, and has demonstrated efficacy in allograft protection^{93,94}. In summary, adoptive transfers of regulatory immune cells as cell-based therapies hold great promises for clinical applications for transplantation tolerance induction and maintenance.

5. Impact of infection on transplantation tolerance

Infections can transiently or permanently alter the host immune system⁹. As tolerance strategies are beginning to show promises in clinical transplantation, an important area of ongoing research is to examine their efficacy and stability during various infections. Cytomegalovirus (CMV) infection and latency is highly prevalent in the general population and is a common opportunistic infection post-transplant. Our lab has examined its impact on

ECDI-SP induced transplantation tolerance using models of murine CMV (MCMV) infection. In this section, we will summarize our own work in the context of research by others to discuss the relationship between infections and transplantation tolerance (Figure 3).

5.1 Acute infection and transplantation tolerance

Recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) expressed either on surface or within intracellular vesicles of innate immune cells initiate the first line of host defense, leading to the killing of infectious microbes²¹². Upon PAMP recognition, PRRs signaling results in secretion of proinflammatory cytokines and chemokines, which in turn leads to further downstream gene expression (e.g. IFN-stimulated genes) promoting recruitment of neutrophils, activation of macrophages, and maturation of DCs²¹³. Toll-like receptors, one of the most important subset of PRRs, can recognize a wide range of PAMPs and interfere with transplantation tolerance²¹⁴. In transplant settings, activation of TLR signaling pathways promotes heightened alloimmune responses and results in tolerance impairment in an inflammatory cytokine-dependent manner^{215–217}. Likewise, inflammatory cytokines such as IFN- α/β and IL-6 produced during acute infections can also directly disrupt transplantation tolerance^{112,215,218–220}.

Transplantation tolerance during the induction period is vulnerable to acute infections. It has been reported earlier that acute *lymphocytic choriomeningitis virus* (LCMV) infection can overcome tolerance induction by CD28/CD40 combined blockade or anti-CD154/DST^{221,222}. We recently report that acute MCMV infection abrogates tolerance induction by donor ECDI-SPs in a type I IFN-dependent manner in a murine islet transplant model¹¹². Circulating level of IFN- α increases immediately following MCMV infection and peaks in 2–4 days. Heightened IFN- α directly impairs the differentiation of immunosuppressive MDSCs, and promotes accumulation of inflammatory monocytes that are capable of cross-presenting donor antigens. The collective outcome is that tolerance induction by donor ECDI-SPs is abrogated. Importantly, blocking type 1 interferon signaling during MCMV infection rescues MDSC function and restores transplant tolerance¹¹². Similar findings have been reported for *Listeria monocytogenes* (LM). Perioperative LM infection prevents heart and skin allograft acceptance by anti-CD154/DST in mice in a type-I IFN-dependent manner. Consequently, LM infection prevents anti-CD154/DST-mediated prolongation of heart and skin allograft survival in IFN α 1^{+/+} recipients, but not in IFN α 1^{-/-} recipients²²⁰. In acute *Staphylococcus aureus* (SA) infection, IL-6 production induced by this bacteria plays a direct role in prevention of skin allograft acceptance²¹⁸. Eliminating IL-6 or administering a single dose of methylprednisolone to modulate IL-6 production during SA infection restores immune tolerance and graft acceptance²¹⁸. Together, these findings underscore the crucial role of proinflammatory factors in interfering with tolerance induction after acute infections. Proinflammatory factors generated early after acute infection serve to prime a series of specific immune responses, including promoting maturation of APCs and activation of T cells, as well as inhibiting regulatory immune cells, eventually leading to heightened alloimmune responses and graft rejection.

