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REVIEW ARTICLE

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Regulatory T cells in the induction and maintenance of peripheral transplantation tolerance

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Abstract It is now possible to induce donor-specific transplantation tolerance in adult rodents using non-depleting monoclonal antibodies against T cell co-receptor and co-stimulation molecules or by immunisation with tolerogenic antigen-presenting cells. It is a common finding of all these models of peripheral tolerance, as well as of various mouse models of autoimmune disease, that regulatory $CD4^+$ T cells are the principal mediators. There are currently no specific markers for regulatory T cells, but in some autoimmune models their activity has been associated with the expression of activation markers such as CD25 and CTLA4, or anti-inflammatory cytokines such as IL-10 and TGF-β. $CD4^+CD25^+$ T cells from both naïve and tolerised donors are able to transfer tolerance to grafts in lymphopenic recipients, and this may be directly applicable to bone-marrow transplantation. The challenge is now to understand the biological principles that allow such immune re-programming so that they can be safely applied to clinical organ grafting.

Keywords Transplantation · Tolerance · Regulation · CD4 · CD25

Chimerism and central tolerance

For many years, the classical experiments of Medawar and colleagues [1] were used as a paradigm for the induction of tolerance in the adult. Over the next 40 years, approximately, it became clear that if one could eliminate the mature immune system, usually by total body irradiation, and then introduce long-lived donor antigen-carrying cells (usually bone marrow) and allow the haemopoietic system to regenerate as a chimera, the recipient was generally able to accept donor-type organ grafts. The predominant mechanism was found to be a continuous clonal elimination of donor antigen-specific T cells in the thymus [2], providing an absolute unresponsiveness both in vivo and in vitro. The problem with this approach has always been the need to ablate the mature immune system. Numerous studies in allogeneic bone-marrow transplantation have shown us that in practice this requires lethal irradiation as well as immunosuppressive or lympholytic agents, even if the donor and recipient are siblings matched for major histocompatibility (MHC) loci [3]. Although there has been a recent revival of interest in donor chimerism as a means of generating tolerance, this has generally not proven to be easily practicable.

Immunosuppression and chronic rejection

One might ask why we need to induce tolerance at all. With appropriate cocktails of conventional immunosuppressive drugs we are able to control the acute rejection of renal allografts very effectively, with a successful outcome higher than 90% at 1 year. The

problem is, however, that by 10 years, more than 50% of the grafts will have been lost through a process that is still poorly understood but is generally thought to be a form of chronic rejection. In addition, the use of long-term immunosuppression risks the development of serious infections and tumours, and each drug is associated with specific toxicities. Some of the immunosuppressive agents in common use are probably even counter-tolerogenic [4]. Therefore, we need to identify which, if any, of the currently available immunosuppressive agents are compatible with tolerance induction, either when used alone or in combination with emerging tolerogenic therapies.

Immune re-programming with non-depleting monoclonal antibodies

Although many effector systems play a role in the rejection of allogeneic grafts, including B cells, NK cells, activated macrophages and polymorphs, it is clear that all rejection is absolutely dependent on T cells. The majority of peripheral T cells can be divided into those expressing CD4, which recognise antigen peptides in association with MHC-II, usually associated with helper T cell activity, and those expressing CD8, which recognise MHC-I that traditionally include the cytotoxic effector T cells.

Monoclonal antibodies against the T cell antigen CD4 can be used in vivo to block immune responses in rodents, but, surprisingly, the immune system records such aborted immune challenges as tolerogenic events [5]. Although CD4 antibodies used alone induced tolerance only in certain MHC-matched skin-graft combinations, they were able to induce indefinite acceptance of MHC-mismatched cardiac grafts in some recipient strains of mice [6]. It has been possible to extend the range and reliability of tolerance induction by the addition of other non-depleting antibodies, especially to CD8 and CD40L(CD154), and under these conditions, donor-specific lifelong tolerance can be induced to fully MHC plus multiple minors mismatched skin grafts [7].

