

Review

Mechanisms of Mixed
Chimerism-Based Transplant
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Immune responses to allografts represent a major barrier in organ transplantation. Immune tolerance to avoid chronic immunosuppression is a critical goal in the field, recently achieved in the clinic by combining bone marrow transplantation (BMT) with kidney transplantation following non-myeloablative conditioning. At high levels of chimerism such protocols can permit central deletional tolerance, but with a significant risk of graft-versus-host (GVH) disease (GVHD). By contrast, transient chimerism-based tolerance is devoid of GVHD risk and appears to initially depend on regulatory T cells (Tregs) followed by gradual, presumably peripheral, clonal deletion of donor-reactive T cells. Here we review recent mechanistic insights into tolerance and the development of more robust and safer protocols for tolerance induction that will be guided by innovative immune monitoring tools.

Challenges in Translating Transplantation Tolerance to the Clinic

Immune tolerance is a state of unresponsiveness of the immune system to specific tissues or cells. Tolerance to self prevents autoimmunity. The immune system is educated to discriminate between self and non-self via an intricate set of central and **peripheral immune tolerance** (see [Glossary](#)) mechanisms. Regarding T cell tolerance, which is the major emphasis of this review, **central tolerance** refers to the deletion of reactive clones within the thymus during negative selection. By contrast, peripheral T cell tolerance encompasses several mechanisms that occur outside the thymus, including peripheral deletion, anergy/**exhaustion**, and the suppressive function of Tregs.

In solid-organ transplantation, establishing donor-specific immunological tolerance, which avoids the complications of long-term immunosuppression (infections, malignancies, cardiovascular disease, renal failure, etc.), has long been the ultimate goal. Although many approaches have successfully achieved immunological tolerance across MHC barriers in rodents, very few have been translated to humans. The most advanced approach is the addition of hematopoietic cell transplantation (HCT) to kidney allografts in conditioned recipients [referred to henceforth as combined kidney and hematopoietic cell transplantation (CKHCT)], which has yielded very encouraging results. This strategy has offered proof of concept that operational tolerance can be induced in humans through **mixed chimerism**, defined as a state wherein donor and recipient hematopoietic cells coexist at levels sufficient to be detected by standard techniques such as flow cytometry (generally 1–99%) [1–3]. Toxicities related to the conditioning procedures and the occurrence of opportunistic infections and GVHD in some of the protocols indicate a need for further refinements before routine use of these approaches can be considered. Furthermore, the achievement of tolerance has not yet been uniform in these protocols. Achievement of this goal will benefit from further mechanistic insights, especially in humans and large-animal models.

Trends

A current clinical tolerance protocol achieving sustained full chimerism across HLA barriers in humans is likely to depend on some level of graft-versus-host (GVH) reactivity that counteracts the host-versus-graft response. This is associated with a significant risk of life-threatening GVH disease (GVHD).

By contrast, tolerance induction through transient mixed chimerism involves sparing and early expansion of regulatory T cells (Tregs) and progressive deletion of donor-specific T cells in the periphery.

A better understanding of the molecular pathways involved in peripheral tolerance is paving the way for refined tolerance-inducing protocols that will use biological and pharmacological agents to synergistically achieve tolerance without relying on GVH reactivity.

Treg therapy, T cell proapoptotic agents, and costimulatory blockers are entering into the clinic and have the potential to make chimerism-based tolerance strategies safer and more robust.

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Also, the lack of reliable biomarkers to identify donor-specific tolerance is a hurdle to structured immunosuppression tapering in clinical transplantation [4,5]. However, recent advances in identifying biomarkers of transplant tolerance [4,6] and especially in measuring and tracking the recipient antidonor alloreactive repertoire [6–8] are likely to facilitate such efforts in the future.

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A better grasp of the complex synergistic tolerogenic pathways in rodents and the recent availability of new tools to monitor alloreactivity in humans have helped in deciphering tolerance mechanisms in clinical settings, paving the way for new protocols that could avoid the need for profound lymphoablation of the recipient. We aim herein to review our current understanding of tolerance mechanisms in clinical and highly relevant preclinical models. We also discuss some new approaches with potential to further enhance tolerogenic mechanisms in humans based on recent clues gained from experimental models.

Tolerance Mechanisms Associated with Sustained Mixed Chimerism

Central Tolerance

Sustained Mixed Hematopoietic Chimerism

One of the first avenues explored for sustained tolerance was based on the use of hematopoietic stem cell (HSC) transfers to achieve durable chimerism. In rodent models, intrathymic deletion of donor-reactive immature T cells is a central feature of this approach for tolerance induction [9]. Durably engrafted donor HSCs supply circulating T cell/dendritic cell (DC) precursors that populate the recipient thymus, where they contribute to the pool of thymic DCs leading to the deletion of newly developing donor-reactive T cells [9]. When recipient T cells are globally depleted before transplantation, this central deletion is the major mechanism both inducing and maintaining tolerance, with no meaningful contribution from regulatory mechanisms [10]. Chimerism in thymic DCs was also detected and correlated with tolerance in chimeric nonhuman primates (NHPs) [11] and swine [12,13]. In humans, two clinical CKHCT protocols achieved durable chimerism. Success with one regimen, involving a combination of total lymphoid irradiation and antithymocyte globulin, has been reported only for HLA-identical transplants, whereas the other involved extensively HLA-mismatched donors [14,15]. In the latter, induction of sustained chimerism used the combination of fludarabine, cyclophosphamide and total-body irradiation and substantial numbers of donor T cells were administered, with a significant risk of GVHD (see below) [14,15]. Blood T cells from chimeric patients in both studies failed to proliferate *in vitro* against recipient or donor cells, consistent with (but not specifically indicative of) intrathymic deletion of donor-reactive clones [14,15]. Other approaches have been successfully used in experimental models to promote central tolerance to an allograft, including thymic transplantation and the transfer of thymus-homing DC precursors, but their translational potential remains to be defined (Box 1).