In contrast to the induction phase of tolerance, established tolerance in recipients exhibits a remarkable resistance to acute viral infections. In an anti-CD154/DST transplantation tolerance model, LCMV infection at progressively later time points after tolerance induction and transplantation shows a progressively less significant impact on skin graft survival²²¹. As such, survival of skin allografts in tolerized recipients infected with LCMV approximately 2 months after tolerance induction and transplantation was indistinguishable from that in controls²²¹. Similarly, acute LCMV infection has been reported to be insufficient in breaking stably established tolerance induced by other strategies, such as CD28/CD40 combined blockade and Tr1 cell adoptive transfer^{222,223}. In our experience, delayed MCMV infection after transplantation tolerance induction by donor ECDI-SPs precipitates fewer rejection events (Luo, unpublished data). However, bacterial infections, such as acute LM infection, have been shown to disrupt established tolerance²¹⁹. Similar to preventing tolerance induction by LM infection, proinflammatory cytokines, specifically IL-6 and IFN- β generated during acute LM infection, also underlie the loss of established tolerance following the infection in a mouse cardiac transplantation tolerance model using anti-CD154/DST²¹⁹. It is worth noting that IL-6 or IFN- β alone is not sufficient to break established tolerance by CD154/DST even at higher concentrations than observed during LM infection^{219,220}. These results again highlight the importance of proinflammatory cytokines in breaking stably established tolerance, but further suggest that a combination of multiple proinflammatory cytokines is necessary to disrupt an established tolerance, in contrast to the readiness of disrupting the induction of tolerance.

Although a fraction of the tolerant allografts may survive an acute infection, the quality of their tolerance has been described to be “eroded”²²⁴. In a study using acute LM infection in a mouse cardiac transplantation tolerance model, the infection was shown to lead to full rejection in only ~40% of previously tolerant recipients, with another ~30% of recipients undergoing a “rejection crisis” around 7–14 days post infection characterized by a transient slow-down of the heartbeat and a transient enlargement of the heart allograft, and the remaining 30% showing no clinical difference from uninfected recipients²²⁴. Among the surviving allografts, graft-infiltrating cell numbers increased and exhibited a loss of tolerance gene signature on day 8 post infection. Although a broad restoration of these tolerance genes could be observed by day 30 post infection, the expression levels of 234 genes that were highly expressed in tolerance but downregulated in rejection were only partially restored²²⁴. Furthermore, while anti-PD1 alone had no significant detrimental effect on the stability of tolerance in uninfected recipients, it readily precipitated graft rejection in tolerant recipients surviving an episode of acute LM infection²²⁴. These findings indicate that even though an infection may not clinically abrogate the established tolerance, the quality of the tolerance may still be in fact compromised following the infection.

Taken together, acute pathogen infections have deleterious impact on tolerance induction and maintenance via different mechanisms. Induction of tolerance is more susceptible to perturbation by concurrent infections than established tolerance, but the quality of established tolerance may still be affected even in the absence of overt graft rejection, rendering it more vulnerable to future challenges. In addition, proinflammatory cytokines induced by acute infections play a crucial role in tolerance disruption in both the induction

and maintenance phases of tolerance. Blocking and/or neutralizing these cytokines to dampen the inflammatory responses have been shown to be protective of allograft tolerance in the setting of infections. However, signals triggered by these proinflammatory cytokines may also be critical for pathogen clearance and survival of the host. Therefore, future studies targeting downstream pathways specific for alloimmune responses to prevent graft injury while preserving host anti-pathogen immunity will be highly beneficial.

5.2 Chronic infection and transplantation tolerance

Majority of the effector T cell population differentiated to control an acute infection undergoes apoptosis after clearance of the infection. However, a small subset can differentiate into memory T cells which, upon re-exposure to the same pathogen, rapidly expand and activate effector functions to clear the pathogen²²⁵. Memory T cells are retained long-term even in the absence of their cognate antigens, primarily through two common gamma-chain cytokines IL-7 and IL-15²²⁶. The development of memory T cells and their key functions can, however, be altered in settings of persistent antigen stimulation such as chronic infections²²⁷. During chronic infections, effector T cells gradually progress into an exhausted state characterized by a decreased proliferative capacity and loss of production of effector cytokines such as IL-2, TNF- α and IFN- γ ^{228,229}. In addition, exhausted T cells also express more inhibitory receptors on their surface, such as PD1, CTLA-4 and TIM. The number of inhibitory receptors expressed on T cells positively correlates with the severity of exhaustion^{230,231}.

