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The impact of infection and tissue damage in solid organ transplantation

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Preface

Investigations over the past two decades are revealing the complexity in the regulation of the innate immune response, and how it, in turn, modulates adaptive immunity. Microbial exposure and tissue damage that accompany transplantation result in the release of both pathogen- and damage-associated molecular patterns, as well as the generation of cross-reactive alloreactive T cells. Here, we review these triggers of innate and adaptive immunity in the context of transplantation, and discuss the many ways infections and tissue damage might impact on alloreactivity and the outcome of transplanted allografts.

Introduction

Solid organ transplantation is often the only therapeutic option for end-stage organ failure but life-long immunosuppression is necessary to prevent rejection of allogeneic transplants. High precursor frequencies of T cells that recognize allogeneic MHC molecules (termed direct allorecognition) and peptides from donor MHC molecules or minor histocompatibility antigens presented on recipient antigen-presenting cells (APCs) (termed indirect allorecognition) contribute to the vigour of the allogeneic response (**Box 1**). Observations from experimental models indicate that events associated with clinical transplantation, such as ischaemia–reperfusion injury and infections, can enhance alloreactivity and promote rejection episodes through the release of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), as well as through alterations in the alloreactive T cell repertoire.

Despite the relative frequency of infections and acute rejection especially in the early transplant period[1], definitive clinical proof that infections stimulate alloreactivity is lacking. In a previous review[2] we discussed the difficulty in making this causal link: the immunosuppression used to prevent or treat transplant rejection episodes increases the susceptibility to developing infections and also reduces some of the pro-inflammatory responses normally elicited by the infections. Furthermore, some infections trigger the release of endogenous corticosteroids that can compromise the pro-inflammatory response to infection[3-6]. The prophylactic use of antibiotics, anti-fungal and anti-viral drugs can also

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reduce the impact of infections on alloreactivity. Finally, tapering of immunosuppression to treat some types of viral infections[7] may increase the likelihood of rejection, which then makes the definitive demonstration of the direct participation of infections in transplant rejection extremely challenging. Nonetheless, emerging paradigms from experimental models of how infections can enhance immune responses in the context of transplantation may lead to renewed investigations in the clinic into whether infections prevent long-term graft acceptance that is attained by continued pharmacological immunosuppression or by immunological tolerance.

In this review, we discuss how infections and tissue damage may impact on the clinical outcomes of solid organ transplantation, describe the emerging mechanistic insights based on experimental models, and consider the therapeutic implications arising from these insights.

Timing of infections relative to transplantation

Infections prior to transplantation include those implicated in the cause of organ failure, such as hepatitis B virus (HBV) or HCV for liver damage, and those associated with end-stage disease — such as bacterial and fungal infections in patients with cystic fibrosis and in patients with end-stage renal failure on haemodialysis or peritoneal dialysis [8, 9]. Infections in the post-transplant period are shaped by the type of immunosuppression regimen used, whether there are infections within the donor organ or recipient, and the use of prophylactic anti-microbial agents. These infections are typically divided into those within the first year of transplantation when the immunosuppression regimen is most intensive and those after the first year. Pathogenic bacterial and candidal infections arising from the donor or recipient, as well as surgical complications, are most prevalent in the first 4 weeks of the post-operative period, whereas opportunistic infections (such as pneumocystis pneumonia and oral candidiasis) and activation of latent viral infections (such as cytomegalovirus (CMV), Epstein–Barr virus (EBV), BK polyomavirus, HBV and HCV) occur more frequently at 6–12 months post-transplantation. Even when the dose of immunosuppressive therapy is tapered, transplant recipients retain an increased risk of community-acquired respiratory viral and urinary tract infections that can lead to secondary bacterial or fungal infections, as well as increased risk of late viral infections (such as CMV, EBV, HBV, HCV, and herpes simplex virus (HSV) infections) (review[1]). Both the type and timing of the infection influences its impact on alloreactivity and allograft outcome (see **Figure 1**).

Infections prior to transplantation

An important concept introduced almost 15 years ago is that alloreactive memory T cells are generated not only through sensitization with alloantigen (due to pregnancies, transfusions or previous transplants), but also through viral infections that promote the induction of memory T cells that cross-react with allogeneic MHC through direct allorecognition[10] (**Figure 2**). This cross-sensitization has been termed heterologous immunity (**Box 1**). The structural basis for heterologous immunity is not completely understood, but experimental evidence support two main hypotheses. The first hypothesis is molecular mimicry where a T cell receptor (TCR) binds directly to allogeneic MHC molecules presenting endogenous (self) peptide in a manner similar to its binding to viral antigens presented by self-MHC

molecules. The second hypothesis is based on the inherent plasticity of some TCRs, which enables them to mould their ligand-binding loops, thereby using distinct docking modes for recognition of endogenous peptide– allogeneic MHC versus microbial peptide–self-MHC complexes (reviewed[11, 12]). Importantly, the affinity of TCR for allogeneic MHC molecules can, in some cases, be greater than for self-restricted recognition, further suggesting a functional role for these cross-reactive T cells. For example, the EBV-specific LC13 TCR recognizes syngeneic HLA-B*0801 bound to the EBV epitope FLRGRAYGL with a 10-fold lower affinity than that for the allogeneic HLA B*4402 bound to the self-peptide EEYLQAFTY (reviewed[11]). Similarly, the mouse 2C TCR binds to syngeneic H-2K^b presenting the self-peptide dEV8 from NADH-ubiquinone oxidoreductase with an affinity almost 100 fold lower than that for the allogeneic H-2L^d molecule bound with the self-peptide p2Ca from α-ketoglutarate dehydrogenase (reviewed[11]).

Memory T cells are known to have qualitatively different requirements for activation and to generate immune responses that are faster and more vigorous compared with naïve T cells (reviewed[13]). Although T cells with alloreactive specificity exist equally within the naïve and memory T cell compartment[14], it is likely that memory T cells play a more important role in transplant rejection because of their ability to more rapidly expand and express effector molecules such as interferon-γ (IFN*γ*), granzyme B and perforin, as well as their relative resistance to select immunosuppressive and tolerance pathways. Indeed, Heeger *et al.*[15] reported that the pre-transplant frequency of donor-specific memory IFNγ-producing lymphocytes in renal transplant patients correlated with the risk of post-transplantation rejection episodes. These observations were confirmed in mice[16] and more recently in non-human primates[17] where memory T cells were shown to confer resistance to long-term graft acceptance.

Initial experimental demonstration of the importance of heterologous immunity in transplant rejection involved sequential viral infections that generated a population of alloreactive memory CD8$^+$ T cells in mice. These animals then became resistant to the induction of transplant tolerance by co-stimulation blockade and donor-specific transfusion (DST) or by bone marrow chimerism protocols [18]. Data demonstrating the participation of virus-specific T cells in an alloresponse came from studies where mouse memory lymphocytic choriomeningitis virus (LCMV)-specific CD8$^+$ T cells were generated *in vivo* by LCMV infection[19]. These LCMV-specific T cells were isolated using LCMV peptide–self-MHC tetramers and were shown to be cross-reactive with alloantigen and to induce the acute rejection of skin allografts when transferred into severe combined immunodeficiency (SCID) mice.