The fact that such tolerance is lifelong and independent of the presence or absence of a thymus shows that the adult immune system can indeed be re-programmed in the periphery to accept donor antigens as if they are self. While mice, which are centrally tolerant, are unable to proliferate or generate cytotoxic T cells in vitro against donor antigen-presenting cells because the T cells have been clonally deleted in the thymus, peripheral tolerance induction has rarely generated any observable change in vitro. Donor antigen-specific proliferation, cytotoxicity and Th1 or Th2 cytokine assays are usually similar to those seen in primed (i.e. rejecting) recipients [8]. This lack of a clear in vitro correlate of tolerance has been a major hurdle in the understanding of the cellular and molecular mechanisms.

Regulatory T cells in transplantation tolerance

In the absence of appropriate in vitro readouts, much effort was put into in vivo systems to study peripheral tolerance. It soon became clear that powerful $CD4^+$ regulatory T cells enforce tolerance after adoptive transfer into secondary recipients. Although tolerance is dominant in this situation [9], with the secondary $CD4^+$ T cells themselves becoming tolerant and regulatory ('infectious tolerance'), there is no elimination of the antigen-specific effector T cells: depletion of the regulatory $CD4^+$ population can reveal primed, $CD8^+$ T cell-mediated rejection, suggesting that there is an element of active suppression [10]. The mechanisms of such suppression remain elusive, even if we apply modern technology to follow T cell activity in situ. For example, it has recently been shown that donor graft antigen-specific $CD8^+$ T cells can proliferate normally in tolerant recipients (using CFSE tracking studies—see Fig. 1), although they fail to develop effector functions and do not reject the graft [7].

Linked suppression of graft rejection

Possibly the most convincing and perhaps the only reliable assay for regulatory cell activity in transplantation models is that of linked suppression [11, 12] in the original recipient. Although the tolerant mouse can reject third-party grafts, demonstrating that there is no non-specific immunosuppression and that tolerance has overall donor specificity, a graft from an F1 cross between the donor and a third party is only rejected slowly or may be fully accepted. Acceptance of this F1 graft then leads to full tolerance of the third-party graft, with no other external manipulation of the immune system. This suggests that having the two antigens brought closely together, either on the same antigen-presenting cell or target tissue, elicits donor-directed regulatory $CD4^+$ T cells to drive tolerance in third-party specific T cells.

Linked suppression is not only highly significant for the understanding of the mechanisms of tolerance, it also has important therapeutic implications. It may not be necessary to induce tolerance to every transplantation antigen in order to achieve graft acceptance if there is sufficient regulatory T-cell activity against a proportion of the graft antigens. It might therefore be possible to induce strong regulatory T-cell tolerance to certain common MHC antigens in advance of a transplant becoming available, perhaps reducing the requirement for aggressive tolerogenic therapies at the time of grafting.