Limited information is available on T cell exhaustion in transplantation despite an extensive literature on this topic in cancers and chronic viral infections. The protective role of exhausted T cells in transplantation has been shown in a bm12 to B6 single MHC class II-mismatched heart transplant model²³². In this case, CD4⁺ T cell exhaustion induced by impaired leucocyte recruitment prevents chronic allograft vasculopathy and rejection in this model²³². Recent clinical data in kidney transplant patients indicate that PD-1⁺CD57⁻ exhausted T cells in peripheral blood mononuclear cells (PBMCs) increase after lympho-depletion induction therapy, and their presence correlates with a better allograft function²³³. This population produces markedly reduced levels of IL-2, IFN- γ , and TNF- α compared to their PD-1⁻ counterparts, supporting their functional features of exhaustion²³³. Another clinical trial directly links exhausted T cells induced by chronic hepatitis C virus (HCV) infection with operational tolerance, which was notably induced in 50% of HCV infected adult liver recipients with successful immunosuppression withdrawal³⁹. The operational tolerance in these recipients is associated with intrahepatic overexpression of type I interferon and immunoregulatory genes, along with expansion of circulating exhausted PD1/CTLA4/2B4 positive HCV-specific CD8⁺ T cells³⁹. In addition to T cell exhaustion, DC function may also be affected by chronic HCV infection. In one study, monocyte-derived DCs isolated from PBMCs of HCV infected patients were found to be poor stimulators of T cell proliferation in allogeneic MLRs²³⁴. They failed to secrete IFN- α in response to poly(I:C) but secreted a significant amount of IL-10 instead²³⁴. These findings indicate that persistent viral infections may exert immunoregulatory effects that can contribute to restraining alloimmune responses. However, despite its potential benefit for allograft tolerance, chronic viral infection in and of itself is a threat to host health²³⁵. For instance,

mortality of HCV(+) patients is significantly higher than that of HCV(-) patients^{236,237} following kidney or lung transplantation. Therefore, the utility of chronic infection-induced T cell exhaustion in transplantation tolerance warrants further studies.

5.3 Latent infection and transplantation tolerance

Latent infection is a metastable, nonproductive infection state of viruses that is capable of subsequent reactivation to repeat the infection cycle²³⁸. In clinical transplantation, reactivation of latent viruses is common due to the loss of virus-specific immunity caused by potent immunosuppressive regimens. Reactivation of latent viruses is often associated with poor post-transplant outcomes^{239,240}. In a clinically relevant mouse model of kidney transplantation, the Abecassis group recently reported that reactivation and dissemination of latent MCMV requires “two hits”: the first hit is transcriptional activation of the latent MCMV in the donor kidney allograft triggered by ischemia reperfusion injury during graft procurement and implantation; the second hit is persistent immunosuppression that impairs host anti-viral immunity that facilitates uncontrolled replication of the reactivated MCMV and its ultimate dissemination to distant organs²⁴¹. According to this model, immunosuppression plays a critical role in latent virus replication and dissemination. Therefore, transplant tolerance, the state which the need for immunosuppression can be completely eliminated, is highly beneficial for transplant patients in preventing viral reactivation and viral illnesses from latent infections.