Counteracting Rejection Using GVH Reactivity

Balance between Host-Versus-Graft (HVG) and GVH Immune Responses

Some allograft types, such as liver and, especially, intestine, have high lymphoid cell loads and the potential to induce GVHD. However, GVH responses are not synonymous with GVHD, as GVH responses confined to the lymphohematopoietic system [**lymphohematopoietic GVH responses (LGVHRs)**] can destroy recipient hematopoietic cells without causing GVHD and can balance out HVG-reactive T cells [16–18]. The recent observation that high levels of peripheral blood T cell mixed chimerism occur commonly, without GVHD, in recipients of intestinal allografts, and the association of this chimerism with lack of graft rejection [20] led us to propose that a LGVHR may similarly counteract HVG responses in these patients, promoting hematopoietic chimerism and preventing rejection. In line with this hypothesis, immunosuppression withdrawal in a liver transplant recipient induced the conversion of mixed to **full donor chimerism**, despite the lack of GVHD [19]. This case report underscores the role of graft-borne GVH-reactive T cells in neutralizing HVG-reactive T cells and in promoting transplant tolerance

Box 1. Alternative Experimental Approaches to Induce Transplant Tolerance through Central Mechanisms**Thymus Transplantation**

An alternative experimental strategy to promote central tolerance involves combining thymus and organ transplantation from the same donor [112,113]. The powerful tolerance-inducing capacity of this approach was demonstrated in the highly disparate pig-to-mouse [114] xenogeneic combination and then in humanized mice (i.e., immunodeficient mice reconstituted with human immune cells) after the engraftment of porcine tissue [115,116]. Vascularized thymic lobe transplantation from juvenile donors to thymectomized young recipients induces T cell tolerance across fully allogeneic barriers in swine [112,113]. So far in humans, allogeneic thymi have been transplanted, only in the form of cultured thymic tissue, in congenitally athymic infants [117,118]. Tolerance to simultaneously grafted parathyroid grafts sharing donor class II HLA alleles [119] suggests the potential of this approach to promote tolerance in humans. Although the deletion of newly developing thymocytes is a major mechanism by which thymic grafts promote tolerance [120], the generation of Tregs with specificity for the donor is an important mechanism for the suppression of non-ablated, preexisting donor-reactive T cells [115,121].

Donor APCs Homing to the Thymus

In addition to the DCs that arise intrathymically from a common T cell/DC precursor, some subsets of thymic DCs originate extrathymically and subsequently colonize the thymus, where they promote tolerance toward antigens loaded in the periphery. This includes immature CCR9-expressing plasmacytoid DCs (pDCs) endowed with the ability to home to the thymus, mediate antigen-specific thymocyte deletion [122], and induce Tregs in mice [123]. A similar subset of thymus-resident pDCs, driving the development of Tregs, was also identified in human thymi [124]. Importantly, donor-derived thymic DCs injected into the circulation can colonize the thymi of allogeneic mice and prolong skin allograft survival by reshaping the thymocyte repertoire and deleting donor-reactive clones [125]. In addition to these pathways, the direct presentation of donor-derived peptide–MHC complexes in the thymus could be promoted by the migration of donor-derived exosomes to the thymus, where they coat recipient cells [126]. Cross-dressing (i.e., transfer of intact donor peptide–MHC complexes onto recipient APCs) is a phenomenon of unexpectedly large magnitude following organ transplantation [126,127]. The potential of cross-dressed thymic DCs to mediate central tolerance remains to be addressed.

[19,20]. Furthermore, we found, in intestinal transplant recipients, that expanded intragraft GVH-reactive T cells may have attenuated the HVG response locally, as high GVH:HVG clonal ratios in the graft were associated with slower replacement of graft T cells by the recipient and less rejection [7]. Notably, the expansion of GVH-reactive clones in the graft was found to occur early in association with recipient replacement of graft mucosal antigen-presenting cell (APC) populations [7].

Role of GVHRs in Clinical Mixed Chimerism Protocols

The perennial challenge in clinical HCT has been the reliance on GVH reactivity both to counterbalance HVG reactivity and to mediate graft-versus-tumor (GVT) effects, as this GVH reactivity is often associated with GVHD, especially when HLA barriers are traversed. GVH reactivity can be controlled as a beneficial (mediating GVT effects) LGVHR that does not migrate to the epithelial GVH target tissues but promotes full donor chimerism if inflammatory stimuli associated with conditioning have subsided by the time that GVH-reactive T cells are administered in a delayed donor lymphocyte infusion [16,17,21]. We have aimed to develop clinical protocols that are completely devoid of GVHD risk, which we consider unacceptable when HCT is added to organ transplantation solely for the purpose of tolerance induction (i.e., in the absence of malignancy or any other indication for HCT). Our transient chimerism protocol was first shown to meet this criterion in recipients of HLA-mismatched transplants for malignancy [22] before being adapted for the induction of renal allograft tolerance across HLA barriers in the absence of malignancy [1–3]. These protocols include peritransplantation treatment with a depleting anti-CD2 antibody that eliminates donor, in addition to recipient, T cells, thereby avoiding any GVHR from the HCT and permitting robust maintenance of recipient HSCs (Figure 1). By contrast, other clinical tolerance induction protocols that are associated with sustained donor chimerism may rely on GVH reactivity to achieve this outcome

Glossary**Activation-induced cell death**

(AICD): a programmed cell death process by which T cells undergo apoptosis following activation in a manner controlled through the interaction of death factors (FASL and TRAIL) and their receptors; also known as the extrinsic apoptosis pathway. AICD involves a death-inducing signaling complex and caspase 8.

Central tolerance: the mechanism by which newly developing T cells are rendered non-reactive to self antigens as the result of intrathymic deletion of those with the greatest reactivity to self.

Exhaustion: a state of T cell dysfunction defined by poor effector function, a hallmark transcriptomic pattern, and sustained expression of inhibitory receptors; usually results from chronic antigen stimulation in a suboptimal environment.

Heterologous immunity: refers to the phenomenon by which T cells that were generated during an earlier infection can recognize and mount a memory T cell response against allogeneic antigens through TCR crossreactivity.

Intrinsic apoptosis pathway: also named the mitochondrial pathway; activated in response to various types of intracellular stress, including nutrient starvation. Mitochondrial outer membrane permeabilization and cytochrome c release are key points of no return in the intrinsic apoptosis pathway, leading to caspase 9 activation.

Lymphohematopoietic graft-versus-host response (LGVHR):

GVH reactivity that eliminates recipient lymphohematopoietic cells without trafficking to epithelial GVHD target tissues (skin, intestine, and liver) and therefore without causing clinical GVHD.

Mixed or full chimerism: mixed hematopoietic chimerism refers to a state wherein recipient and donor hematopoietic cells coexist in an individual, with a donor cell frequency ranging from >1% to <100%. Full chimerism denotes donor reconstitution close to 100%.

Peripheral tolerance: the mechanisms that occur outside the thymus to prevent mature T lymphocytes from mounting an efficient immune response against a given set of antigens. Mechanisms

(Figure 1) [2,14,15]. In one study the full donor chimerism achieved in most patients is not in keeping with the maintenance of recipient HSCs that would be expected following non-myeloablative conditioning [2,14,15]. Despite full donor leukocyte chimerism, however, when donor and recipient blood types were mismatched most of the patients retained the recipient's blood type [14]. Recipient HSCs may survive without contributing to chimerism when their progeny are destroyed by a LGVHR, consistent with the immunoprotection of the HSC niche that has recently been described [23]. The observation of persistent recipient erythropoiesis in the presence of full donor leukocyte chimerism in humans similarly suggests the persistence of recipient HSCs whose HLA-expressing progeny are destroyed by a GVHR while HLA antigen-deficient erythrocytes survive [14]. Unfortunately, this approach has been associated with

include anergy, exhaustion, ignorance, deletion, and suppression.