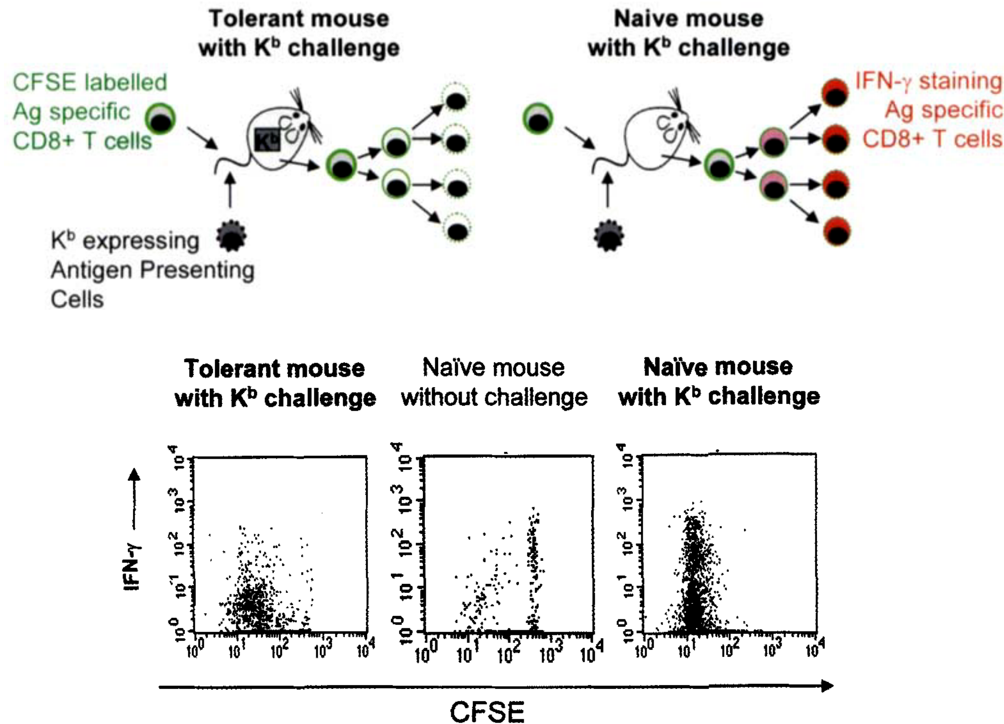


Fig. 1 Peripheral tolerance blocks the function but not the proliferation of graft specific CD8$^+$ T cells. CBA mice (H-2^k) were made tolerant of B10 skin (H-2^b) by treatment with non-depleting CD4, CD8 and CD154 antibodies [7] or were left untreated (*naïve*). Once the antibodies had cleared, the mice were given H-2 K^b expressing antigen presenting cells and CFSE labelled T cells i.v. from mice transgenic for a TCR specific for the H-2 K^b molecule. After 3 days, the spleen cells were removed and analysed by multicolour flow cytometry, gating on the transgenic TCR$^+$ cells. The CFSE staining is halved each time a cell divides, so that the T cells in the H-2 K^b challenged naïve or tolerant mice both show low levels of staining compared with the naïve cells without challenge. In addition, the cells had been briefly activated (6 h) in the presence of brefeldin A, permeabilised and stained for intra-cytoplasmic IFN-γ. The dividing cells in the H-2 K^b-challenged naïve mouse showed strong staining for IFN-γ, while those from tolerant animals remained negative, indicating an inhibition of their function (that could be confirmed by the failure to reject the graft or generate cytotoxic T cells in vitro), but not their proliferation

Regulatory T cells in autoimmune models

In parallel with the discovery of regulatory CD4$^+$ T cells in transplantation tolerance, it has been found that there is an intrinsic regulation of the autoimmune response within the CD4$^+$ T-cell compartment. Originally it was found that if CD4$^+$ T cells were separated on the basis of CD45RB expression and adoptively transferred into lymphopenic recipients, the CD4$^+$CD45RB^{high} T cells caused an autoimmune disease, while replacement of the missing CD4$^+$CD45RB^{low} T cells suppressed it (reviewed in [13]). Meanwhile, Sakaguchi et al. [14], Takahashi et al. [15], Suri-Payer et al. [16] McHugh and Shevach [17], and others, have shown that murine CD4$^+$CD25$^+$ T cells could not only suppress polyclonal T-cell proliferation in vitro, but were able to regulate autoimmunity in vivo. It was also shown that the CD4$^+$CD25$^+$ T cells are predominantly found within the CD4$^+$CD45RB^{low} population [18]. Additionally, the thymus was shown to contain even more powerful regulatory capacity entirely within the CD4$^+$CD25$^+$ population [19, 20], and this has led many to suggest that there is a dedicated lineage of 'professional', self-antigen specific, regulatory T cells that are generated within the thymus [20, 21, 22, 23, 24]. Human CD4$^+$CD25$^+$ peripheral blood T cells and thymocytes [25] also have a similar suppressive activity in vitro to that seen in the mouse, and these cells have generally been described as both anergic and IL-10 producing [26, 27, 28, 29, 30, 31, 32].