Our lab recently explored the dynamic between latent MCMV infection and transplantation tolerance using a donor MCMV-positive (D+) to recipient MCMV-negative (R-) allogeneic murine kidney transplantation model (Luo, unpublished data). The ability of donor ECDI-SPs to induce transplantation tolerance is found to be preserved even in the presence of latent MCMV infection in the kidney allograft. Interestingly, we found that MCMV reactivation and its dissemination are sequential events that can be successfully uncoupled by the induction of transplantation tolerance. Though MCMV reactivation is readily detectable in all kidney allografts undergoing transplantation after sustaining ischemic reperfusion injury, MCMV replication and dissemination can be successfully prevented in tolerant recipients in which chronic immunosuppression is no longer required. Further mechanistic studies reveal that cytotoxicity of MCMV-specific CD8 T cells is preserved in tolerant recipients but significantly impaired in chronically immunosuppressed recipients. Therefore, our findings indicate that both tolerance induction and the ability to develop anti-virus immunity can be preserved in recipients transplanted with MCMV latently-infected donor organs. However, one limitation of this D+/R- transplantation model is that it does not allow studies of the impact of latent MCMV infection on the recipient immune system *before* transplantation, which may affect subsequent tolerance induction and MCMV dissemination. Indeed, Cook et al has reported that tolerance induction can be prevented by latent MCMV infection in a D-/R+ allogeneic murine cardiac transplantation model²⁴². Furthermore, the latent virus from recipients (R+) was found to be transmitted to the donor grafts (D-) after transplantation, although it remained unclear whether this was due to viral dissemination, or simply graft infiltration by MCMV-containing recipient leukocytes²⁴². Therefore, knowledge up to date suggests that dynamics between latent virus infection and transplantation tolerance may be largely dependent on the particular transplant scenario: in

the D+/R- setting, both graft tolerance and virus-specific immunity are preserved, leading to long-term graft acceptance and absence of virus dissemination. However, in the D-/R+ setting, pre-existing anti-viral immunity may prevent tolerance induction, and its effect on virus dissemination remains unknown.

6. Intestinal microbiota and transplantation tolerance

Numerous studies indicate that human microbiota has significant influences on multiple organ systems and disease processes ²⁴³. The composition of microbiota amongst people varies considerably, some of which have been implicated in health disorders such as autoimmune diseases ^{244,245}. Microbiota can also be protective against disease processes. For example, polysaccharide A (PSA) produced by commensal *Bacteroides fragilis* has been shown to induce Tregs and produce IL-10, both of which play a crucial role in protection against colitis ²⁴⁶. Several other genus of commensal bacteria, such as *Clostridium* and *Lactobacillus*, have also been found to induce Treg expansion in the colon ^{247–249}. Therefore, by having a remarkable impact on host immune system, the intestinal microbiota likely can also affect organ transplant outcomes.

6.1 Intestinal microbiota and organ transplant outcomes

Development of immunosuppression medications, such as calcineurin inhibitors, cyclosporine, tacrolimus, and purine analogues, have been instrumental in prolonging organ survival post-transplant ²⁵⁰. However, there are also many complications associated with lifelong immunosuppressive therapy. Risks of infection, metabolic disorders, *de novo* malignancy and cardiovascular events are all significantly increased in transplant recipients on immunosuppression treatment ^{251,252}. Therefore, alternative means of intervention that will increase immune tolerance for allogenic transplant without indefinite immunosuppression are highly desirable. The main focus of this section is to review existing literature on the influence of intestinal microbiota on the outcome of organ transplantation, and the therapeutic potentials of fecal microbiota transplantation (FMT) for allograft tolerance.

6.1.1 Clinical studies—With the advancement of sequencing technologies, researchers are able to identify and isolate bacteria groups that are strongly associated with transplant outcomes. Many clinical studies have been conducted in liver, small intestine, and bone marrow transplant patients with an attempt to understand the effect of microbiota on transplant outcome and post surgery infection risks. Sun and his colleagues sequenced and analyzed fecal samples from nine liver transplant (LT) recipients with improved liver function and portal venous hypertension post-liver transplant, and discovered that *Actinobacillus*, *Escherichia*, and *Fusobacteriaceae* were significantly decreased in post-LT fecal samples compared with pre-LT samples. In contrast, populations of *Clostridium* cluster XIVa, *Corynebacterium*, and *Blautia* (formerly known as *Clostridia*) were expanded in post-LT in comparison to pre-LT fecal samples. These changes allowed the post-LT fecal microbiota composition to return to resemble those in healthy subjects ²⁵³. In a small bowel transplant study, Oh and his colleagues studied the ileal microbiota during stages of non-rejection, pre-rejection, and active rejection. In their study, substantial reduction in