Based on the structural understanding of direct alloreactivity, there is no reason to believe that only viruses can generate heterologous immunity. Indeed, infection of mice with the parasite *Leishmania major* was shown to generate memory T cells that are cross-reactive with allogeneic MHC molecules and prevent the induction of transplant tolerance to subsequent skin grafts[20]. Additionally, some bacterial species such as *Staphylococcus* and *Streptococcus* spp. produce toxins with superantigen properties, which allow them to activate up to 20% of the T cell repertoire in mice and may also contribute to the generation of alloreactive memory T cells[21]. The ability of infections prior to transplantation to

sensitize alloreactive T cells may be particularly important in light of the potentially richer history of infections in patients waiting for a transplant. Indeed, cross-reactivity between virus-specific T cells and allogeneic HLA molecules has been demonstrated with human T cells[14], and a remarkable 80% of T cell lines and 45% of T cell clones specific for EBV, cytomegalovirus (CMV), varicella zoster virus or influenza virus cross-react with allogeneic HLA class I or II molecules[22].

Nonetheless, despite widespread acceptance that heterologous immunity is a demonstrable barrier to successful allograft acceptance in mice, some degree of caution is necessary in extrapolating from studies of T cell cross-reactivity to pathogenicity, and in assuming that heterologous immunity contributes significantly to the T cell pool that mediates organ allograft rejection in humans. Indeed, it was observed recently that the adoptive transfer of EBV-, CMV- and/or adenovirus-specific cytotoxic T lymphocytes (CTLs) did not result in greater graft-versus-host disease in human recipients of HLA-mismatched CTLs (with demonstrated broad alloreactivity) compared with patients that received HLA-matched CTLs[23]. One possible explanation for this observation is that memory T cells remain dependent on TCR signalling and are therefore susceptible to calcineurin inhibitors, which are essential components of clinical transplantation immunosuppression regimens[24]. Thus the impact of heterologous immunity and of memory T cells in general may be diminished in clinical transplantation.

In June 2011, belatacept, which is a modified CTLA4–immunoglobulin fusion protein that provides co-stimulatory blockade, was approved as an alternative to calcineurin inhibitors for the prophylactic treatment of rejection in adult kidney transplant recipients[25]. The relative resistance of memory T cells to co-stimulation blockade raise the possibility that belatacept-based regimens may not adequately control memory T cells, and thus their role in clinical rejection may become more apparent.

Infections in the post-transplant period

Pathogenic bacterial, fungal and viral infections are prevalent in the peri- and post-transplant periods as a result of surgical insult and immunosuppression. Studies evaluating defined categories of bacterial infections in transplant recipients have been able to link incidence of bacterial infections with transplant rejection (reviewed[2]). Infections with *Chlamydia pneumonia*[26] and *Simkania negevensis* [27], and colonization of the allograft with *Pseudomonas aeruginosa*[28] or *Aspergillus* spp.[29] in lung transplant patients has been associated with development of acute rejection or bronchiolitis obliterans syndrome (BOS). Furthermore, urinary tract infections, especially those occurring late after renal transplantation when prophylactic antibiotics have been discontinued, have been associated with chronic rejection and renal graft loss[30]. When infections occur locally in the transplanted organ, graft injury can result from immune responses directed at the pathogen, from reactivation of alloreactivity and/or from activation of T cells reacting to cryptic auto-antigens exposed following cell damage. Despite these reports, unambiguous clinical evidence for a causal relationship between infection and transplant rejection is currently lacking.

The question of whether bacterial infections can have direct effects on alloreactivity has been addressed in a number of experimental settings. We have shown that systemic infections with the intracellular bacterium *Listeria monocytogenes*[31] or that extracellular bacterium *S. aureus*[32] induced skin allograft rejection in mice treated with DST and a CD154-specific antibody to mediate co-stimulation blockade. Interestingly, another common nosocomial infection *Pseudomonas aeruginosa* had no effect on graft acceptance in mice[32], confirming the clinical observations that only specific bacterial infections are associated with increased incidence of rejection. These studies suggest that this is due to the different pro-inflammatory cytokines triggered by each bacterial strain[32] (see **Figure 2** and below).

Local effects of viral infections are most evident with respiratory viral infections involving influenza virus, parainfluenza virus, rhinovirus, metapneumovirus and respiratory syncytial virus, which can be associated with acute rejection and the development of BOS in lung transplant patients[33]. While these clinical associations are not definitive and do not demonstrate a causal relationship, they are nonetheless consistent with experimental observations that demonstrate causality[34-39]. Respiratory infection with Sendai virus at 15–30 days post-transplantation of allogeneic orthotopic trachea in mice has been shown to enhance the development of chronic rejection[34]. CMV is another frequently occurring infection in the clinic and is associated with increased rates of rejection and graft loss, especially in the absence of prophylactic antivirals[35]. The broad cell tropism of CMV suggests that both local and systemic effects of CMV infection are likely to impact on early alloreactivity and long-term graft outcome. Acute infections of mice with MCMV in the peri-transplant period across different allogeneic combinations[36], as have the re-activation of latent MCMV infection[37]. Furthermore, the impact of infection on graft survival in mice was greater with some types of viral infections, such as LCMV and Pichinde virus, compared with other viruses, and when infection occurred at or shortly after transplantation[38,39]. Williams et al.[39] reported that the ability of LCMV infections at the time of transplantation to trigger rejection was not due to the direct participation of LCMV-specific T cells but rather to the stimulation of innate immunity, namely enhanced CD28- and CD40-independent maturation of dendritic cells (DCs) leading to the generation of alloreactive T cells (see **Figure 2**).

Although clinical data suggest an association between infection and graft outcomes, it is difficult to definitely prove cause and effect. Experimental models have permitted such a demonstration, and the mechanisms by which infections affect graft outcome are just being delineated (**Figure 2** and see below). Caveats regarding the extrapolation of data derived from mice to the clinic include differences in experimental and natural routes of infection and the use of immunosuppressant and prophylactic therapies in humans, which may alter the way the infections affect alloreactivity.

Infections and alloreactivity

Sensing infections

Molecular patterns expressed on microorganisms or released from damaged cells are recognized by various pathogen-recognition receptors (PRRs) on or in haematopoietic cells

(**Box 2**). PRR-mediated signals can enhance APC maturation, upregulate expression of costimulatory ligands, increase antigen presentation, and induce production of pro-inflammatory cytokines (reviewed[40]). Different cytokines drive differentiating T cells toward distinct effector phenotypes such as T helper 1 (Th1), Th2 and Th17 cells. In turn, these effector Th cells activate other myeloid cells, such as macrophages, eosinophils and neutrophils that may participate in rejection. In addition, PRRs on T cells can have direct co-stimulatory effects and promote cell survival, whereas those on regulatory T (Treg) cells may either promote or abrogate the suppressor function of these cells (reviewed[41, 42]). PRRs are also expressed by various epithelial and endothelial cells, such that infections or tissue damage may also be sensed by these parenchymal cells (reviewed[42]).

