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Transplantation tolerance
Clinical potential of regulatory T cells

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The major challenge in transplantation medicine remains long-term allograft acceptance, with preserved allograft function under minimal chronic immunosuppression. To safely achieve the goal of sustained donor-specific T and B cell non-responsiveness, research efforts are now focusing on therapies based on cell subsets with regulatory properties. In particular the transfusion of human regulatory T cells (Treg) is currently being evaluated in phase I/II clinical trials for the treatment of graft versus host disease following hematopoietic stem cell transplantation, and is also under consideration for solid organ transplantation. The purpose of this review is to recapitulate current knowledge on naturally occurring as well as induced human Treg, with emphasis on their specific phenotype, suppressive function and how these cells can be manipulated in vitro and/or in vivo for therapeutic purposes in transplantation medicine. We highlight the potential but also possible limitations of Treg-based strategies to promote long-term allograft survival. It is evident that the bench-to-beside translation of these protocols still requires further understanding of Treg biology. Nevertheless, current data already suggest that Treg therapy alone will not be sufficient and needs to be combined with other immunomodulatory approaches in order to induce allograft tolerance.

Introduction

Following on the pioneering work of Medawar and colleagues more than 50 years ago, extensive data obtained in rodents and large animal experimental transplantation models have led to a better understanding of the mechanisms leading to graft rejection and transplantation tolerance.[1,2] The advent of powerful immunosuppressive drugs that can control the rejection process since the mid 1980s has allowed the development of clinical transplantation with increasing success. Consequently, solid organ transplantation (SOT) has become the therapy of choice for end-stage organ diseases. Patient and allograft survival, as well as rates of acute allograft rejection episodes in the first year after transplantation have steadily improved. In contrast,

concerns remain on the long-term outcome. Besides morbidity and mortality associated with chronic immune suppression and drug exposure, there is an inexorable loss of transplanted organs due to chronic allograft dysfunction.[3,4] This process is mediated by both immunological (inadequate immunosuppression) and non-immunological (drug-related toxicity) factors.[5] Thus, the ultimate goal in transplantation medicine remains the induction of specific tolerance to donor alloantigens and long-term graft acceptance with minimal immunosuppressive drug exposure. In this perspective, the development of tolerogenic protocols and in particular of regulatory cell-based therapies has stimulated much interest in recent years. In this review, we discuss the mechanisms of immune tolerance to self-antigens and how they could be exploited in the transplantation setting. In particular we scrutinize current knowledge on regulatory T cells (Treg) and their potential for cell-based immunotherapy in SOT.

Central and Peripheral Tolerance

The recognition of the allograft major histocompatibility complex (MHC)-mismatched antigens by circulating alloreactive T cells is the primary event that ultimately leads to graft rejection. T cells are therefore the main target of current immunomodulatory strategies. Transplantation tolerance is defined by a state of sustained donor-specific T and B cell non-responsiveness with preserved graft function, with no (operational tolerance) or only minimal (near-tolerance) chronic immunosuppressive drugs. The immune system is capable of distinguishing between self- and non-self antigens, leading to specific protective cell-mediated and humoral responses in the absence of autoimmunity. Several different experimental protocols were successful in inducing transplant tolerance to in rodents and large animals, demonstrating the possibility to exploit the mechanisms that normally maintain immune homeostasis and tolerance to self-antigens to induce tolerance to alloantigens. Furthermore, clinical reports of occasional "tolerant" recipients who had stopped taking their immunosuppressive drugs but still experienced prolonged allograft survival, suggest that immunological tolerance may indeed be a goal that can be achieved in transplantation medicine.[6-9]

Immune tolerance is principally mediated via central and peripheral mechanisms. Central tolerance normally leads to the intrathymic deletion of T cells recognizing thymus-expressed autoantigens with high avidity, so that potentially deleterious