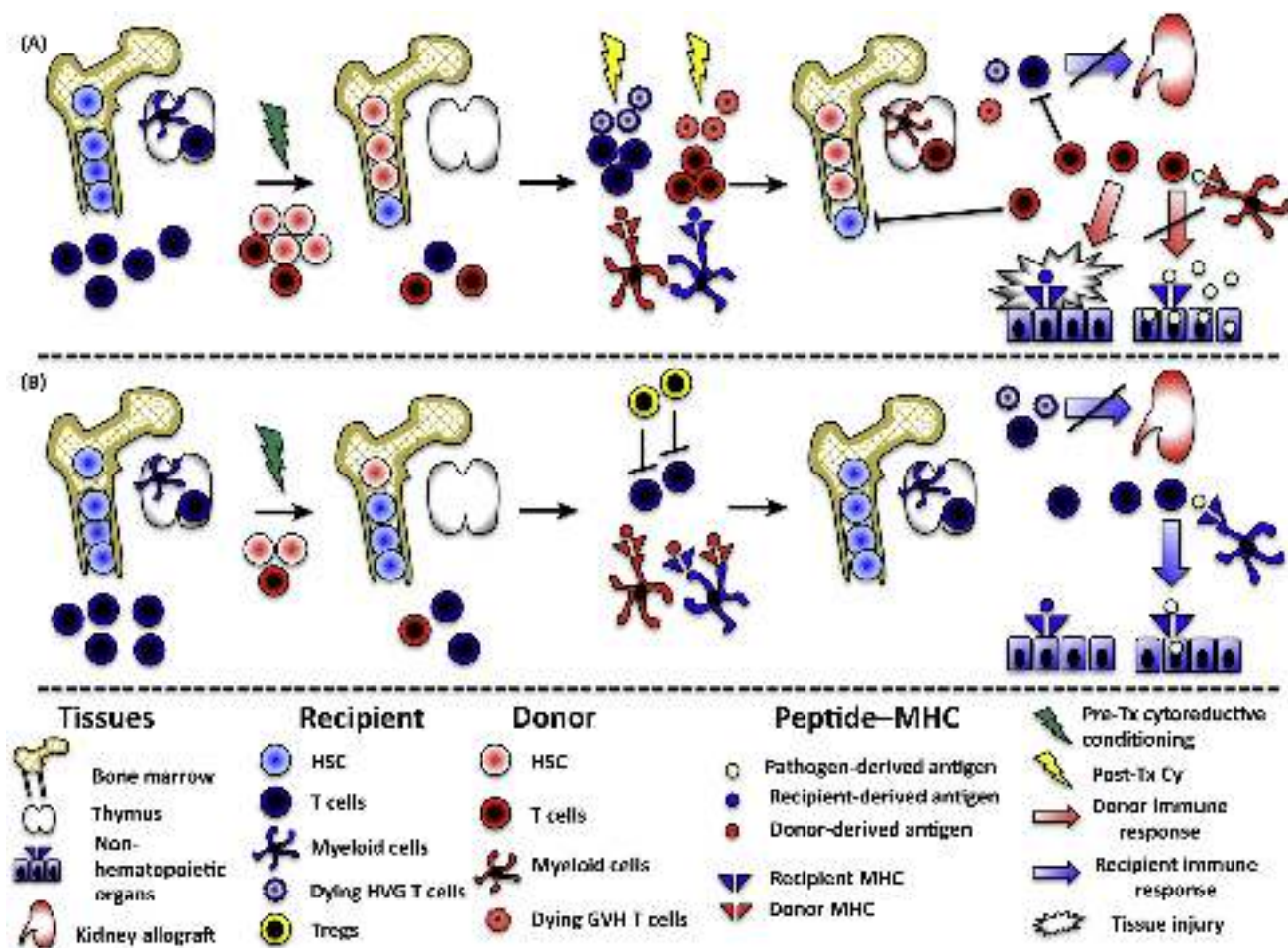


Figure 1. Mechanisms Involved in Clinical Chimerism-Based Regimens for Induction of Tolerance across HLA Barriers. (A) Sustained full chimerism. A large number of donor CD34⁺ hematopoietic stem cells (HSCs) (up to $17 \times 10^6/\text{kg}$) and donor T cells ($3.8 \times 10^6/\text{kg}$) plus 'facilitating cells' are administered to recipients along with the kidney allograft after non-myeloablative conditioning (green lightning symbol). Graft-versus-host (GVH) reactivity may drive the expansion of donor T cells that destroy recipient T cells and hematopoietic cells, creating HSC niche space in the bone marrow. The GVH response is partly attenuated by post-transplantation cyclophosphamide (Cy) treatment (yellow lightning). Durably engrafted donor HSCs supply the thymus with T cell precursors and dendritic cell progenitors that induce central donor-specific tolerance. These conditions allow sustained full chimerism. Donor-derived T cells can mediate GVH disease (GVHD) yet may fail to eliminate infectious organisms from the recipient's tissues, as post-transplantation pathogen-specific immune responses are restricted to donor MHC. (B) Transient mixed chimerism. Unfractionated bone marrow and kidney allograft are transplanted following non-myeloablative donor and recipient T cell-depleting induction (green lightning). Early regulatory T cell (Treg) expansion, driven by lymphopenia and relative Treg sparing by the conditioning regimen combined with the presence of donor antigen, prevents the activation of host-versus-graft (HVG)-reactive T cells and creates a microenvironment that supports peripheral deletion of donor-reactive T cells. Individuals with mixed or transient chimerism preserve efficient anti-infectious immune responses.

significant clinical GVHD in several cases, resulting in the death of one (Ildstad *et al.*, Abstract 450.1, 2016 Congress of The Transplantation Society, Hong Kong). In another CKHCT protocol, efforts to extend tolerance induction from the HLA-matched to the HLA-mismatched setting utilized dose escalation of donor T cells. While more sustained mixed chimerism in HLA haplotype-matched recipients of this protocol was achieved with a 50-fold increase in the donor T cell dose compared with that in HLA-matched recipients (50 versus 1×10^6 /kg), successful immunosuppression withdrawal has not yet been reported [15]. Collectively these studies emphasize that GVHR may serve as a potent contributor to the induction of durable chimerism but is associated with GVHD risk (Figure 1). Using an approach of *ex vivo* donor T cell depletion combined with *in vivo* recipient T cell depletion, we have previously obtained proof of principle in hematologic malignancy patients that durable mixed chimerism can be achieved across HLA haplotype barriers without relying on GVH reactivity [22]. However, the anti-CD2 monoclonal antibody (mAb) needed for this regimen subsequently became unavailable, two of four patients lost chimerism, and the protocol has not been evaluated in patients who had not undergone previous cytoreductive therapy for lymphoma. Thus, the reliable achievement of durable chimerism across HLA barriers without risk of GVHD in humans remains elusive. Given the ample demonstrations that lifelong mixed chimerism can be achieved without relying on GVH reactivity in animal models, we believe this remains an attainable and desirable goal in humans. As discussed below, Tregs have the potential to advance this goal.