Clinical evidence that Toll-like receptor (TLR) signals promote alloreactivity has been suggested by genomic studies of TLR polymorphisms and allograft outcome, where loss-of-function mutations in *TLR2*, *TLR4*, *TLR9* and the TLR accessory molecule *CD14* are associated with better graft survival but also with increased incidence of infections[43-46]. The role of TLRs in promoting allograft rejection has been confirmed experimentally in recipient mice deficient in TLR signalling — due to the deletion of the myeloid differentiation primary response gene 88 (*MYD88*) and/or *TRIF* genes — which display delayed rejection kinetics, or with TLR agonists that enhance rejection and prevent graft acceptance[47-51]. The mechanisms by which TLRs and infections affect the alloimmune responses independently of cross-reactivity can be divided into bystander cytokine and cell-intrinsic effects.

Bystander cytokine or chemokine effects

Accumulating experimental and clinical data support a paradigm of different infections eliciting different classes of immune responses. Virus and intracellular bacterial infections tend to drive Th1 cell-, cytotoxic $CD8^+$ T cell- and NK cell-mediated responses, extracellular bacteria trigger both Th1 and Th17 cell-mediated responses, and fungal infections promote predominantly Th17 cell-mediated responses [52]. These observations are complemented by studies of patients with genetic defects in cytokine signalling who demonstrate susceptibility to signature infections. For example, genetic deficiencies in signal transducer and activator of transcription 1 (STAT1), IL-12Rβ or IFNγ expression result in impaired Th1-type responses and the predisposition to mycobacterial and, to a lesser extent, viral infections[53]. Lack of Th17 cell differentiation because of STAT3 loss-of-function mutations or autosomal dominant deficiencies of IL-17RA are associated with susceptibility to candidiasis and *S. aureus* abscesses[54]. Thus, different classes of host defence responses to specific infections may result in differentially primed APC populations and cytokine microenvironments that direct specific effector fates of alloreactive T cells (see **Figure 2**).

Type I IFNs are produced following viral and intracellular bacterial infections, as well as some extracellular bacterial infections, and their immune-enhancing effects are well documented (reviewed[55]). Treatment of recurrent hepatitis C virus infection in transplant recipients with IFNα is associated with enhanced viral clearance but also with increased risk of allograft rejection[56]. Experimental studies with the synthetic ligand for TLR3 polyinosinic:polycytidylic acid (poly I:C) confirmed the ability of type I IFNs to prevent

skin and islet graft acceptance and to precipitate rejection in mice[57]. IFNβ treatment was shown to prevent the deletion of alloreactive $CD8^+$ T cells, which normally occurs during the induction of tolerance, and to facilitate their priming in the presence of CD154-specific antibodies[57]. We demonstrated that live *Listeria monocytogenes* infection prevent skin and heart graft acceptance in mice treated with CD154-specific antibodies and DST[58]. The pro-rejection effects of *L. monocytogenes* infection were lost in IFNAR-deficient recipient mice and IFNβ alone was sufficient to precipitate rejection in recipients treated with CD154-specific antibodies and DST. Thus, systemic infections that lead to the early production of type I IFNs can confer a CD154-CD40-independent activation and differentiation of Th1 cells that mediate allograft rejection.

Systemic infection with *S. aureus* triggers early IL-6 production that is dependent on recipient expression of TLR2 and MYD88[59]. We reported that the pro-rejection effect of *S. aureus* infection at the time of transplantation in mice was dependent on IL-6 production by recipient cells[32]. Furthermore, IL-6 was sufficient to precipitate rejection by enabling naïve alloreactive T cells to escape suppression by CD154-specific antibodies and DST and to differentiate into alloreactive Th1 cells. In a fully mismatched cardiac transplantation model, administration of CpG oligodeoxynucleotides (ODNs), a TLR9 agonist, also triggered IL-6 production and prevented tolerance induction by CD154-specific antibodies[60]. This graft rejection was correlated with the accumulation of Th1 and Th17 cells in cardiac allografts, and was prevented by the simultaneous inhibition of IL-6 and IL-17. Thus, IL-6 production in response to extracellular bacteria or TLR agonists can facilitate the bystander differentiation of alloreactive T cells into Th1 and Th17 cell phenotypes.

How might cytokines produced in response to infections affect immune responses to the allograft through bystander mechanisms? Although cytokines released during APC–T cell interactions have been long thought to signal only locally, recent observations by Perona-Wright *et al.*[61] suggest that certain cytokines may be able to permeate and modify the majority of lymphocytes within a given lymph node. The authors showed that inoculation of *Heligmosomoides polygyrus* or *Schistosoma mansoni* eggs resulted in permeation of the draining lymph node with IL-4 and STAT6 phosphorylation in essentially all $CD19^+$ B cells and most of the $CD4^+$ and $CD8^+$ T cells within that lymph node, whereas infection with *Toxoplasma gondii* resulted in IFN*β* production and STAT1 phosphorylation in the majority of B and T cells in the reactive lymph node[61]. Importantly, IL-4 conditioning of the lymph node prevented subsequent Th1 cell polarization by *Yersinia pestis*-pulsed DCs injected 7 days later. These effects may be relevant in explaining how infections that trigger strong cytokine production might be able to enhance and skew the effector fate of ongoing alloimmune responses.

Infections within the allograft may also directly lead to enhanced alloreactivity as suggested by a recent report that community-acquired respiratory viral infections in lung transplant recipients are associated with increased levels in the lung of the IFN*β*-inducible CXC-chemokine ligand 9 (CXCL9), CXCL10 and CXCL11, chemokine ligands for the CXC-chemokine receptor 3 (CXCR3)[62]. CXCR3 ligands produced by bronchial epithelial and peribronchial mononuclear cells recruit activated lymphocytes that are able to mediate chronic lung allograft rejection[62, 63]. Similarly, CXCL9 and CXCL10 expression was

markedly elevated in the urine of renal transplant recipients experiencing BK viral infection compared with non-infected recipients[64], and these are also chemokines associated with acute and chronic renal allograft rejection[64, 65]. Thus, cytokines and chemokines produced in response to infection may serve to incite alloreactivity either within secondary lymphoid organs or within the allograft itself .

Cell-intrinsic effects on antigen presentation and T cell priming

In addition to bystander cytokine effects, infections can have direct effects on the uptake and presentation of graft-derived antigens (see **Figure 2**). Early studies demonstrated that MYD88- and TRIF-dependent signals in DCs promote their maturation and migration to draining lymphoid organs, thereby facilitating the activation of alloreactive T cells[48, 51]. More recently, TLRs have been shown to have additional effects on antigen presentation. Normal tissue homeostasis requires the rapid clearance of dying apoptotic cells in the absence of inflammation, as part of embryonic development and normal tissue turnover[66]. The switch from the homeostatic phagocytosis of apoptotic cells to the induction of host defence immunity has been shown to be regulated by TLRs within the phagosome[67, 68]. Phagocytosis of *Escherichia coli*-infected apoptotic neutrophils, or apoptotic B cells carrying LPS, resulted in the engagement of TLRs within the phagosome, triggering MYD88-dependent signals that greatly increased the maturation kinetics of the phagosome[67,69]. This resulted in the more efficient delivery of the phagocytic cargo for degradation, for loading of the antigenic peptides on to MHC class II molecules and ultimately, for their presentation to $CD4^+$ T cells. Torchinsky *et al.*[70] reported that infection-induced TLR engagement in the presence of apoptotic cells also favoured the development of Th17 cells. Apoptotic signals in the absence of PRR signals triggered the production of anti-inflammatory mediators such as IL-10, transforming growth factor-β (TGFβ) and prostaglandin E2; however, when apoptotic signals were integrated with TLR signals, the secretion of the Th17-promoting cytokines IL-6, IL-23 and TGFβ was induced[70]. Alloreactive $CD4^+IL\text{-}17^+$ cells have been observed in both acute and chronic transplant rejection in humans[71-73], but whether these Th17 cells are generated by cognate mechanisms involving the uptake of apoptotic allogeneic cells containing microbial PAMPs, in addition to bystander cytokine effects, remains to be elucidated.