antigen-reactive T cells will not reach the periphery. Since the early observations by Medawar and colleagues,[1,10] a large number of experimental as well as clinical studies have confirmed that central tolerance against alloantigens can be achieved in transplant recipients by the induction of full (donor cells reconstituting recipient's hematopoietic compartment) or mixed (donor cells coexisting with recipient cells) hematopoietic chimerism.[11-16] In these settings, cells of donor origin, either spontaneously released by the allograft (liver transplantation) or infused in therapeutic protocols (hematopoietic stem cells, HSCT or bone marrow transplantation, BMT), can migrate to the recipient's thymus and induce clonal deletion of donor-reactive host T cells. However, to achieve donor cell engraftment and full or even mixed hematopoietic chimerism in recipients with a fully functional immune system, some degree of cytoreductive conditioning is needed, unless very high doses of donor stem cells or BM are administered. Thus, these approaches are relatively toxic and not easily feasible, precluding their application in routine clinical practice. Furthermore, although encouraging data have been obtained in pre-clinical studies (non-human primates, pigs),[17,18] and some small trials in humans,[14-16] in most cases mixed chimerism-based strategies have been difficult to translate successfully to large animal models. This indicates that central deletion may not be the only mechanism involved in the induction of robust tolerance in humans.

As not all antigens are expressed in the thymus (for instance tissue-specific antigens) and therefore evade central tolerance, peripheral tolerance usually prevents auto-immunity by various mechanisms. Peripheral tolerance to self- and non-self antigens can be achieved by deletion of activated T cells, T cell anergy and active regulation.[19] The encounter of naive T cells with antigen-presenting cells (APC), mainly DC, modulates their differentiation into various subsets of polarized effector and regulatory T cells and is a major component in the nature of T-cell responsiveness. In particular, the type of DC and the local cytokine microenvironment determine the outcome of the immune response towards immunity or tolerance. Thus, alloreactive effector T cells could be either controlled by other cells with regulatory/suppressive functions or by modulating the immunogenicity of APC and the local cytokine milieu.

Increasing evidence suggest the existence of interactions between central and peripheral mechanisms to promote the induction and maintenance of transplantation tolerance. Indeed, the thymus is not only responsible for clonal deletion of autoreactive T cells but also for active peripheral regulation through the generation of natural Treg. Moreover, peripheral DC can migrate to the thymus to present donor-derived antigens and therefore participate in the education of the immune system towards alloantigens and the induction of central tolerance.[20]

Regulatory T Cells

Regulatory cells are defined by their functional ability to suppress immune responses. This concept was initially proposed following the observation in experimental transplantation models that donor-specific transplantation tolerance could be transferred to

a new naïve host via the adoptive transfer of cells. In the 1970s, Gershon first described thymus-derived cells in mice that suppressed the response to an antigenic challenge with the injection of sheep red blood cells. He then developed the concepts of "infectious tolerance" and "suppressor cells".[21] However, due to poor experimental reproducibility in vitro, the "suppressor cells" fell into disrepute, although transferable tolerance remained a robust concept in vivo.[22] Convincing evidence for the existence of Treg came in the mid 1980s from experiments in rodent models of autoimmune disease.[23-25] Further experiments by Sakaguchi and colleagues allowed the identification of a subset of naturally occurring CD4+ T cells with constitutive expression of the IL-2 receptor α-chain (CD25) that was essential for the prevention of autoimmunity.[26] Since these early data, a huge body of literature has accumulated showing that CD4+CD25+ Treg are involved in the control of a wide variety of immune responses. Besides the naturally occurring CD4+CD25+ Treg that have been identified in non-manipulated animals and humans, uncommitted naïve CD4+ T cells can be skewed towards T cells with regulatory functions in the peripheral immune system under specific in vitro or in vivo conditions.

Foxp3+ T Regulatory Cells

Naturally occurring CD4+CD25+Foxp3+ Treg (nTreg) derive from the thymus and constitute 3–10% of the naïve peripheral CD4+ T cell population in humans. As CD25 is also upregulated on the surface of activated effector T cells, other specific markers are needed to identify nTreg. To date, the best marker of nTreg is the intracellular expression of the transcription factor forkhead box P3 (Foxp3). Foxp3, the cytokine IL-2 as well as CD25 as a component of the IL-2 receptor, are essential for the development, function and survival of nTreg, because mutations or polymorphisms in the genes encoding these molecules predispose to autoimmune or lymphoproliferative diseases.[27-30] Furthermore, the specific role of Foxp3 in the function of nTreg was highlighted by the fact that retroviral transduction of *Foxp3* into CD4+CD25- T cells converts them to functional Treg that are able to suppress proliferation of other T cells in vitro and inhibit the development of autoimmune diseases mediated by pathogenic effector T cells in in vivo experimental models.[31] It was also shown that sustained Foxp3 expression is required to confer the suppressive capacity of Treg. While Foxp3 expression constitutes a lineage specification of bonafide nTreg in mice, human activated effector T cells can transiently express Foxp3.[32,33] Thus, more specific markers for human nTreg are still sought-after and the precise molecular role of Foxp3 in nTreg remains to be fully understood.