Tolerance Mechanisms associated with Transient Mixed Chimerism

For clarity, tolerance mechanisms are discussed separately in this section, although growing evidence suggests that they are intimately interconnected. Indeed, the ultimate stage of T cell exhaustion is physical deletion [24], while recent data support the role of Tregs in promoting CD8⁺ T cell exhaustion [25]. Furthermore, apoptotic body uptake by phagocytic cells can induce local transforming growth factor beta (TGF- β) secretion, which in turn promotes the generation and intragraft expansion of Tregs [26].

Peripheral Deletion

Evidence for Peripheral Deletion in Clinical CKHCT Protocol

In mice achieving mixed chimerism and donor-specific tolerance with costimulation blockade, specific deletion of T cells expressing donor-reactive T cell receptors (TCRs) occurs in the periphery [27–30]. Moreover, in humans and in NHPs with transient chimerism and long-term tolerance after CKHCT, thymic deletion of donor-reactive T cells is unlikely to be the major mechanism of tolerance. However, proliferation and cytotoxicity assays demonstrate donor-specific hyporesponsiveness in these patients [1,6,31]. Functional evidence suggested that regulatory mechanisms played an early, but not a long-term, role in this systemic hyporesponsiveness to the donor [31]. Together these findings suggested that peripheral mechanisms control the antidonor response but did not indicate whether the clones persist as quiescent T cells (anergic, exhausted) or become progressively deleted. To address this important issue, we established a method for identifying and tracking the donor-reactive TCR repertoire [6]. HVG-reactive clones were identified via a pretransplantation mixed lymphocyte reaction (MLR), where recipient antidonor T cells are identified by dilution of the dye CFSE, followed by high-throughput sequencing of TCR β CDR3 regions and comparison with unstimulated pretransplantation T cells to identify MLR-expanded clones as donor-reactive. Predefined HVG and non-HVG clones (those that were detected in the unstimulated pool and did not expand in the MLR) could then be quantified in post-transplantation blood [6] and biopsy [7] samples. This method allowed the demonstration of gradual deletion of the clones with the strongest alloreactivity in tolerant patients, suggesting peripheral deletion as a significant tolerance mechanism (Figure 1). Importantly, such deletion did not occur in the one patient in whom tolerance induction failed and the graft was rejected on immunosuppression withdrawal [6]. Remarkably, this non-tolerant patient exhibited complete donor-specific unresponsiveness in

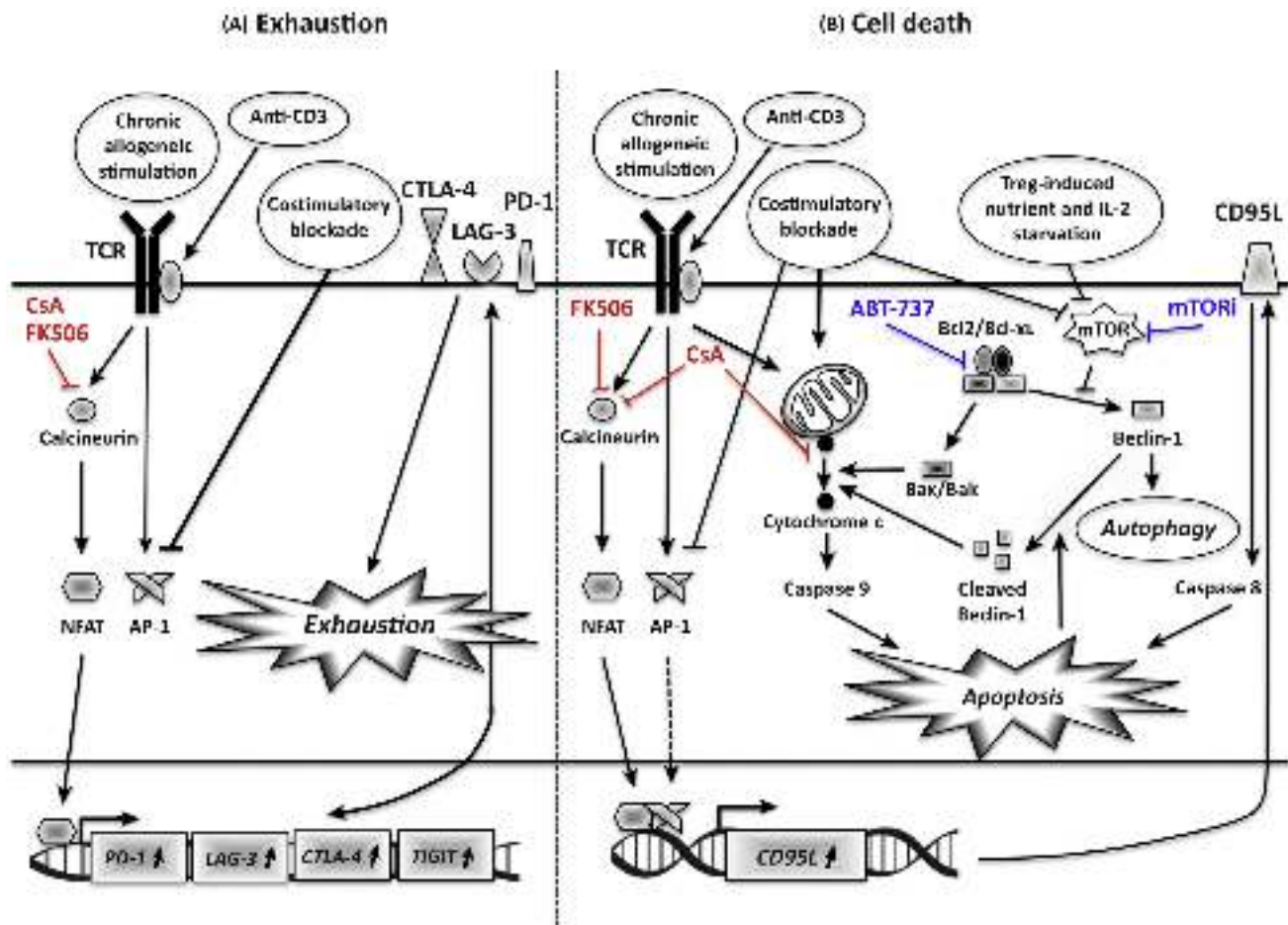
all *in vitro* assays, suggesting that the donor-specific T cells were 'anergic' under the conditions of these assays but that this anergy did not translate to a robustly tolerant state *in vivo*. These sobering findings reinforce the unreliability of functional *in vitro* assays in identifying a tolerant state and suggest that tracking of donor-specific TCRs may have the potential to distinguish tolerant from non-tolerant patients. This possibility deserves further exploration in view of the need for reliable predictors of 'spontaneous' allograft tolerance that would permit weaning off immunosuppression in appropriate patients without increasing the risk of graft rejection.