Indirect allorecognition (**Box 1**) requires alloantigens to be taken up by recipient APCs and presented on MHC class I or class II molecules to alloreactive T cells. This indirect pathway can play an important role in acute rejection, and it is also considered to be critical in late rejection when the donor-derived APCs capable of direct presentation to alloreactive T cells have been largely eliminated. Infections within the allograft may result in the simultaneous uptake of alloantigens and PAMPs into the same phagosome, thereby allowing for more effective alloantigen presentation and the promotion of rejection [67-69](see **Figure 3**). These newly described effects of PPRs on antigen presentation complement the well-established enhancement of MHC and co-stimulatory molecule expression on APCs during infection that result in enhanced T cell priming (review[74]). Furthermore, it is possible that self-antigens exposed following cell death are presented in the context of PRR signalling, thereby permitting the activation of autoreactive T cells that can contribute to transplant rejection through direct killing or through the production of pro-inflammatory cytokines (see

Figure 3). This process may explain the detection of autoreactivity — quantified as increased T cell frequencies or antibody titres to the self-proteins collagen type V, Kα1 tubulin and vimentin [71, 75, 76] — in patients undergoing acute or chronic rejection.

Effects on established tolerance

A state of transplantation tolerance has not been reliably achieved in human transplantation, although there are promising reports of spontaneous acquisition or deliberate induction of transplantation tolerance in the clinic[77-79]. Understanding the impact of infections on established tolerance will be necessary for predicting the longevity of the tolerance state but because of the limited number of tolerant patients, studies addressing this issue have necessarily involved the use of animal models. The *in vitro* observations by Pasare and Medzhitov[80] introduced the concept that pro-inflammatory cytokines, specifically IL-6, provide an important stimulus that releases effector T cells from suppression mediated by Treg cells. These observations raised the possibility that infections, especially those that elicit IL-6, may be able to confer a loss of established tolerance that is maintained by Treg cells suppressing alloreactive T cells. In an experimental mouse model of heart allograft tolerance, we reported that *L. monocytogenes* infections occurring 60 days after transplantation can overcome established tolerance and precipitate the acute rejection of established heart allografts[31]. Signalling in recipient mice through type I IFN receptors and IL-6 was necessary for the loss of tolerance, and the combination of IFNβ and IL-6 was sufficient to induce rejection. IL-6 induced the proliferation of alloreactive T cells from tolerant mice, whereas IFNβ enhanced IFNγ production by these cells[31]. These data suggest that IL-6 and type I IFN work synergistically to permit previously suppressed alloreactive T cells to escape regulation, become activated and acquire effector function.

Our and other groups have demonstrated that reversing established tolerance is more difficult than preventing the induction of tolerance. Indeed, LCMV infection within the peritransplantation period prevented the induction of transplant tolerance in mice, but had no effect when infection occurred at 50 days post-transplantation, when tolerance had been established[38]. We reason that acquired mechanisms of donor-specific tolerance are in place in tolerant recipients, and that these have to be overcome for alloreactive T cells to become activated. These observations thus bode well for the persistence of tolerance once established, but also underscore the importance of carefully monitoring the state of tolerance, which may be overcome by infections that trigger PRRs and pro-inflammatory cytokine production.

Immunosuppressive effects of infections

Despite the well-characterized pro-inflammatory and pro-rejection effects of infections from experimental models, clinical data suggest that some infections may have systemic immunosuppressive effects. For example, CMV infection is often associated with increased systemic immunosuppression, predisposing transplant recipients to opportunistic superinfections with fungi, bacteria and other viruses (reviewed[81]). Increased rates of co-infections or reactivation of human herpes virus 6 (HHV6) and HHV7 have been reported, and the course of HCV infection is accelerated in liver transplant patients who are infected with CMV. In addition, up to a 7–10-fold increase in EBV-associated lymphoproliferative

disease has been reported for CMV-infected versus non-infected transplant recipients (reviewed[35]). Finally, although experimental and some clinical studies report an association between CMV and acute rejection, others studies do not[82], in spite of a reduction of immunosuppression that is often instituted to treat CMV disease. These observations collectively raise the possibility that CMV infections may also have immunosuppressive effects.

The basis for the paradoxical ability of infections to reduce systemic immunity is suggested by experimental studies demonstrating the increase in circulating glucocorticoids during viral infections[3, 5]. Infection with MCMV or respiratory influenza virus were reported to trigger a transient increase in serum glucocorticoids, which blunted pro-inflammatory cytokine production and suppressed systemic immune responses thereby permitting a more severe secondary bacterial infection[3]. Notably, adrenal corticosterone release is triggered by both viral and bacterial infections[6], suggesting that the release of endogenous glucocorticoids during a wide range of acute infections may transiently induce systemic immunosuppression, resulting in a simultaneous increase in the severity of secondary infections and a reduction in alloreactivity.

In summary, experimental data suggest that infections can augment alloreactivity in multiple ways: infections prior to transplantation generate heterologous memory T cells that differentiate into potent effector cells mediating rejection, whereas infections after transplantation can stimulate alloreactivity through PRR engagement and the production of pro-inflammatory cytokines. Infections can also trigger a counter-regulatory immune–adrenal response resulting in elevated glucocorticoid levels that temper systemic immune responses. Thus a complex and dynamic relationship can exist between infections and alloreactivity.

Tissue injury and DAMPs

Tissue damage arises during the process of brain or cardiac death of the transplant donor, cold ischaemia (due to storage and transport of procured donor organs) followed by reperfusion in the recipient, warm ischaemia and as a result of the surgical procedure. This damage in the absence of infection, as well as damage as a result of infection, cause the release of DAMPs (**see Figure 3**). An increasing number of DAMPs have been identified, including extracellular matrix fragments, self-nucleic acids, histones and high mobility group box protein 1 (HMGB1) (reviewed[73, 83]. Notwithstanding possible contamination with microbial products, DAMPs can bind to an increasing number of PRRs, including TLR2, TLR3, TLR4, TLR7, TLR9 and receptor for advanced glycation end products (RAGE, an HMGB1 receptor).

A broad range of PPRs has been implicated in ischaemia–reperfusion injury. In experimental models, ischaemia–reperfusion injury is reduced in hosts deficient in TLR2, TLR4, RAGE or the intracellular PRRs nucleotide-binding oligomerization domain 1 (NOD1) and NOD2[85-87]. The NLRP3 inflammasome is expressed in kidney cells, and its absence also protects from kidney ischaemia–reperfusion injury by reducing cellular necrosis and apoptosis[88]. In a clinical setting, Kruger *et al.*[89] reported that human kidneys expressing

TLR4 mutations that confer diminished affinity for HMGB1 express lower levels of tumour necrosis factor (TNF) and CCL2 (also known as MCP1), higher levels of hemeoxygenase 1, and exhibited a higher rate of immediate graft function compared with kidneys expressing wild-type *TLR4* alleles.