Human naturally occurring Treg. As Foxp3 expression is not a reliable phenotype in human nTreg and cannot be used as a selection marker to isolate living cells due to its intracellular expression, numerous surface markers have been identified to help discriminate between activated effector T cells and nTreg. These include CD127, CD45RA/RO, inducible costimulatory protein (ICOS) and HLA class II. The IL-7 receptor α-chain (CD127) was shown to be downregulated on human peripheral nTreg and the combined use of CD4+, CD25+ and

Table 1. Generation of Foxp3^{+} iTreg

	Positive factors	Negative factors
Cytokines	TGFβ,[45] IL-2,[95] leukemia inhibitoy factor (LIF)[96]	IL-4, IFNγ, IL-21, IL-6.[96,97]
Costimulation	PD-1/PD-L1.[98]	OX40,[99] T cell-immunoglobuline mucin protein-1 (Tim-1)[100]
Proliferation	Rapamycin (mTOR inhibitor)[101,102]	PI3K-AKT pathway activation (mTOR activation)[101,102]
Others	Retinoic acid,[103] IDO,[104,105] activation of the aryl hydrocarbon receptor[106]	

Superscript numbers are references.

CD127^{low} markers results in a highly purified population of suppressive cells, as opposed to CD4^{+}CD25^{+}CD127^{hi} T cells that have been associated with pathogenic antigen-specific immune responses including chronic allograft rejection.[34,35] It appears that human nTreg display at least two different states of activation: resting/naive (CD45RA^{+}Foxp3^{low}) and activated/differentiated (CD45RA^{-}CD45RO^{+}Foxp3^{hi}).[36,37] The proportion of these subpopulations differs between cord blood and adult peripheral blood, and in patients with immunological diseases. Activated/differentiated CD45RO^{+} nTreg have been described to be mainly ICOS positive whereas resting nTreg can be ICOS positive or negative. ICOS is a T cell costimulatory receptor and an activation marker and appears to define functionally distinct nTreg populations: ICOS[+] nTreg produce IL-10 whereas ICOS^{-} nTreg mediate suppression predominantly via transforming growth factorβ (TGFβ).[38] While HLA class II expression on CD25^{hi} nTreg doesn't fully match ICOS or CD45RO, it was associated with a functionally distinct subset of "terminally activated" Treg. To date, the link between resting, activated, terminally activated and possibly memory nTreg remains unclear.[39]

Induced Treg. Induced or adaptive Treg (iTreg) can be generated from naive T cells in vitro or induced in the periphery in vivo independently from thymic selection.[40,41] Two main subtypes of CD4^{+} iTreg have been described: Tr1 cells producing IL-10 and TGFβ induced Foxp3^{+} iTreg. Tr1 cells are defined by their signature suppressive cytokine IL-10 but can transiently upregulate Foxp3 expression upon activation. They can be generated in vitro or in vivo by repeated antigenic stimulation in the presence of IL-10 and IFNα. Tr1 cells exert suppression mainly via the production of IL-10 and to a lesser degree by TGFβ secretion, as well as by modulating DC activation and cytokine production.[42] Tr1 and nTreg might synergize to control alloresponses as nTreg can induce naive T cells to differentiate into Tr1 cells in vitro in the presence of allogeneic DC.[43] In the initial experiments by Groux et al. Tr1 cells were able to suppress the development of colitis in SCID mice when co-transferred with CD4^{+}CD45RB^{hi} T cells.[44] Subsequently, it was shown that Tr1 cells were also involved in the regulation of immune responses in transplantation, autoimmunity, inflammation and tumor progression.[42]