Firmicutes and expansion in Proteobacteria were detected during active rejection. Populations of *Enterococcaceae* and *Lactobacillaceae* also decreased during active rejection, while *Corynebacterium durum* and *Clostridium irregulare* were more abundant during the non-rejection stage²⁵⁴. In two bone marrow transplant studies, researchers identified that increased abundance of Lactobacillales and decreased percentage of *Clostridiales* were associated with exacerbated inflammation seen in graft versus host disease in both bone marrow transplant recipients^{5,255}. These clinical studies show that certain genus of bacteria such as *Lactobacillus* and *Clostridium*, both belong to the phylum of Firmicutes, appear to be associated with positive transplant outcome and a decreased rate of rejection.

6.1.2 Preclinical studies—Recent research identified a close association between microbiota and transplant rejection in mouse models with comparable findings to clinical studies. Alegre's group has achieved substantial progress in this area in showing that changes in microbiota has a significant effect on allograft survival^{5,256}. In one study, they showed that germ-free mice or mice treated with prophylactic antibiotics exhibited prolonged survival of major antigen-mismatched skin grafts and MHC class II-mismatched cardiac allografts. When these germ-free mice were inoculated with fecal matters from conventional mice, skin graft rejection was accelerated. 16S rRNA sequencing showed that antibiotic treatment reduced *Clostridium coccoides* population, but increased *Lactobacillus* spp. This shift of ratio in microbiota correlated with an increased allograft survival and a decreased alloimmunity²⁵⁷. Spontaneous variations in microbiota also has an effect on transplant outcomes. C57BL/6 mice from different vendors carry different microbiota even though their genetic makeup is identical. When B6 mice from two different vendors underwent skin graft transplant, a significantly different survival curve was obtained. When the two groups of mice were cohoused or received fecal matter transplant (FMT) from one another, their survival curves blended with each other. Genetic analysis of microbial community profiles in mice from different vendors before and after cohousing or FMT experiments also confirmed distinct composition of microbiota before intervention and convergence of microbiota composition after cohousing or FMT. These findings suggest that normal variations of natural commensal microbiota in healthy individuals could precondition the immune system to respond differently to transplanted allogeneic organs. Analysis of 16S rRNA sequencing of pre-cohousing or pre-FMT groups versus the post-cohousing or post-FMT groups revealed one bacterial genus, *Alistipes*, to be associated with prolonged skin allograft survival²⁵⁶. These studies warrant repeating in clinical transplant patient populations, as information acquired will allow characterization of human microbiota composition that can precondition the human immune system to enhance post-transplant outcome and promote long-term allograft survival.

6.2 Mechanisms of regulation of alloimmune responses by intestinal microbiota

Although the aforementioned studies did not propose or examine specific molecular pathways that might be responsible for the influence of microbiota on transplant outcome, there are several papers discussing potential mechanisms by which microbiota regulates T cells differentiation in mouse models of autoimmune diseases. One of the most thoroughly studied effect of commensal microbiota on the immune system is PSA secreted by *Bacteroides fragilis*. Studies show that PSA can signal both plasmacytoid DCs (pDCs) and

conventional DCs (cDCs) to stimulate CD4⁺ T cells to secrete IL-10. However, only pDCs significantly increase their expression of TLR2 after incubation with PSA. Cognate interaction between MHC II and PSA-activated pDC (with a high level of expression of TLR2) is crucial for the production of IL-10 by CD4⁺ T cells. It is worth noting that PSA oral gavage not only confers protection against 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, but also against myelin proteolipid protein (PLP)-induced autoimmune encephalomyelitis^{246,258,259}. This is an important finding because it indicates that microbiota not only can moderate the immune system locally, it can also modulate the immune system as a whole.