Apoptotic Pathways and Targeted Therapeutics

Deletion of donor-reactive T cells can occur at different stages of development and through different mechanisms. Understanding which mechanisms promote tolerance induction has important implications for the design of immunosuppressive drug combinations that might achieve this goal (Figure 2). While calcineurin inhibitors (CNIs), unlike mammalian target of rapamycin (mTOR) inhibitors, were shown to block **activation-induced cell death (AICD)** and thereby prevent tolerance induction in a costimulatory blockade-based tolerance model, this conclusion was drawn from experiments using one CNI, cyclosporine, but not tacrolimus [32]. Mice constitutively expressing Bcl-x_L were also shown to be resistant to induction of transplantation tolerance through costimulatory blockade [33] and to induction of mixed chimerism with costimulatory blockade [34]. Bcl-x_L and Bcl-2 hamper intrinsic cell death by maintaining mitochondrial integrity but provide little protection from the death receptor (FAS and TRAIL)-induced cell-extrinsic apoptosis pathway (often referred to as AICD) [35]. In a mixed chimerism-based model using costimulatory blockade, constitutive Bcl-x_L expression impaired the early deletion of donor-reactive T cells and prevented the induction of donor-specific tolerance [34], whereas FasL was dispensable for CD4 cell tolerance [36]. A Bcl-2/Bcl-x_L inhibitor (ABT-737), combined with costimulatory blockade and donor bone marrow cells, induces complete peripheral deletion of alloreactive T cells, allowing mixed chimerism induction without cytoreductive conditioning [37]. Furthermore, Bim, a key player in the **intrinsic apoptosis pathway**, is required for induction of mixed chimerism without lymphoablation [37]. Collectively these data suggest that the intrinsic apoptosis pathway may be more important than the extrinsic pathway in peripheral deletion in these murine models. Importantly, while both cyclosporine and tacrolimus interfere with calcineurin-dependent T cell activation, only cyclosporine and not tacrolimus inhibits the mitochondrial permeability transition pore, an end point of the intrinsic apoptotic pathway (Figure 2) [38]. Consistently, in pig models, tacrolimus was found to better facilitate the induction of tolerance than cyclosporine [39]. Cyclosporine derivatives (such as Debio-025 or NIM811) lacking anticalcineurin activity but inhibiting mitochondrial permeability could be useful in investigating the separate roles of the two apoptosis pathways.

Targeted Memory T Cell Depletion

Unlike laboratory mice [40], patients already harbor a large repertoire of memory T cells at the time of transplantation. **Heterologous immunity** and CD8 T cell crossreactivity constitute a significant barrier to immunologic tolerance in large outbred animals and humans. For example, up to 45% of anti-CMV T cell clones have been reported to be alloreactive [41]. Generally, achieving mixed chimerism in NHPs is far more challenging than in rodents, apparently due to the presence of abundant crossreactive memory T cells [42]. NHPs with the greatest frequency of pretransplantation donor-reactive memory T cells fail to become tolerant after non-myeloablative CKHCT [43]. In addition, viral or bacterial infections to which humans may be exposed can prevent or even break established tolerance following transplantation [44,45]. Memory T cells are less dependent on costimulatory signals and are more resistant to suppression by Tregs. Interestingly, the type of pathogen involved in the generation of crossreactive CD8⁺ memory T cells can determine the nature of the dominant memory T cell subset, whose susceptibility to costimulation/integrin blockade varies greatly [46]. Central memory T cells are



Trends in Immunology

Figure 2. Approaches to Inducing Exhaustion and Death of Host-Versus-Graft (HVG)-Reactive T Cells. (A) Prolonged inefficient antigenic stimulation or anti-CD3 therapy results in an altered balance between nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1), inducing an exhaustion program as defined by the expression of several inhibitory cell surface receptors, including programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3 (LAG-3), TIM3, TIGIT, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and a hyporesponsive state. Both cyclosporin A (CsA) and FK506 inhibit NFAT-dependent T cell exhaustion. (B) The two main apoptosis pathways, namely the extrinsic (caspase 8 dependent) and the intrinsic (caspase 9 dependent), involved in the peripheral deletion of HVG T cells following tolerance-inducing protocols are depicted. The complex crosstalk between autophagy and mitochondrial apoptosis is illustrated. Autophagy is initiated in response to metabolic cues (nutrient deprivation) and/or costimulatory blockade, both sensed by mammalian target of rapamycin (mTOR). If this process is insufficient, it triggers cytochrome c release and mitochondrial apoptosis. Furthermore, caspase activity hampers Beclin-1-dependent autophagy, fueling a positive amplification loop. Although both calcineurin inhibitors (CsA and FK506) block NFAT-dependent activation–cell death (extrinsic), CsA inhibits, unlike FK506, mitochondrial depolarization and cytochrome c release. By contrast, mitochondrial apoptosis is enhanced by mTOR and Bcl2/Bcl-x_L (ABT-737) inhibition. Abbreviation: mTORI, mTOR inhibition.

more sensitive [46], possibly because of lower Bcl-2 expression on cytokine stimulation, than effector memory T cells [47]. Attempts to eliminate donor-reactive memory T cells have yielded promising results in NHPs [48,49]. Both alefacept, a fusion protein that antagonizes LFA-3/CD2, and a humanized anti-CD8 mAb successfully restrain the expansion of memory T cells after kidney transplantation and allow tolerance induction via a delayed mixed chimerism protocol [48,49]. Also, the use of a Bcl-2/Bcl-x_L inhibitor, currently developed in clinical oncology, restores the efficiency of costimulation blockade in mice whose immune system has been primed by donor cells and allows mixed chimerism-based donor-specific tolerance across memory T cell barriers [47]. However, the model of adoptive transfer of memory T cells used in these studies may be less challenging than induction of tolerance in a native host with