In the presence of TGFβ, in vitro TCR-mediated stimulation of peripheral CD4^{+}CD25^{-} naive T cells was shown to generate CD4^{+}CD25^{+}Foxp3^{+} T cells with all the phenotypical and functional characteristics of nTreg.[45,46] In vivo, Foxp3^{+} iTreg could also be generated from naive T cells in the periphery with TGFβ playing a pivotal role.[40,41,47] It is unclear at present whether Foxp3^{+} iTreg differ from TGFβ-secreting Th3 cells previously described

in oral tolerance protocols.[48] Various positive and negative stimuli were reported to influence the induction of Foxp3 expression in CD4^{+} T cells (**Table 1**). However, in contrast to nTreg, Foxp3 expression in iTreg seems to be less stable as reflected by their histone methylation, acetylation and microRNA status.[49-51] The in vitro regulation of Foxp3 in iTreg as well as their dynamics after transfer in vivo has been analyzed.[52] Removal of TGFβ in vitro led to a loss of Foxp3 expression after a few days and, after adoptive transfer into wild-type mice most iTreg downregulated Foxp3 within 2 days only. Some studies have suggested that while TCR stimulation of human CD4^{+}CD25^{-} T cells in the presence of TGFβ induces high levels of stable Foxp3 expression, these iTreg are neither anergic nor suppressive and produce effector cytokines. Furthermore, while the presence of regulatory cytokines such as TGFβ and antigen-presentation by immature DC was shown to favor the generation of antigen-specific iTreg in vitro and in vivo, in a pro-inflammatory environment naïve T cells would rather differentiate into Th17 cells as suggested by recent data.[53-56] Thus, in vitro differentiated human iTreg might not be stable phenotypically and functionally, implying that in vivo transfer of iTreg for therapeutic purposes may give unexpected results and should be considered with caution.

Mechanisms of Suppression

There are numerous mechanisms of nTreg-mediated suppression that can be mainly subdivided into two categories: dependent on cell-cell contact and/or mediated by cytokines. The respective relevance of theses mechanisms are still a matter of debate which might be explained by differences in the experimental systems (in vitro vs. in vivo, rodent vs. human cells) and disease models that have been used. In vitro, nTreg were shown to inhibit the activation of effector CD4^{+}CD25^{-} T cells predominantly by cell-cell contact dependent mechanisms. Naturally occurring Treg constitutively express on their surface important molecules for their suppressive function such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), membrane-bound TGFβ latency-associated peptide (LAP), glucocorticoid induced tumor necrosis factor receptor (GITR), CD4-related lymphocyte activation gene 3 (LAG-3), galectin-1 and CD39.[57-63] Moreover, after activation, human nTreg were shown to be able to directly kill CD4^{+} and CD8^{+} T cells via the secretion of perforin and granzyme B.[64] The role of regulatory cytokines such as IL-10 a TGFβ and more recently IL-35 in nTreg-mediated suppression of immune pathologies has mainly been described in in vivo experimental models.[58,65,66] While the effector mechanisms of nTreg is still

debated, there is less controversy, at least in in vitro models, on the resulting effect on the responding co-cultured CD4⁺CD25⁻ T cells, namely inhibition of the transcription of IL-2.[67] IL-2 is key in nTreg homeostasis as these cells are highly dependent on exogenous IL-2 for growth in vitro and in vivo. Experiments using transgenic mice have demonstrated that IL-2, although dispensable for nTreg development, was essential for their peripheral maintenance and competitive fitness.[68,69] Different groups have also highlighted that IL-2 production by activated effector T cells was responsible for the maintenance of the peripheral pool of nTreg in vivo.[70,71] Moreover, there is evidence for IL-2 "sequestration" by nTreg in the regulation of T-cell responses in vivo, i.e., nTreg would compete with activated effector T cells for the available IL-2 to maintain their peripheral pool.[72,73] Collectively these data suggest the presence of an autoregulatory loop during immune responses where nTreg respond and expand via IL-2 to the inflammation that they regulate.[74] However, it remains unclear which stimuli trigger nTreg suppression in vivo. It was originally thought to be dependent on specific TCR activation[75] but recent in vitro experiments using cells from TCR-transgenic mice unravelled TCR-independent mediated suppression.[76]