Another microorganism that has been well studied in the context of immunomodulation is segmented filamentous bacteria (SFB). SFB is a non-culturable *Clostridia*-related host-specific species. They are potent inducers of Th17 cells in the lamina propria in the ileum^{260,261}. SFB adhere tightly to the ileal mucosa and Peyer's patches, where adaptive T cell responses are initiated. Serum Amyloid A is then induced by SFB colonization which acts on DCs to stimulate Th17 cell differentiation. SFB plays an important role in protecting host from microbial pathogens such as vancomycin-resistant *Enterococcus* (VRE), *Clostridium difficile*, and *C. rodentium* through induction of Th17 cytokines. However, SFB could also play a role in aggravating inflammatory bowel diseases by polarizing self-reactive CD4⁺ T cells towards the Th17 lineage.

Many *Clostridium* species have been found to play an important role in regulating Tregs. Specific pathogen free (SPF) mice given vancomycin to target gram-positive bacteria have significantly lower numbers of Tregs in the colon, suggesting that gram-positive bacteria are important for Treg homeostasis²⁴⁹. *Clostridium leptum* and *Clostridium coccoides* have long been recognized to play a crucial role in maintaining immune homeostasis and prevent inflammatory bowel disease^{262,263}. When GF mice are colonized with a cocktail of 46 strains of *Clostridium*, there is a significant expansion of Tregs population in the colon. SPF mice inoculated with *Clostridium* feces also demonstrate a higher IL-10 production and attenuated symptoms from dextran sodium sulfate (DSS)-mediated colitis when compared with control mice. Interestingly, many Foxp3⁺ Tregs observed in these *Clostridium*-colonized mice are negative for Helios, implying that these Tregs are not thymus-derived nTregs, but rather peripherally induced by *Clostridium* to differentiate into Tregs (iTregs). Supernatants from *Clostridium*-colonized intestinal epithelial cell culture yield high levels of TGF- β and IDO, both of which are important for Treg differentiation^{249,264,265}. In addition to *Clostridium*, other bacteria such as *Bifidobacteria*, *Lactobacilli*, *Bacteroides* spp., *Bacteroides fragilis* also induce Treg differentiation^{266–268}. As such, bone marrow derived DCs (BMDCs) incubated with *Lactobacillus* promote Treg expansion and suppress inflammation via IL-10 secretion through MyD88 and TLR2 signaling pathways. Likewise, increased abundance of *Lactobacillus* and certain species of *Clostridium* may also promote allograft survival and suppress allograft immunity through Treg induction and high IL-10 production, thereby promoting transplantation tolerance. Such potential beneficial effects of microbiota on transplantation tolerance warrant future studies.

Numerous studies have reported potential correlations between many other bacterial species of the gut microbiota and various disease processes^{243,247,269–271}. Gevers and his

colleagues found that increased abundance in Enterobacteriaceae, Veillonellaceae, Fusobacteriaceae, and Pasteurellaceae, and decreased percentages of Bacteroidales, Clostridiales, and Erysipelotrichales have a strong correlation with exacerbation of Crohn's disease²⁶⁹. Kasper's group conducted a study of mice colonized with 53 individual bacterial species from the human microbiota in an attempt to identify the immunomodulatory effects of these bacteria²⁴⁷. Their analysis confirmed that SFB does indeed upregulate Th17 cells in the small intestine as previous research have identified^{260,261}. However, they found that many bacteria from different species are also capable of inducing Tregs in the colon, unlike the previously suggested predominant role of *Clostridium* species in this process²⁴⁹. Their work also identified *Veillonella* as a strong inducer for IL10-producing CD4⁺ T cells and *L. rhamnosus* as a suppressor of pDCs. These studies therefore offer us many exciting opportunities for immunomodulation using microbes that have not been explored previously. However, a clear causative relationship between the microbes of interest and the observed immune findings must be established first.