The better transplantation outcome of poorly matched donor kidney allografts from living donors than well-matched grafts from deceased donors[84] suggest that brain death and cold ischaemia may impact the development of alloreactivity. The ability of DAMPs to enhance alloreactivity has been demonstrated in experimental mouse models using minor MHC-mismatched donor–recipient combinations, which trigger a less robust alloimmune response compared to fully MHC-mismatched allografts. DAMPs such as HMGB1, hyaluronan and heparan sulfate, and more recently, haptoglobin, have been shown to enhance alloresponses and graft rejection[90-92] (reviewed [93]). Although these data demonstrate that DAMPs can contribute to allograft rejection in limited MHC-mismatched situations, their contribution to alloreactivity and pathogenicity is unclear in situations of full MHC-mismatched allografts.

DAMPs appear to require different co-receptors and accessory molecules from those used by PAMPs, and as a consequence, elicit different signalling and inflammatory factors compared to PAMPs (reviewed[83]) and can cooperate to stimulate inflammation and immune responses. For example, HMGB1 synergizes with the TLR4 ligand lipopolysaccharide, TLR9 ligand CpG ODNs or the TRL1–TLR2 ligand Pam_3CSK_4 to elicit pro-inflammatory cytokine production [94]. Thus in transplantation, the cross-talk between DAMPs arising from tissue damage and PAMPs possibly arising from the translocation of bacteria and/or bacterial products from the intestine, or from post-surgical infections, may synergistically contribute to development of alloreactivity and allograft rejection.

Implications for therapy

Can our understanding of how infections alter alloreactivity be translated into improving the outcome of transplantation without incurring decreased protective immunity to infections? The control of memory alloreactive T cells, whether having arisen by heterologous immunity or other means, by blocking adhesion molecules has successfully been demonstrated in non-human primate transplantation models. Antibodies directed against lymphocyte function-associated antigen 1 (LFA1) (efalizumab), VLA4 (natalizumab) and the LFA3–immunoglobulin fusion protein (Alefacept) effectively prevented acute rejection of kidney or islet transplants in mice and non-human primates but also diminished protective memory to subsequent infections[95-98], underscoring the potential limitation of this approach. Indeed, efalizumab and natalizumab have been associated with increased risk of progressive multifocal leukoencephalopathy (PML), and efalizumab has been withdrawn from the US market [98]. These infectious concerns prompt the consideration of alternative approaches to minimize the impact of alloreactive memory T cells, such as through the selection of donor-recipient pairs with minimal pre-transplant memory donor-specific T cell reactivity[17].

A number of PRR agonists and antagonists targeting PRRs are being developed for the treatment of cancer, autoimmunity and inflammatory diseases and the prevention of viral and bacterial infections (**Table 1**; reviewed[99]). In particular, compounds that reduce

inflammation or tissue damage may potentially be useful in the setting of transplantation. To temper concerns of reduced protective immunity to infections, the blocking of PRRs has been explored primarily in experimental models of ischaemia-reperfusion injury, where the necessity to block TLRs and RAGE[85-87] is anticipated to be transient. Partial MD2–TLR4 and TLR2 antagonists being developed as a treatment for endotoxaemia and agents that block HMGB1–RAGE signalling, including soluble RAGE and RAGE- or HMGB1-specific monoclonal antibodies, may attenuate ischaemia–reperfusion injury. A pharmacological derivative of the TLR5 agonist flagellin that prevents stress-induced apoptosis has been shown to protect against acute ischaemic renal failure in mice[100]. Even if these strategies are effective at limiting ischaemia-reperfusion injury, it is currently unclear how these strategies can be safely used in transplant recipients in the immediate post-transplantation because this is also the time when infection rates are highest.

Pro-inflammatory cytokines released in response to viral or bacterial infections can enhance alloreactivity and allograft rejection, and the inhibition of type I IFN signalling or IL-6 has been shown to promote both the induction and maintenance of transplantation tolerance in animal models[31, 32, 58,60]. The IL-6 receptor-specific monoclonal antibody tocilizumab is already approved for use in patients with rheumatoid arthritis, and a recently completed Phase 2 clinical trial with the IL-6-specific antibody sirukumab in rheumatoid arthritis also showed promise in terms of efficacy and safety[101]. In a small study of 8 patients, toclizumab was effective in treating corticosteroid refractory graft-versus-host disease but infections were the primary adverse events[102]. Indeed, both type I IFNs and IL-6 have pleiotropic effects and play important roles in the control of viral, bacterial and fungal infections, so targeting these cytokines in a safe and efficacious manner in transplant patients who are already immunosuppressed will be challenging.

The ideal therapeutic approach is to block innate immune responses that lead to the stimulation of alloreactivity but not protective immunity to infections. Interestingly, the immunosuppressant rapamycin was shown to increase the magnitude and quality of the effector and memory $CD8^+$ T cell responses to infection but inhibited the alloreactive $CD8^+$ T cell response[103]. Rapamycin has been shown to inhibit maturation of DCs and their secretion of pro-inflammatory and anti-inflammatory cytokines, to enhance their antigen presentation by inhibiting autophagy, and to inhibit T cell proliferation (reviewed[104]). Whether these checkpoints explain the differential effects of rapamycin on alloreactivity and protective immunity is unknown, nonetheless these observations provide important proof of concept that graft-specific responses may be selectively attenuated while preserving pathogen-derived responses.

Conclusions and perspective

The complex effects of infections and tissue damage on the outcome of transplantation are starting to be appreciated, not only in the generation of memory alloreactive T and possibly B cells, but also in their role as a source of PAMPs and DAMPs that can promote innate immune responses. The tools to analyse the complex feedback circuits between the immune responses and host microbiota (**Box 3**) or infections are just being developed, with some pathways known and some postulated to have an impact on alloreactivity. Further study of

how these circuits impinge on alloresponses will likely continue to change our understanding of organ rejection and tolerance, and provide insights into how the effects of infection on alloreactivity can be controlled without compromising protective immunity.

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Glossary

Minor histocompatibility antigens (miHAs)	In the context of transplantation, miHAs are polymorphic peptides that are recognized by donor T cells. miHAs are derived from polymorphic alleles of genes in which the donor and recipient differ. Both GVHD and graft versus leukemia effects are induced in response to these polymorphic antigens.
Ischaemia–reperfusion injury	Cellular damage caused by the return of a blood flow to a tissue after a period of inadequate blood supply. The absence of oxygen and nutrients causes cellular damage and restoration of the blood flow results in inflammation.
MHC tetramers	Fluorescently labelled tetravalent complexes of MHC class I or class II molecules complexed with antigenic peptide. They can be used to identify antigen-specific T cells by flow cytometry.
SCID mice (Severe combined immunodeficiency mice)	Mice with a defect in DNA recombination that results in absence of B and T cell development. Such mice are incompetent at rejecting tissue grafts from allogeneic and xenogeneic sources.
Superantigen	A microbial protein that activates all T cells expressing a particular set of Tcell receptor (TCR) Vβ chains by crosslinking the TCR to a particular MHC regardless of the peptide presented.
Graft-versus-host disease (GVHD)	Tissue damage in a recipient of allogeneic tissue that results from the activity of donor lymphocytes recognizing the tissues of the recipient as foreign. GVHD varies markedly in extent, but it can be life-threatening in severe cases. Damage to the liver, skin and gut mucosa are common clinical manifestations.
Bronchiolitis obliterans syndrome (BOS)	A fibroproliferative process of the small airways that results in multifocal bronchiolar obliterations, which is presumed to reflect chronic allograft rejection. BOS is the major factor that limits the survival of lung transplant recipients.