Besides CD4⁺CD25⁻ and CD8⁺ T cells, nTreg can modulate the effector function of other immune cells including DC, monocytes and B cells as well as NK cells.[77-80] By their constitutive surface expression of CTLA-4, nTreg can downregulate CD80/CD86 on APC and induce the expression of indoleamine-2,3-dioxygenase (IDO) in DC. IDO exerts immunomodulatory effects through multiple mechanisms including depletion of tryptophan (which is required for normal T cell functions) and production of tryptophan-derived catabolites that promote T and NK cell proliferative arrest and anergy. IDO-expressing immunoregulatory DC have been shown to promote immune tolerance, including in transplantation experimental models.[81,82] Thus, by inhibiting the immunogenicity of other effector cells, nTreg could favor a tolerogenic environment that could promote the development of iTreg, contributing to the maintenance of tolerance (a phenomenon referred to as "infectious tolerance").[83] Altogether, the crosstalk between nTreg and alloantigen-presenting DC is important in determining the outcome of the immune response, tipping the balance in favor of regulation rather than immunity (**Fig. 1**).[84-86] Besides regulating APC function, previous in vitro and in vivo studies also reported a potential role of nTreg in inhibiting NK cell effector functions and innate immunity. This was mediated by nTreg membrane-bound TGFβ which downregulated NKG2D receptors on the NK cell surface.[87]

Treg-Based Immunotherapy in Transplantation

Approaches to treat autoimmunity and to prevent allograft rejection have focused historically on potent immunosuppressive drugs that block the activation and expansion of pathogenic effector T cells. Although very potent, currently available drugs require chronic administration associated with toxicities and the impairment of protective immune responses against pathogens or tumors. The great progresses in our understanding of the basic processes that control immune tolerance as well as more recent

characterization of professional nTreg open the door to their therapeutic application, either by enhancing their activity in autoimmune diseases, allograft rejection and graft versus host disease (GVHD), or by blocking their suppressive activity in tumor immunity and in vaccine development. In the transplantation setting, there is evidence that in many experimental protocols where robust peripheral tolerance can be achieved, immunoregulatory mechanisms dependent on donor-specific Treg are critical in the induction and maintenance of the tolerant state.

The possibility of using nTreg in immunotherapeutic protocols is however mainly limited by cell number. Indeed, the peripheral pool of nTreg only accounts for a small proportion of peripheral T cells in healthy individuals. For efficient suppression of alloreactive T cell responses, the pool of Treg needs to be expanded to obtain a peripheral Treg/T effector cells ratio that favors regulation.[19]

Expansion strategies. Currently, three main approaches are being explored for Treg expansion in the perspective of therapeutic protocols: ex-vivo nTreg expansion, ex-vivo conversion of naïve T cells to iTreg and in vivo expansion of nTreg and/or induction of iTreg. The first method requires selection of highly purified nTreg prior to in vitro expansion for subsequent adoptive transfer. Purity is a critical issue as even a few contaminating effector T cells might expand in vivo and cause unwanted immune pathologies. As discussed, different surface markers have to be combined to purify human nTreg from the peripheral blood, including CD25ʰⁱ and CD127ˡᵒʷ expression, CD45RA⁺, CD27, CD39, CD49b, folate receptor 4 (FR4) or PD-1.[88-91] Good manufacturing practice (GMP) accepted isolation strategies are based on CliniMACS (Miltenyi®) protocols, using antibody cocktails with magnetic microbeads and columns. However, these immunomagnetic techniques do not allow the same broad multiparameter selection as compared to flow cytometry cell sorting. Thus, these approaches may lead to poor nTreg purity and still need to be optimized. Once selected, nTreg have to be expanded to the yields needed for clinical application and transfer into patients. We and others have described robust protocols to expand nTreg in vitro in great numbers without loss of their suppressive function.[92-94] In brief, these strategies are based on the use of donor-derived APC, recipient-derived APC pulsed with donor antigens or surrogate APC (such as anti-CD3/CD28 coated beads) in the presence of high amounts of exogenous IL-2.