It is indeed difficult to establish the causal relationship between a single bacterial species and an observed clinical outcome using the massive dataset generated from sequencing the microbiota from a large number of subjects. Therefore, it should be noted that Surana and Kasper recently proposed a new method to identify causal microbes through triangulation of microbe-phenotype relationships²⁷². This method aims to map microbe-phenotype relationships in animals with overlapping microbiotas but with statistically significant phenotypical differences. Such microbiome environment and phenotypes can be achieved through co-housing mice with different microbiota and distinctive phenotypes to generate hybrid-microbiota animals that are microbially related to their parental strains but with intermediate phenotypes that are still distinct from parental phenotypes. For example, the lab generated two types of gnotobiotic mice, one colonized with a mouse microbiota (MMb) and the other with a human microbiota (HMb). HMb mice consistently survived better than MMb mice when both groups were induced to have colitis by dextran sodium sulfate (DSS). The authors then co-housed HMb and MMb mice for one or three days to get hybrid-microbiota mice. The one-day and the three-day co-housed groups of HMb and MMb mice exhibited two intermediate phenotypes of DSS-induced colitis distinct from parental HMb and MMb mice. Microbiome-wide association studies (MWAS) in both parental and hybrid-microbiota animals yielded more than one hundred bacterial taxa that could be responsible for the exacerbated phenotypes of DSS-induced colitis. However, when they compared MWAS results across the groups, only one bacterial taxon, Lachnospiraceae, was consistently present in all MWAS analysis. Surana and Kasper then sequenced the Lachnospiraceae and identified a previously unknown bacterial species, *Clostridium immunis*, to be the protective species against DSS-induced colitis. The same method of microbe-phenotype triangulation was used to successfully illustrate that *R. gnavus* and *L. reuteri* can induce ileal Reg3 γ expression²⁷². This method is therefore highly useful in ascertaining the immunomodulatory effects of specific microbes in human subjects, particularly given the complex microbiota compositions in different subjects.

6.3 Clinical implications of intestinal microbiota for transplantation

Taken together, intestinal microbiota possesses immunomodulatory capacities that may be engaged to modulate transplant immunity and outcomes. FMT has been utilized as an effective treatment option for many chronic diseases in recent years^{273–275}. Kakihana and colleagues performed FMT in four patients undergoing acute graft-versus-host disease (aGVHD) after allogeneic stem cell transplantation²⁷⁵. All four patients responded to FMT with improvement of gastrointestinal symptoms and reduced steroid dosage. There was also no severe adverse event such as gastroenteritis from FMT even though these patients were on heavy immunosuppressants. Therefore, FMT as a potential therapeutic option for modulating rejections in organ transplantation should be further explored.

Plasmacytoid DCs may also be an interesting therapeutic target through microbiota manipulation for organ transplantation rejection. As discussed earlier, pDCs are bone marrow-derived antigen presenting cells specializing in viral recognition through TLR7 and TLR9^{276,277}. When pDCs are activated by the synthetic TLR9 ligand CpG, there is an increase in IFN- α production. pDCs can also produce large quantities of type I IFN, IL-12, IL-6, and TNF- α in response to actual viral infections, which promote T-helper 1 (Th1) polarization of CD4⁺ T cells^{276,278}, CD8⁺ T cell cytolytic activity⁴¹, and long-term T cell survival and memory^{41,46,279,280}. However, the role of pDCs in transplant rejection remains controversial. On the one hand, multiple studies have demonstrated that alternatively stimulated pDCs (not through TLR7 or TLR9) can act as tolerogenic DCs to induce Tregs^{160,246,281}. On the other hand, an increased number of pDCs has been found in the tubulointerstitium of renal biopsies at the time of rejection, suggesting that they may play a role in alloimmunity and promote acute and chronic rejection²⁸². The group further demonstrates that TLR9 ligation on pDCs in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly induces their phagocytic capacity, especially in the presence of CMV-infected human kidney proximal tubular epithelial (HK2) cells. It should be noted that at baseline, pDCs show very limited phagocytic activity when co-cultured with apoptotic cells. However, when pDCs are incubated with CMV-infected HK2 cells and apoptotic cells, their phagocytic capacity is greatly increased. More interestingly, when pDCs are activated by CpG in the presence of HK2 cells, there is also an increase in the expression of co-stimulatory molecules CD86 and CD83, and a decreased expression of the co-inhibitory molecule PD-L1. These findings are highly clinically relevant in the context of kidney transplantation, as many studies have suggested a correlation between CMV infection and allograft rejection including our own work^{283,284}. However, intestinal microbiota may alter the pathways of pDC activation. Specifically, introducing *Bacteroides fragilis* in these patients may stimulate pDCs to produce IL-10 instead of IFN- α or IL-12. This may in turn mitigate alloimmunity and consequently mitigate acute and chronic rejection in kidney transplantation, particularly in the setting of CMV infections. On the other hand, in patients whose pDCs are more prone to develop an inflammatory response regardless, it may be beneficial to introduce *L. rhamnosus* to reduce the overall pDC number.