Donor-specific transfusion (DST).	A treatment for inducing transplant tolerance, which involves infusion of spleen cells into a transplant recipient from a donor that also provides the organ or tissue for the recipient.
Glucocorticoids	A group of compounds that belongs to the corticosteroid family. These compounds can either be naturally produced (hormones) or synthetic. They affect metabolism and have anti-inflammatory and immunosuppressive effects. Many synthetic glucocorticoids (for example, dexamethasone) are used in clinical medicine as anti-inflammatory drugs.
haptoglobin	A plasma protein that can bind free haemoglobin in the bloodstream.
Endotoxaemia	This is caused by the presence in the blood of lipopolysaccharide (endotoxin), which is derived from Gram-negative bacteria. It results in systemic activation of the inflammatory response, the development of shock, multi-organ failure and death. Models of endotoxaemia are used in experimental settings to induce systemic inflammation, but they do not necessarily mimic human sepsis.

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Box 1: Basic vocabulary and concepts in transplantation

Autologous, syngeneic and allogeneic grafts

Autologous grafts are transplants from one individual to him/herself, usually skin grafts for burns. Syngeneic grafts are between genetically identical individuals and allogeneic grafts between genetically distinct individuals of the same species.

Acute and chronic rejection

Acute and chronic rejection are defined by both the timing of rejection (early versus late after transplantation) and the mechanism of rejection, that is T cell- or antibody-dependent acute injury or a more progressive loss of function involving vasculopathy and both immune-dependent (T cells and antibodies) and independent factors (for example, drug toxicities).

Direct and indirect allorecognition

Direct allorecognition describes the ability of the recipient's T cells (educated in the thymus to be self-peptide–MHC restricted) to recognize allogeneic peptide–MHC complexes from the donor either via molecular mimicry between self-peptide–MHC and allogeneic peptide–MHC complexes or via new T cell receptor (TCR) docking contacts on allogeneic MHC molecules presenting donor peptide.

Indirect allorecognition refers to recipient T cell recognition of peptides that are processed from allogeneic MHC or other donor non-MHC molecules with polymorphic differences with host proteins (termed minor histocompatibility antigens), and presented on self-MHC molecules of the recipient.

Heterologous immunity

Heterologous immunity refers to the ability of antigen-experienced T cells of a given specificity to cross-react with a different antigen, most commonly microbial antigen-specific memory T cells that cross-react with allogeneic MHC molecules. Because memory T cells are more difficult to suppress compared with naïve T cells, heterologous memory following infections has been hypothesized to be a significant barrier to the induction of transplantation tolerance.

Box 2 Pathogen-recognition receptors(PRRs)andpathogen-associated molecularpatterns (PAMPs)

Toll-like receptors (TLRs): The cell surface TLRs TLR1, TLR2, TLR4, TLR5 and TLR6 recognize components of extracellular bacteria and fungi; TLR2, TLR4 and TLR13 recognize viral structural proteins; endosomally located TLR3, TLR7 and TLR8, and TLR9 recognize viral double-stranded RNA, single-stranded RNA and single-stranded DNA, respectively (reviewed[40, 74]).

C-type lectin receptors: Dectin-1, dendritic cell-specific ICAM-2 grabbing, non-integrin (DC-SIGN), mannose receptor and macrophage-inducible C-type lectin (Mincle) are transmembrane C-type lectin receptors that recognize classes of carbohydrates expressed on the surface of fungi (reviewed[40, 74]).

Cytosolic PRRs: These PRRs sense cell-intrinsic viral and intracellular bacterial infections and serve primarily to trigger the secretion of type I IFNs and other factors that recruit and activate DCs[74]. These cytosolic PRRs include retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation factor 5 (MDA5) that recognize viral RNA, absent in melanoma 2 (AIM2), DNA-dependent activator of IFN-regulatory factors (DAI) and IFNγ- inducible protein 16 (IFI16) that recognize viral DNA[40], and the family of nucleotide oligomerization domain (NOD)-like receptors (NLRs) that recognize a wide range of PAMPs and damage-associated molecular patterns (DAMPs), from flagellin and bacterial toxins to particulate antigen, aluminium salts, RNA and low K^+ concentrations (reviewed[105]).

Sensing of live versus dead microorganisms: Live microorganisms have a superior ability to activate innate immune responses than attenuated or dead microbes. Recently, Sander *et al.*[106] reported that the mammalian innate immune system directly senses microbial viability through the detection of viability-associated PAMPs (which they termed vita-PAMPs), identified as prokaryotic messenger RNA (mRNA). The prokaryotic mRNA is recognized by cytosolic receptors that activate NOD-, LRR- and pyrin domain- containing 3 (NLRP3) and the TLR adaptor molecule TRIF, resulting in the production of active interleukin-1β (IL-1β) and increased levels of interferon-β (IFNβ). Thus vita-PAMPs provide a mechanism by which the innate immune system modulates its response appropriately to bacterial viability and infectivity.

Box 3 Microbiota and transplantation

Another potential source of pathogen-associated molecules patterns is commensal microbiota in the host and from donor organs such as the lung, intestine and skin.

Donor microbiota: A putative role for commensal bacterial signals at the time of transplantation in promoting transplant rejection is suggested by the clinical and experimental observations that the lung and intestine, which harbor significant commensal bacterial loads, are more prone to rejection than more sterile organs like the heart or kidney (http://optn.transplant.hrsa.gov/latestData/step2.asp?). However, a causal role of local donor commensal microflora in enhancing the immunogenicity of colonized organs remains to be proven.

Recipient microbiota: The bacterial composition of the intestine can shape the repertoire of Treg cells, dictate the effector differentiation of T cells and modulate disease-associated immune responses locally, for example in inflammatory bowel disease, or distally, for example in autoimmune arthritis (review[107, 108]). In turn, the immune system can modify the pathogenicity of certain bacteria, as exemplified by the ability of interferon-γ (IFN*γ*) to enhance the virulence of *Pseudomonas aeruginosa*, transforming it from an indolent colonizer to an invasive pathogen within the intestinal tract[109]. Analysis of a potential role of recipient microbiota in alloimmune responses is at its infancy, but a recent study[110] analyzing ileal microbiota composition following intestinal transplantation indicated that the ratios of phylum *Firmicutes* to *Probacteria* spp. were significantly decreased in the effluents of patients undergoing acute rejection compared to non-rejecting and pre-rejecting samples. The study did not determine whether the change in microbiota was a cause or a consequence of the rejection process or could be a useful biomarker to predict patients at risk of rejection, it nonetheless underscores the importance of developing further studies to address these questions.