The second approach is based on the induction of iTreg in vitro from naïve CD4⁺ T cells as described in **Table 1** or by forcing *Foxp3* expression by viral transduction.[31,95-107] Finally, the third strategy consists in expanding nTreg and/or de novo generation of iTreg in vivo. This would alleviate the need for GMP cell isolation and cumbersome ex-vivo manipulations, thus rendering the therapy more clinically applicable. Blocking the T cell costimulatory signaling pathways (CD28:CD80/CD86, CD154:CD40, OX40:OX40L, ICOS:ICOSL, CD27:CD70) at the time of transplantation and donor-antigen encounter has been shown to facilitate donor-specific iTreg conversion and/or preferential proliferation of nTreg, while inducing anergy of alloantigen-specific effector T cells.[108] Current studies suggest that effector T cells and nTreg have qualitative and quantitative
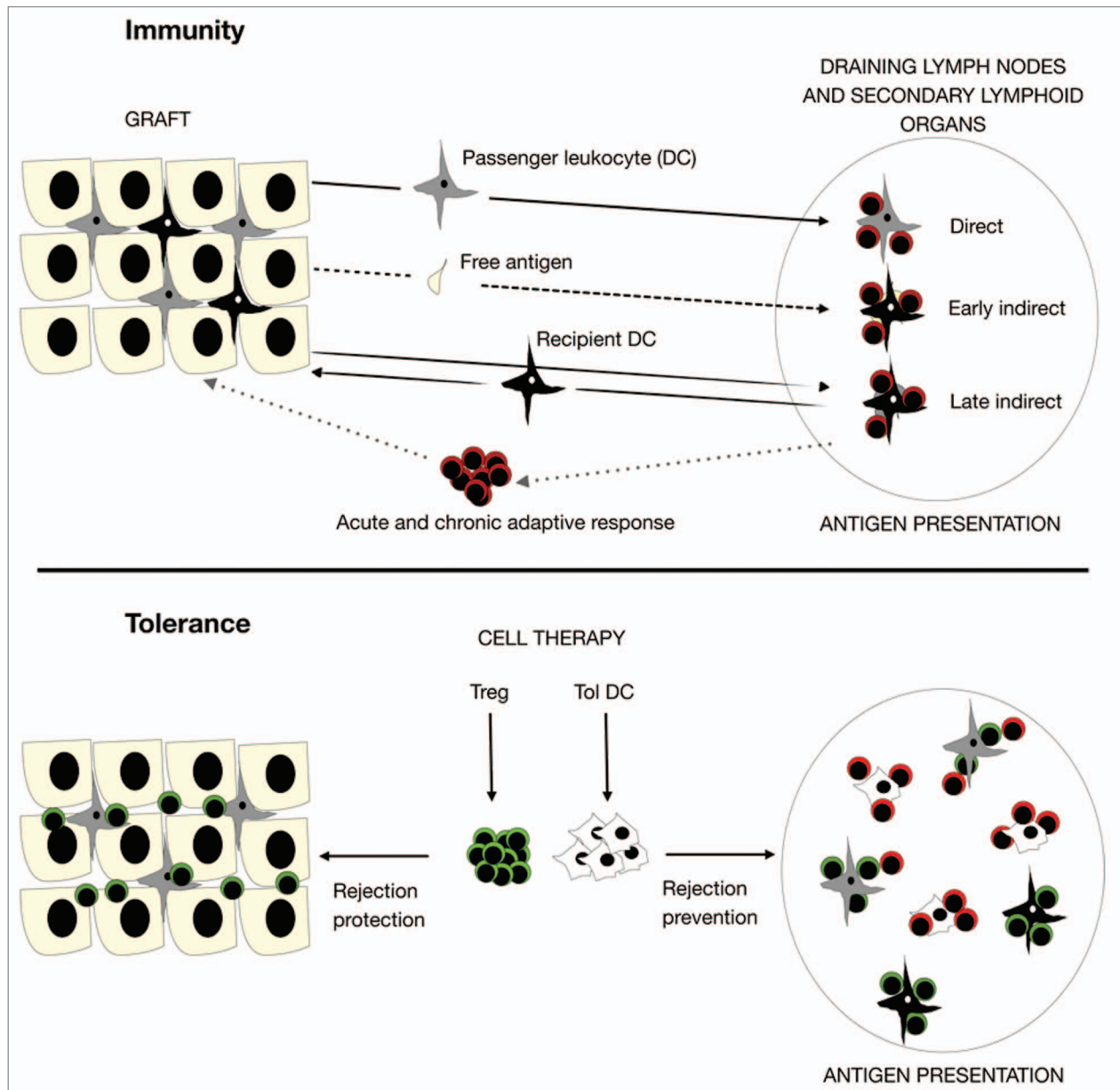
Figure 1. Pathways of allorecognition, allograft rejection and mechanisms to induce transplantation tolerance.