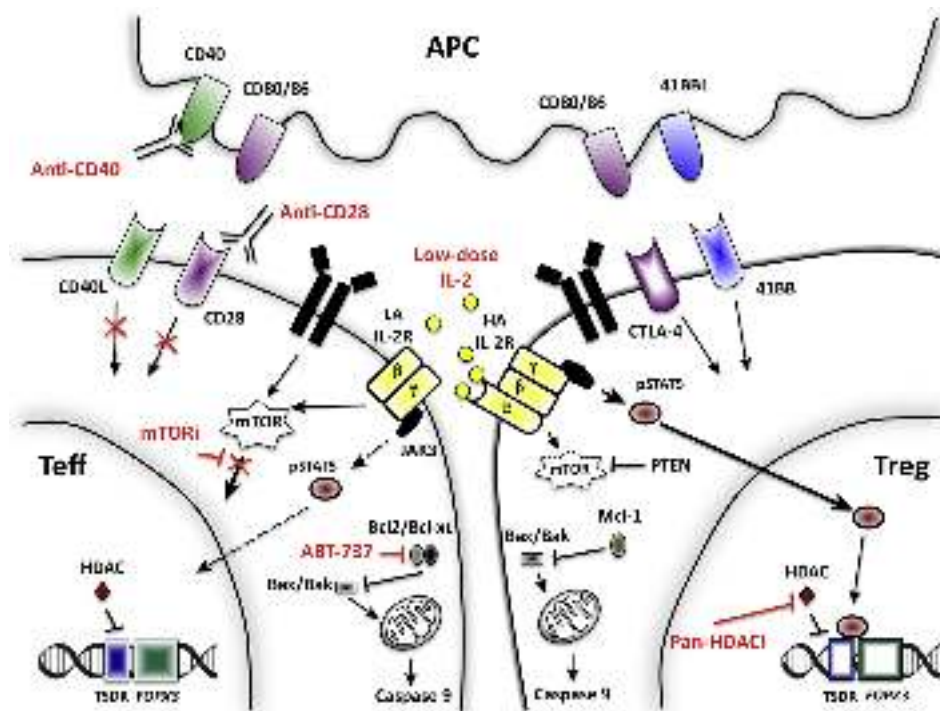
alloreactive memory T and B cells, in which induction of mixed chimerism is inhibited by B cell-dependent T cell memory, even after exhaustive T cell depletion and costimulatory blockade [50].

Skewing the Balance toward Regulation

A critical goal of tolerogenic protocols is to tip the balance toward suppression by targeting effector cells while sparing Tregs [51]. Recent studies demonstrate that Mcl-1 rather than Bcl-2 expression is critical for the survival and fitness of Foxp3-expressing Tregs, in contrast to naïve, effector, and memory T cells [52]. Building on this insight into murine Treg biology, Cippa *et al.* demonstrated that this differential effect of selective Bcl-2/Bcl-x_L inhibition creates a more favorable environment for Tregs, which are required for tolerance induction by ABT-737 (Figure 3) [53].

Energy and Exhaustion

Energy and T cell exhaustion are two states of qualitative impairment of T cell function [54]. The former may occur quickly after TCR stimulation when the integration of costimulatory signals



Trends in Immunology

Figure 3. Pharmacological and Biological Agents with Potential to Tip the Balance toward Tolerance. Low-dose IL-2 selectively activates high-affinity (HA) IL-2 receptor (IL-2R)-expressing regulatory T cells (Tregs) but has limited effect on effector T cells (Teffs). IL-2R signaling relies mostly on the Jak3–STAT5 pathway in Tregs. By contrast, the PI3K–Akt–mammalian target of rapamycin (mTOR) pathway is highly dispensable for the generation, expansion, and survival of Tregs but not of Teffs. Hence, mTOR inhibition (mTORi) favors Tregs over Teffs. Similarly, the critical role of the antiapoptotic Bcl2/Bcl-x_L genes in hindering mitochondrial apoptosis in Teffs but not Tregs makes the former more vulnerable to Bcl2/Bcl-x_L inhibition. Exposure of FOXP3⁺ Treg cells to pan-histone deacetylase (HDAC) inhibition (HDACi) promotes the stability and function of Tregs through acetylation of the FOXP3 gene. Tregs are unaffected by anti-CD40 antibody as they do not upregulate CD40L expression. Abbreviations: APC, antigen-presenting cell; LA IL-2R, low-affinity IL-2 receptor; NFAT, nuclear factor of activated T cells; PD-1, programmed cell death protein 1; PTEN, phosphatase and tensin homolog; TCR, T cell receptor; TSDR, Treg-specific demethylated region.

results in an inhibitory signal, leading to the inability of T cells to produce IL-2 or proliferate [54]. There are several forms of anergy, some of which may be reversed by exogenous IL-2. CD8⁺ T cell exhaustion requires chronic stimulation resulting from an overwhelming antigen load and/or prolonged exposure to an environment that is suboptimal for their response, such as lack of CD4⁺ T cell help and high levels of inhibitory signals [24,55]. The ability to reverse T cell exhaustion with checkpoint inhibitors has provided a major advance in cancer immunotherapy [54,56]. A handful of key transcription factors are involved in shaping the transcriptomic landscape of exhausted T cells, including eomesodermin (Eomes), T-box 21 (Tbet), PR domain zinc protein 1 (Blimp1), forkhead box O1 (Foxo1), nuclear factor of activated T cells (NFAT), and von Hippel–Lindau tumor suppressor (VHL). Interestingly, the core transcriptional program of exhausted CD8⁺ T cells only partly overlaps with that of exhausted CD4⁺ T cells [57]. T cell exhaustion has been identified as one of the main immune escape mechanisms in the settings of chronic infection and malignancies [24,55]. The limited studies of exhaustion in clinical transplantation include studies in HCV-infected liver transplant recipients [55]. Chronic exposure to alloantigens and the blunting of the immune response by immunotherapies provide the conditions that promote T cell exhaustion, which is therefore likely to be a mechanism involved in other tolerance settings [55].

Administration of IL-2 breaks transient mixed-chimerism-based tolerance in NHPs and leads to rapid allograft rejection. In this setting IL-2 disrupts peripheral tolerance, resulting in expansion of CD8⁺ alloreactive memory T cells [58]. This finding suggests that alloreactive T cells persist in the anergic (or suppressed) state and can be reactivated on IL-2 injection. Different forms of anergy are suggested by the observation of decreased but persisting donor-specific clones 2 years after transplantation in tolerant CKBMT patients [6]. These clones did not cause rejection, in contrast to those in a patient in whom tolerance induction failed and who showed no reduction in donor-reactive T cell clones following the transplantation yet showed donor-specific anergy under *in vitro* allostimulation conditions [6].