It should be noted that even though several studies have shown a clear correlation between certain bacterial groups and extended transplant allograft survival, microbiota likely plays a

secondary role in transplant rejections in clinical scenarios. The primary cause for organ rejection is the MHC disparity between the donor and the recipient. Published data to date has demonstrated that manipulation of microbiota alone can lead to extended allograft survival, but not allograft tolerance. In the study where the presence of *Alistipes* was shown to significantly prolong skin allograft survival, it should be noted that the skin transplant occurred between male donors and female recipients with the minor H-Y mismatch on the same MHCs background²⁵⁷. Conceivably, the benefit of microbiota engineering is likely only able to manifest when immune responses driven by MHC mismatches are significantly reduced.

7. Conclusion

A review of current literature reveals that the interaction between infection and transplantation tolerance is a complex one, depending on numerous factors including the stage of infection (acute, chronic, or latent) and the stage of tolerance (induction or maintenance). Based on a comprehensive understanding of this relationship, several interventions are conceivable in preserving the ability to induce and maintain transplantation tolerance in settings of inadvertent infections. However, it is worth noting that mechanisms of tolerance impairment by pathogens are frequently also mechanisms of host defense against pathogens. Therefore, future studies are urgently needed to focus on specific interventions that will both prevent tolerance disruption and at the same time preserve host anti-pathogen immunities.

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8. References

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Figure 1. Effects of infection on alloreactive T cell activation.

Activation of alloreactive T cells require 3 signals: TCR engagement, costimulation, and inflammatory cytokines. These three signals can be altered by infections: pathogen-specific T cells have the potential to cross-react with alloantigens presented by host APCs and become activated to attack the allograft. IL-2 generated during an infection can augment the costimulation signal through inhibiting the expression of anergy-related genes. Type-I IFN and IL-12 induced by infections can bind to their receptors on T cells and serve as the third signal.

Fig. 2. Effects of infection on the regulatory immune cell network.

CD4⁺Foxp3⁺ Tregs play a central role in the regulatory immune cell network. Tregs inhibit the effector T cells (Teffs) through a number of mechanisms including the production of IL-10, TGF- β , and IL-35. Tregs are also important for the expansion and/or maintenance of other regulatory cell populations, including Tol-DCs, Mreg and MDSCs. These regulatory cell populations are themselves capable of directly suppressing Teffs via several unique mechanisms. During an infection, the induced inflammatory cytokines can disrupt this network, block the suppressive function of various regulatory cell populations, and/or promote their differentiation to inflammatory immune cells.

Fig. 3. Impact of infection on transplantation tolerance.

The impact of infection on transplantation tolerance varies according to the acuity of the infection. Generally, acute infections readily prevent the induction of tolerance but slowly erode established tolerance. On the contrary, chronic infections may facilitate transplantation tolerance due to populations of exhausted T cells generated under persistent chronic pathogen stimulation. Latent infection and transplantation tolerance have reciprocal effects on each other. Tolerance may permit reactivation of latent infections but prevent their dissemination, whereas reactivation of latent infections may impair the induction of transplantation tolerance.