differences in TCR stimulation and costimulatory molecules requirements, and thus could be differentially targeted.[109,110] Besides costimulatory blockade, T-cell depletion induction therapies (e.g., anti-CD3, anti-CD52 monoclonal antibodies or polyclonal anti-thymocyte globulins) are used in clinical SOT to prevent acute rejection. These therapies induce profound and durable (weeks to months) reduction of circulating lymphocytes capable of mounting an alloresponse. Recent data suggest that T-cell depletion protocols allow preferential expansion of Treg once lymphocytes gradually repopulate the host, thus skewing the Treg/effector T cell ratio towards tolerance. In these studies, the increased frequency of Treg was neither fully explained by their homeostatic proliferation in a lymphopenic environment nor preferential sparing by the depleting

antibody.[111-113] Although the underlying mechanisms need to be clarified, the induction of apoptotic cells in vivo (as would occur with cell-depleting agents) leads to TGFβ secretion by phagocytes (immature DC, macrophages) involved in clearing these cells, thus favoring iTreg generation and expansion. The uptake of apoptotic cells may also help to maintain DC in an immature state (low level of surface MHC II and costimulatory molecules), favoring tolerance.[114-116] Besides induction therapies, maintenance immunosuppressive drugs such as mammalian target of rapamycin (mTOR) inhibitors (e.g., sirolimus, everolimus), allow preferential expansion of nTreg and iTreg that promote antigen-specific transplantation tolerance.[103,117-119] Finally, as stressed before, in vivo homeostasis and expansion of Treg is highly dependent on IL-2. Thus, the administration of IL-2

could be combined to these immunomodulatory approaches and is under investigation in stringent experimental allotransplantation models.[120-122]

Antigen specificity. Naturally occurring Treg have been shown to have a polyclonal TCR repertoire primarily driven against self-antigens, but with cross-reactivity to transplantation antigens. We and others have shown that, on a cell-per-cell basis, antigen-specific nTreg were more potent suppressors than polyclonal nTreg in organ-specific autoimmune diseases and transplantation experimental models.[92,93,123] Moreover, antigen-specificity dictates trafficking of Treg to the appropriate site i.e., the allograft. A potential benefit of donor-specific Treg in SOT is also supported by the fact that Treg-mediated tolerance was demonstrated to be dependent on a continuous supply of donor-derived alloantigens.[124] Furthermore, the adoptive transfer of polyclonal Treg carries the risk of deleterious non-specific immune suppression. One noteworthy exception would be the infusion of donor polyclonal Treg to prevent GVHD after allogeneic BMT, as the disease is systemic with muti-organ involvement. In line with this concept, in vitro expanded donor polyclonal nTreg have been successfully used in mouse models of BMT to prevent GVHD while allowing graft-versus-leukemia effect and phase I/II clinical trials are underway.[125-127] The benefit vs. risk ratio of infusions of donor-derived iTreg (Tr1 cells) are also being evaluated in HSCT from haploidentical donors.[128] Overall current available data indicate that the transfer of Treg (nTreg or iTreg) would be a feasible strategy in clinical transplantation with no apparent major side-effects.

Donor alloantigens are recognized by recipient CD4+ T cells either as intact MHC class II:peptide complex presented by donor APC (direct pathway of allorecognition) or after being processed and presented by recipient APC (indirect pathway) (**Fig. 1**). A series of experimental and clinical data indicate that the indirect pathway alloresponse is the main driver for chronic allograft rejection.[129] As current immunosuppressive regimens have little effect on preventing chronic rejection, the control of T cells with indirect alloreactivity would promote transplantation tolerance. Treg with indirect allospecificity can be generated in vitro and were indeed shown to prevent acute and chronic rejection of skin and cardiac allografts respectively in rodent models.[92,130-132] We and others have also demonstrated that specificity to a single antigen was sufficient to convey protection for whole tissues expressing at least the same antigen together with other epitopes (a mechanism referred to as "linked suppression").[133] The use of antigen-specific Treg at the time of transplantation may be limited if the donor is cadaveric i.e., not known in advance, as time is required to generate and expand ex-vivo donor-specifc Treg. In the contrary, if a living donor is available (HSCT, kidney, liver transplantation), recipient (or donor in the case of HSCT) T cells could be isolated in advance and manipulated ex-vivo in the presence of donor-derived APC or peptides.