Studies in mice demonstrate that both CD4⁺ and CD8⁺ donor-reactive T cells from recipients of allogeneic BMT with anti-CD40L are donor-unresponsive before being deleted. Tolerance of CD8⁺ T cells may involve exhaustion, as multiple inhibitory pathways, including programmed cell death protein 1 (PD-1) [59], lymphocyte-activation gene 3 (LAG-3), cytotoxic T lymphocyte-associated protein 4 (CTLA-4), and TGF- β , are required, whereas several of these exhaustion-associated molecules are not required for CD4 tolerance [60]. Notably, in the same model NFAT activation is critical in tolerizing CD8 alloreactive T cells yet is completely dispensable for the induction of CD4 tolerance [61]. Recent data shed light on the molecular mechanisms linking costimulatory blockade, NFAT, and exhaustion in CD8⁺ T cells (Figure 2) [57]. Nuclear translocation and activation of NFAT controls T cell fate decisions in a manner that depends on complexes with other transcription factor partners, such as activator protein 1 (AP-1) [57]. TCR activation along with costimulation blockade profoundly unbalances the equilibrium between NFAT and AP-1, resulting in monomeric NFAT that preferentially activates a transcriptomic program driving T cell exhaustion (Figure 2) [57]. Together these results suggest that T cell exhaustion contributes to the state of CD8⁺ T cell hypofunction and deletion observed in the model of mixed chimerism induced by anti-CD40L blockade.

Reduced expression of the AP-1 complex was also noticed in tolerant T cells infiltrating islet allografts after anti-CD3 antibody [62]. Moreover, CD8⁺ T cells isolated from tolerated islet allografts failed to produce IFN γ in response to donor antigens [62]. Single-cell PCR of individual graft-infiltrating CD8⁺ T cells revealed high expression of the inhibitory receptors LAG-3, TGF- β -induced PD-1, and PDL-1 as well as increased expression of Eomes in tolerant compared with control CD8⁺ T cells [62], highly suggestive of T cell exhaustion [55]. Furthermore, downregulated expression of hypoxia-inducible factor alpha (HIF α) in tolerant CD8⁺

T cells [62] is aligned with the low metabolic requirement and the high expression of VHL in exhausted T cells [63]. Similar findings in type I diabetes patients treated with the anti-CD3 mAb teplizumab demonstrate the relevance of these new insights into peripheral CD8 tolerance [64]. A population of hypoproliferative EOMES⁺TIGIT⁺KLRG1⁺CD8⁺ T cells is readily identified in the blood of patients with the greatest response to anti-CD3 treatment [64].

Regulatory Cells

Tregs

Mounting evidence implicates FOXP3-expressing Tregs in clinical transplant tolerance induction. The rare kidney transplant recipients who spontaneously develop a state of operational tolerance exhibit an increased frequency of highly suppressive memory Tregs with a fully demethylated *FOXP3* Treg-demethylated region (TSDR), a hallmark epigenetic change of stable Tregs [65]. Similarly, marked enrichment of FOXP3-expressing Tregs during the early post-transplantation course has been observed following the non-myeloablative anti-CD2 mAb-based CKHCT protocol that leads to transient chimerism [31,66]. Consistently, a recent study suggested donor-specific FOXP3⁺ Treg expansion in tolerant NHPs receiving a T cell depletion/costimulatory blockade-based transient chimerism CKHCT regimen [67]. Mouse models have decisively established a causal link between accumulation of donor-reactive FOXP3⁺ Tregs and transplant tolerance through a variety of experimental approaches including targeted deletion of FOXP3-expressing Tregs, which disrupted well-established tolerance [68,69]. Importantly, Tregs have the capacity to elicit infectious tolerance, permitting the spread of tolerance toward a broad diversity of donor antigens [69].

Numerous strategies that promote Treg expansion or stability are being implemented in the clinic (Figure 3). Dramatic Treg expansion *in vivo* has been observed on low-dose subcutaneous IL-2 treatment in patients with chronic GVHD [70]. However, excessive IL-2 may activate alloreactive effector T cells (Teffs), preventing or breaking transplant tolerance, as recently described in NHPs [58]. In this respect, close monitoring of downstream targets of the IL-2 receptor (IL-2R) pathway (e.g., phosphorylated Stat5) in Tregs and Teffs may help in adjusting IL-2 doses [71]. Further, the development of an IL-2 mutant that fails to recruit proinflammatory cells expressing low-affinity IL2 receptor (i.e., comprising the $\beta\gamma$ IL-2R chains only) is currently underway and appears promising (patent n° US2014/0286898). Histone deacetylases (HDACs) play a critical role in regulating gene expression through epigenetic changes. Pan-HDAC inhibition in BMT patients was associated with a reduced rate of acute GVHD [72] along with marked enrichment of circulating Tregs with highly demethylated TSDRs [73]. HDAC-selective targeting (such as HDAC-6 or -9) has a safer toxicity profile and has yielded promising results in experimental transplantation models [74]. Moreover, HDAC inhibition elicits a synergistic effect with mTOR inhibition in promoting transplant tolerance [75]. The favorable effect of mTOR inhibitors on Treg generation and expansion has been extensively documented and reviewed elsewhere [76].

Costimulatory blockade targeting the CD40–CD40L and CD28–CD80–CD86 pathways has long been used to promote peripheral tolerance in rodent models and has significantly improved mixed chimerism-based tolerance in monkeys by reducing the need for cytoreduction [77]. Fc-silent forms of anti-CD40 (CFZ533) [78,79] and anti-CD40L (BMS-986004) [80] antibodies, which lack the side effects of the respective full-length antibodies (B lymphopenia and thromboembolic events), are currently being developed for clinical applications in transplantation. Growing evidence suggests that costimulation through CD40L is highly dispensable in human Tregs, in contrast to conventional T cells, and that CD40/CD40L blockade may tip the balance toward suppression. Gene expression profiles revealed marked downregulation of CD40L in human Tregs compared with non-Tregs [81]. Moreover, human Tregs fail to upregulate CD40L on activation, in contrast to conventional CD4⁺ T cells [82]. Also of interest,

blocking anti-CD28 antibody treatment was associated with an increased Treg frequency in large-animal models [83] and was extremely potent in preventing alloantibody development by blocking follicular T helper cell activation [84] while preserving CTLA-4-dependent T follicular regulatory T cell function [85].