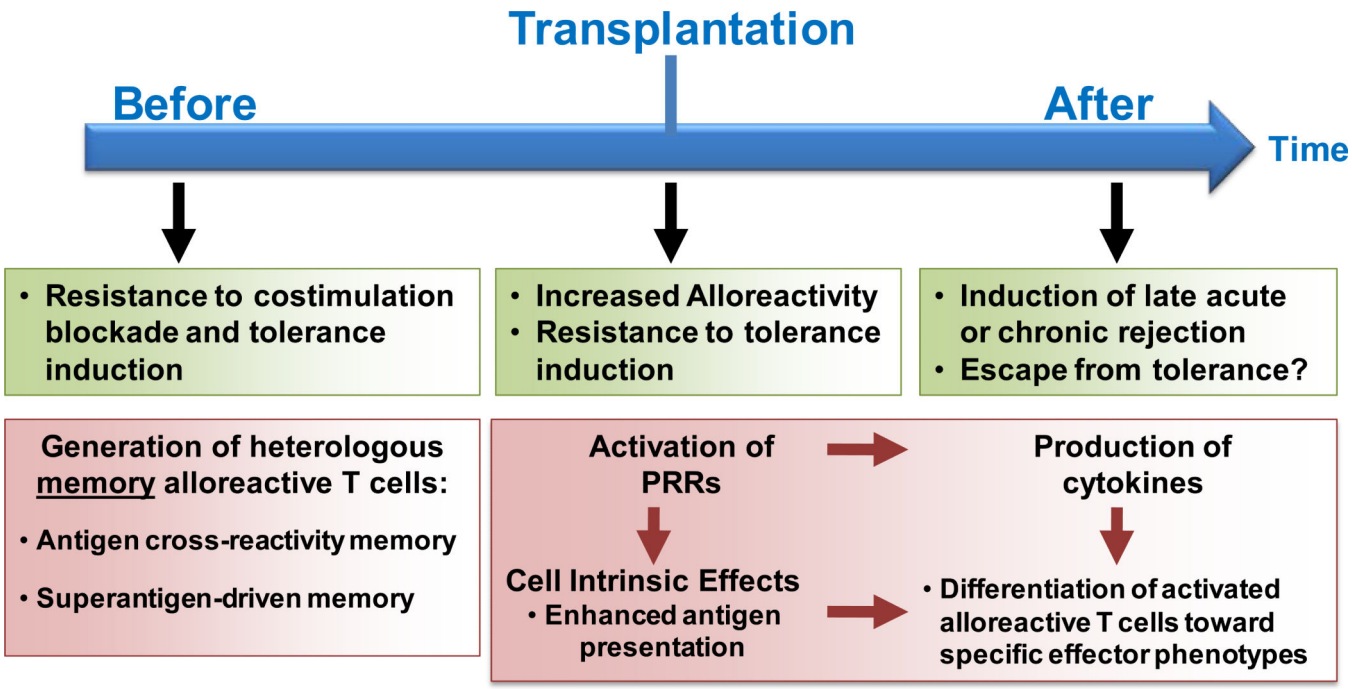

Figure 1. Possible effects of infections before, at and after transplantation

Some T cells specific for microbial peptides presented by self-MHC molecules can cross-react with allogeneic MHC while bacterial superantigens can directly activate large populations of T cells. Therefore, infections experienced before transplantation can give rise to memory alloreactive T cells that may be more resistant to immunosuppression than naïve T cells [15, 18]. Signals and cytokines elicited upon engagement of PRRs on APCs, T cells or parenchymal cells at the time of or after transplantation can result in enhanced priming, survival and expansion of alloreactive T cells, as well as dictate the phenotype of differentiating alloreactive T cells. Infections occurring late after transplantation may, in theory, elicit pro-inflammatory signals that activate tolerant T cells by enabling their escape from immunosuppression and/or peripheral mechanisms of tolerance, thereby precipitating rejection.

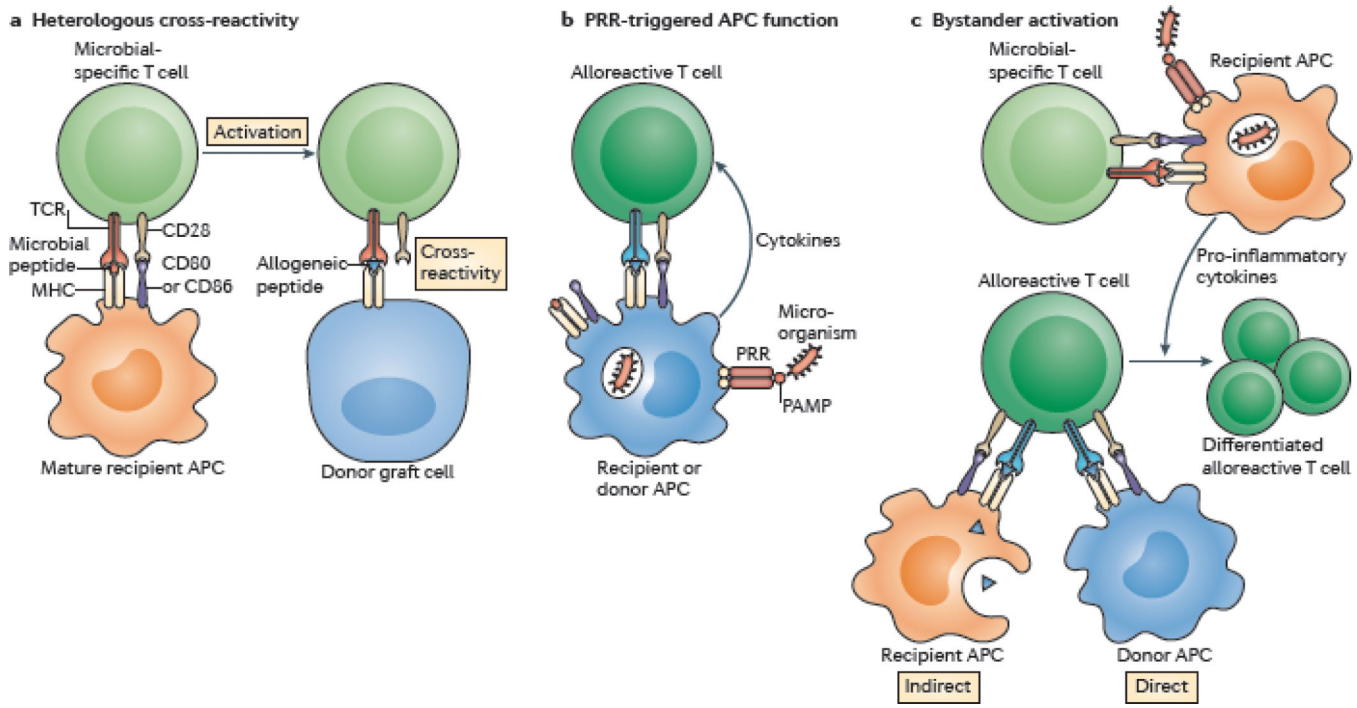

Figure 2. Anti-microbial immune responses can augment and shape alloimmunity

PRR-dependent signals can enhance alloreactivity by at least 3 mechanisms. **a**, T cells specific for microbial antigens and fully activated by APCs matured by infection-derived PAMPs and presenting microbial antigens in response to an infection may cross-react on donor MHC and therefore recognize allogeneic graft cells directly. **b,** The alloantigen can be presented to alloreactive T cells by an APC that is simultaneously receiving PRR signals either from its own infection, or from sensing PAMPs on or released by the microbe. This PRR-mediated stimulation can result in increased processing and presentation of the alloantigen, expression of co-stimulatory molecules, secretion of cytokines and priming of alloreactive T cells. **c**, inflammatory cytokines elicited by PAMPs during an infection may enhance and divert differentiating alloreactive T cells into specific phenotypes that elicit different graft pathologies. DAMPs may play similar roles to PAMPs.