Limitations for clinical application. To date the efficacy of Treg immunotherapy to induce tolerance in SOT has been mainly demonstrated in experimental models of adoptive transfer into lymphopenic animals.[92,130] Our data indicated several limitations of donor-specific Treg under more stringent conditions such

as donor-recipient full MHC-mismatch, in non-lymphopenic recipients or in regulating responses to more immunogenic skin instead of cardiac or islet allografts.[92] In these situations, while the transfer of Treg prolonged allograft survival, it was not sufficient to induce robust tolerance on its own. This highlights the need for adjuvant immunomodulatory therapies to suppress strong immune activation and overcome the rapidly expanding pool of alloreactive T cells early after transplantation. As discussed, Treg transfer could be combined with drugs such as costimulatory blockade (e.g., belatacept) or mTOR inhibitors.[19,120] Another option would be to combine robust central mechanisms of tolerance induction with peripheral transfer of Treg. Protocols based on hematopoietic mixed chimerism i.e., allogeneic BMT together with a solid organ from the same donor are robust experimental approaches for the induction of transplantation tolerance and were recently applied in kidney transplantation pilot clinical trials.[14-16] However, they imply toxic cytoreductive preconditioning of the recipient. Using a MHC-mismatched skin transplantation model in mice, Wekerle and colleagues recently reported that the infusion of recipient polyclonal Treg (both nTreg and iTreg) together with donor BM allowed the induction of long-term mixed chimerism and donor-specific transplantation tolerance. Interestingly, the recipient mice did not receive any cytotoxic drug and were conditioned only with a short-course of costimulation blockers and rapamycin.[134] Thus, Treg therapy may allow mixed chimerism-based strategies to be translated more easily and with fewer risks into clinical practice.

There is experimental and clinical evidence that an inflammatory context such as post-operative ischemia-reperfusion injury of the graft or concomitant infections can trigger acute rejection episodes and prevent the induction of transplantation tolerance. Recent studies have highlighted the effect of the microenvironment in inhibiting or subverting nTreg function, as well as the plasticity of iTreg that could convert into pathogenic Th17 cells.[135] This would partly explain the reported resistance to Treg-mediated suppression under some inflammatory conditions.[136,137] An alternative strategy to promote tolerance would therefore be to skew the immune response away from Th17 or Th1 cells and towards Treg by modifying the microenvironment, for example by blocking critical cytokines.[138] Finally, in a recent experimental study, a fraction of mouse nTreg failed to maintain Foxp3 expression in vivo, produced inflammatory cytokines and their adoptive transfer led to the rapid onset of autoimmunity.[139] Thus, the in vivo homeostasis, lifespan and stability of nTreg and iTreg need to be clarified before clinical trials on Treg transfer can be considered.

Conclusion and Perspectives

Since the first description of suppressor cells in the 1970s, the field of immune regulation has become very complex, reflecting various distinct mechanisms to maintain tolerance. So far, dominant peripheral transplantation tolerance has mostly been associated with Treg. However other T cell subsets have also been described to have immunosuppressive capacities. In particular, CD8+CD28- suppressor T cells, double negative CD4-CD8-

T regulatory cells and NKT cells have been shown to be involved in the maintenance of peripheral allo- and xenotransplantation tolerance.[140] Besides T cells, tolerogenic DC, myeloid-derived suppressor cells, mesenchymal stem cells, embryonic stem cells and more recently B cells were reported to have intrinsic or inducible regulatory properties.[141-143] The biology of all these cells as well as their potential in SOT need further investigation.

While therapies based on regulatory T or non-T cells proved promising in inhibiting donor-reactive T cell responses and promoting long-term allograft survival in experimental models, the bench-to-beside translation for SOT is still to be tested. Indeed, most of our current knowledge on regulatory cells is based on animal studies or restricted to in vitro assays when analyzing human cells. Therefore, many issues on the homeostasis and function of human regulatory cells still need to be addressed. Moreover, before implementing any new tolerance-inducing strategy in clinical practice, it needs to favorably compare to current well established immunosppressive protocols in terms of feasibility, efficacy (rate of acute rejection episodes, long-term graft survival) and toxicity.

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