As an alternative approach to *in vivo* Treg promotion, Tregs can be manufactured as *ex vivo* cellular products for tolerance purposes. However, Treg cell therapy according to Good Manufacturing Practice (GMP) faces several challenges, including the isolation of a pure population that remains stable on expansion, the issue of antigen specificity, and the number of cells required to obtain a therapeutic effect [86]. Concerns about Treg stability [87,88] have converged on the viewpoint that Tregs can be very stable provided they are fully committed, according to the so called 'heterogeneity model' [89]. Consistently, 1 year after the infusion of deuterium-labeled highly purified CD4⁺CD25^{high}CD127^{low} Tregs in humans, deuterium was detected only within the Treg compartment, demonstrating lack of leakage into other lineages [90]. Polyclonal Treg infusion has been implemented in clinical trials as GVHD prophylaxis (<http://clinicaltrials.gov>; NCT00602693) and in new-onset type 1 diabetes patients (<http://clinicaltrials.gov>; NCT01210664), with excellent safety profiles [90–92]. Both polyclonal and donor-specific Treg infusions are being evaluated in several clinical organ transplant trials (reviewed in [86]). The addition of polyclonal Tregs to donor bone marrow allowed the achievement of stable chimerism and tolerance in the absence of cytoreductive conditioning in a rodent model [93]. In efforts to convert a transient chimerism protocol to durable chimerism without relying on GVH reactivity in a preclinical model, we are exploring the addition of expanded polyclonal recipient Treg infusion in NHPs and have observed prolongation of mixed chimerism in association with more robust allograft tolerance. Whereas the original NHP transient chimerism CKHCT protocol relies on the presence of a donor kidney in the early post-transplantation period to promote tolerance, the prolonged chimerism achieved by the addition of expanded recipient Tregs allows the acceptance of a donor kidney grafted as late as 4 months after the BMT, indicating a more robust form of tolerance than that induced by BMT alone [94]. The addition of donor-reactive Treg-enriched recipient cells to liver transplants dramatically increased the success rate of immunosuppressive drug weaning in a pilot study (clinical trial registration number: UMIN-000015789) [95]. Allospecific Tregs offer several advantages over polyclonal Tregs, including greater efficiency in preventing rejection [96] and less potential for toxicity due to global immunosuppression. However, the very low frequency of allospecific Tregs represents a substantial barrier for clinical translation [96]. To tackle this challenge, a recent study used allogeneic HLA-targeting chimeric antigen receptors to redirect human Treg specificity toward the allograft, with promising preclinical results [97].

Regulatory B Cells

A B cell-associated gene expression and flow cytometric signature was identified in two different cohorts of spontaneously operational tolerant kidney transplant recipients and in CKHCT recipients in whom mixed chimerism-based tolerance was successfully achieved [98]. However, these initial studies did not control for the fact that tolerant patients were off immunosuppression while non-tolerant subjects were treated with drugs that could have reduced B cell numbers and/or function. A recent reanalysis revealed that much of this signature was indeed attributable to immunosuppressive drugs used in the non-tolerant subjects [99]. However, consistent with a possible role for B cells in tolerance, B cells from tolerant patients produce IL-10, express inhibitory receptors [100], and restrain T cell proliferation in a granzyme B-dependent manner [101]. In the murine BMT/anti-CD154 model, recipient B cells were required for the tolerization of recipient peripheral CD8 T cells, but this effect did not require IL-10 production by or APC function of the B cells [29,102], suggesting that B cells promote tolerance through additional, unknown mechanisms.

Intrakidney Graft Immunomodulatory Mechanisms

Studies attempting to induce tolerance to various organs via transient mixed chimerism have emphasized the critical role of the kidney allograft itself [42]. The presence of a cotransplanted kidney allograft was found to be required for long-term tolerance to heart allografts following transient chimerism induction in large-animal models [42]. The critical role of the kidney was compellingly demonstrated by the prompt rejection of cotransplanted heart after kidney allograft removal [42]. Moreover, the retransplantation of a tolerated class I MHC-mismatched kidney allograft plus lymphocytes from the tolerant animal leads to acceptance of a donor-matched naïve kidney allograft with systemic donor-specific hyporesponsiveness [103,104]. This 'kidney effect' might be related to its ability to establish an immunologic environment conducive to tolerance. TGF- β and indoleamine 2,3-dioxygenase are abundantly produced by renal parenchymal cells. Furthermore, the PD1 ligand PDL-1, whose role is increasingly evident in transplant immunology [105], is strongly inducible by tubular epithelial cells on alloimmune attack [106]. This finding may explain the observation that T cells from CKHCT patients were unresponsive to donor renal tubular epithelial cells [107] and is reminiscent of the instrumental role of the PD1–PDL1 axis in spontaneous liver allograft acceptance in mice [108]. Moreover, accumulation of FOXP3⁺ Tregs in tolerated grafts has been extensively reported in mice [62,109], and the graft itself was found to be a major site for suppression [69]. Similarly, FOXP3⁺ T cells are detected in biopsies from tolerant CKHCT recipients with transient chimerism [1,66].

Concluding Remarks

Two recent studies have provided proof of concept that transplant tolerance can be induced in humans across MHC barriers through CKHCT. However, the safety profile of a regimen that is associated with full donor chimerism raises concerns. In the other protocol, which is associated with transient chimerism, avoidance of an early engraftment syndrome (i.e., capillary leak syndrome occurring at the time of initial hematopoietic recovery) is a desirable goal. With this method, approaches to making chimerism more durable and less dependent on the kidney itself would allow extension of tolerance to less tolerogenic organs. Ongoing clinical and preclinical studies are moving toward the achievement of both of these goals. Preclinical studies suggest several promising approaches for enhancing peripheral tolerance via deletion, exhaustion, and suppression that may allow further reductions in the need for lymphoablation and cytoreduction in future protocols. Murine studies demonstrating the potency of multiple parallel tolerance mechanisms in promoting robust transplant tolerance [110], and particularly the combination of central deletion and regulation that is achieved with incomplete lymphodepletion and Treg-promoting mixed chimerism regimens [30,111], are useful in guiding these approaches. Together, major new insights into transplant tolerance mechanisms along with the emergence of new immunotherapies and tools for immune monitoring open new avenues for the development of clinical tolerance protocols with reduced risks and greater efficacy (see Outstanding Questions).

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Outstanding Questions

Does the ability of an immune system to be reset for transplant tolerance depend on the recipient's age (thymus output, repertoire diversity, immune senescence)?

Is there a level of heterologous memory T cell response that could not be overcome by targeted lymphodepletion?

Is anti-HLA presensitization an insurmountable hurdle to the induction of mixed chimerism-based tolerance?

Can recurrent autoimmunity be prevented by tolerance regimens that allow allograft acceptance in patients with underlying autoimmune disease?

Can clinical transplant tolerance resist immune-triggering events such as infections and vaccinations?

Can new drugs and cells being used in rodent models be extended to achieve robust tolerance in primates?

Can Tregs be used in combination with minimal host conditioning and donor hematopoietic cell transplantation to achieve durable chimerism without a GVH response in humans?

Would thymus transplantation be sufficient to induce tolerance toward donor antigens recognized through both direct and indirect presentation pathways?

Can alloreactive TCR repertoire tracking provide a biomarker for tolerance and rejection?

What is the role of direct versus semi-direct versus indirect alloreactivity in late graft loss?

Since the prevention of donor-specific antibody generation requires tolerization of indirect allorecognition, reliable tools are needed to monitor this response.

Can exosomes that promote semidirect allorecognition participate in tolerance induction?

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