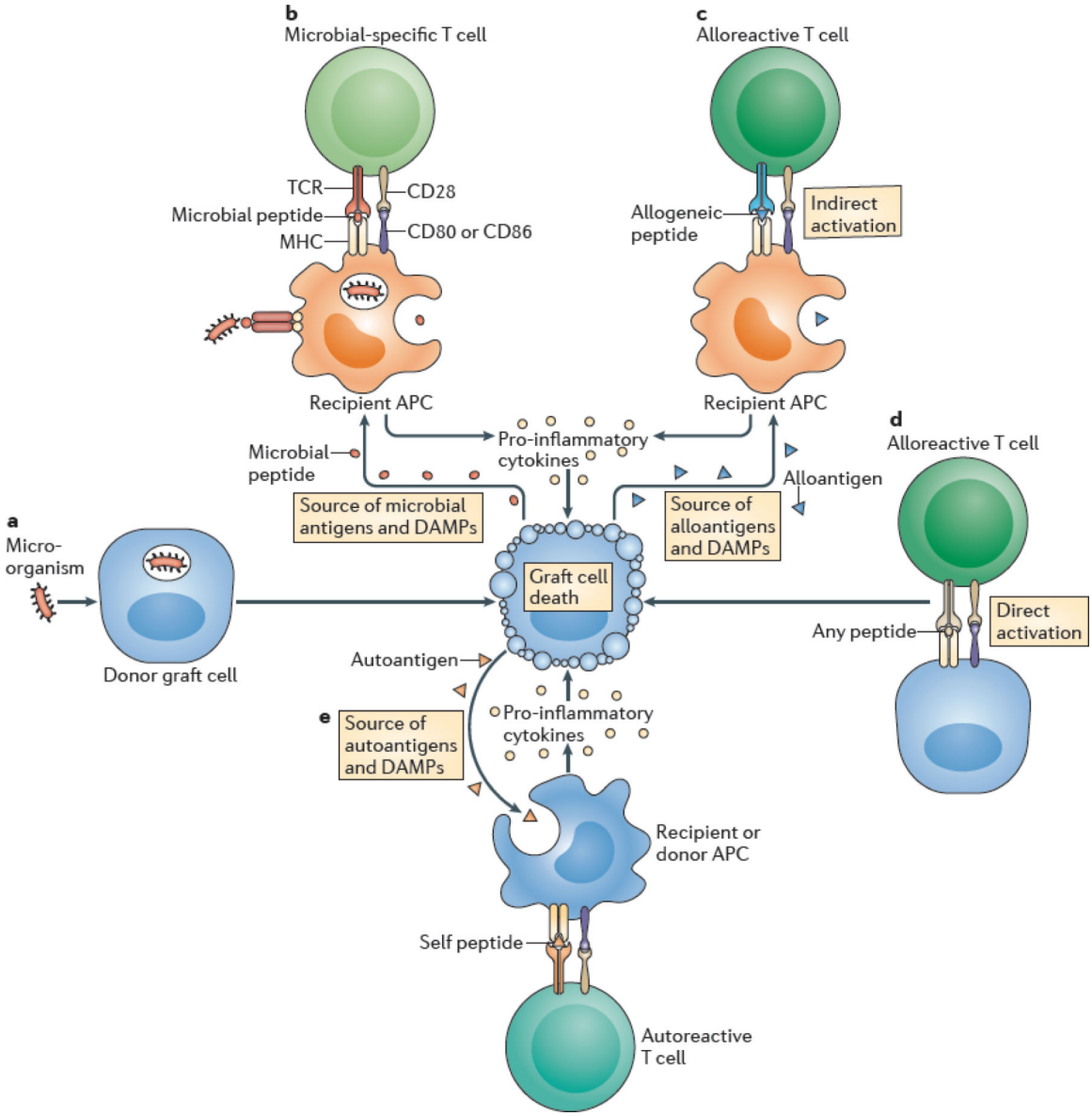

Figure 3. Potential causes of graft damage during microbial infections

Graft destruction during an infection can occur by multiple mechanisms. **a**, microbes with a tropism for the graft organ may have direct cytopathic effects that cause the release of microbial or donor antigens as well as DAMPs. **b**, recipient APCs or endothelial cells (not shown) in the graft may take up and cross-present exogenous microbial antigens to recipient T cells. Activated APCs or T cells may secrete cytokines that are damaging to the graft or injure endothelial cells thereby reducing vascular supply to the graft. Of note, when HLA alleles are shared between the recipient and donor, recipient-restricted anti-microbial T cells may directly engage infected donor cells (not shown in the figure). **c**, infections may augment activation of direct alloreactive T cells by enhancing maturation of donor APCs (not shown) or parenchymal cells resulting in graft damage. **d**, similarly, infections may mature recipient APCs or endothelial cells (not shown) that have taken up donor antigen, resulting in activation of indirect alloreactive T cells and graft injury by toxic soluble factors

that injure blood vessels. **e,** cellular graft damage may release cryptic antigens and DAMPs that then activate autoreactive T cells. Upon re-encounter with these antigens on cross-presenting recipient APCs or endothelial cells, these autoreactive T cells may contribute to graft damage in a phenomenon analogous to epitope spreading.

Table 1

Potential compounds targeting TLRs, HMGB1 or RAGE for transplantation

Compound	Company	Indication	Target*	Drug class	Clinical Phase
IRS-954 (DV-1079)	Dynavax Technologies	SLE, HIV	TLR7 and TLR9 antagonist	Bifunctional inhibitor	Preclinical
NI-0101	NovImmune	Acute and chronic inflammation	TLR4 antagonist	Antibody	Preclinical
OPN-305	Opsona Therapeutics	Inflammation, autoimmunity, ischaemia/reperfusion	TLR2 antagonist	Antibody	Preclinical
OPN-401	Opsna Therapeutics	IBD, rheumatoid arthritis	TLR2 antagonist	Viral-derived Peptide	Preclinical
IMO-3100	Idera Pharmaceuticals	SLE, rheumatoid arthritis, multiple sclerosis	TLR7 and TLR9 antagonist	DNA-based compound	Preclinical
1A6	NovImmune	Colitis	TLR4 antagonist	Antibody	Preclinical
CPG-52364	Pfizer	SLE Poly	TLR antagonist	Quinazoline Derivative	Phase I
CBLB502	Cleveland Biolabs Inc.	Radioprotectant	TLR5 agonist	Flagellin	Phase I
Eritoran	Eisai Pharmaceuticals	Sepsis	TLR4 antagonist	Synthetic Lipodisaccharide	Phase III*
Anti-HMGB1	Abnova	Sepsis	HMGB1	Antibody	Preclinical
PF-04494700	Pfizer	Alzheimer's	RAGE	small molecule Inhibitor	Phase II

Summarized from[99] and Clinical Trials.gov

*Completed but did not meet primary Endpoint