Tr1 cells and IL-10

IL-10 is a cytokine produced by a range of cells both within and outside the immune system, including T cells, macrophages, and keratinocytes. It was originally identified through its ability to inhibit the production of Th1 cytokines, especially interferon- during an inflammatory response. Mice genetically deficient in IL-10, when crossed to susceptible backgrounds, develop an autoimmune pathology including colitis [33], very similar to that obtained in the models of CD4$^+$CD25$^-$ (or CD45RB^{high}) T-cell transfer. A number of groups have now demonstrated that the suppression of inflammatory

bowel disease by $CD4^+CD25^+$ regulatory T cells in vivo is dependent on the presence of IL-10 [34, 35].

It is possible to polarise na $CD4^+$ T cells by antigen (or non-specific anti-CD3) stimulation in vitro in the presence of high levels of recombinant IL-10 [35]. This may also require TGF-β [36], although this is often already present in the serum used for cell cultures. This stimulation produces a mixed population of IL-4 and IL-10 producing Th2 as well as IL-10 only producing Tr1 cells. The latter can be cloned, particularly by use of high levels of solid phase anti-CD3, which generally causes T cell apoptosis but to which Tr1 cells are relatively resistant. Alternatively, the stimulation of naïve T cells in the presence of two drugs, dexamethasone and vitamin D3, and the additional neutralisation of IL-12, IFN-γ and IL-4, can reliably polarise cultures to this IL-10 only-producing Tr1 phenotype [37]. Tr1 cells against the antigen ovalbumin, generated by either method, have been shown to be capable of suppressing autoimmune colitis or experimentally induced acute encephalomyelitis in vivo caused by $CD4^+CD45RB^{low}$ cells, when the animals are given oral ovalbumin as a stimulus for the Tr1 cells [35, 37]. Human Tr1 cells can also be generated in vitro by stimulation in the presence of IL-10 and interferon-α [38].

There have also been reports that IL-10 is required for the regulatory activity that can be transferred after CD4 antibody-induced transplantation tolerance [39], but this tolerance, linked suppression and infectious tolerance cannot be broken by anti-IL10 or anti-IL10R monoclonal antibodies in vivo in the original recipient, even when they are re-challenged with fresh grafts [40]. However, Tr1-like T-cell clones generated in vitro against the male antigen, as presented by MHC-II, are able to block skin graft rejection by either Th1 [41] or Th2 (manuscript unpublished) clones against the same antigen, after adoptive transfer into T cell-deficient recipient mice.

Th3 cells and transforming growth factor-β

Many of the autoimmune models that show a role for IL-10 in suppression also implicate TGF-β [42, 43, 44], a cytokine that has a strong ability to block the differentiation of T cells towards either Th1 or Th2 responses. TGF-β seems to be particularly abundant in the anterior chamber of the eye, and seems to play an important part in the induction of tolerance that can be obtained to both protein and histocompatibility antigens introduced through this route [45, 46].

A $CD4^+$ T-cell subset, sometimes called Th3, has been described, where TGF-β is a major cytokine produced [47]. Mice made deficient in TGF-β1 develop an inflammatory disease [48], and this may be a factor needed for the generation of the IL-10-producing Tr1 population, in a manner similar to that described for $CD4^+CD25^+$ regulatory cells [49]. However, it has recently been shown that $CD4^+CD25^+$ cells from TGF-β1 knockout mice are as effective as those from normal mice at suppressor function in vitro, and that T cells from TGF-β unresponsive $Smad3^{-/-}$ or dominant negative TGF-β type-2 receptor transgenic mice are effective targets of suppression in vitro [50]. Others have demonstrated that $CD4^+CD25^+$ T cells are able to suppress proliferation in vitro even after fixation, ruling out the need for any secreted product in this assay, suggesting that cell contact mechanisms may be sufficient [51, 52]. There have been some reports that this could be due to TGF-β expressed on the surface of regulatory T cells [53, 54], but this remains controversial.

Contact-dependent mechanisms of regulatory T cells

$CD4^+CD25^+$ and Tr1 regulator cells also both constitutively express on their surface CD25, CD152 (CTLA4) and, most recently, the glucocorticoid-inducible TNF receptor (TNFRsf18 or GITR) [41, 55, 56]. All three have been implicated in regulatory function. IL-2 [57] or CD25 [58] knockout mice develop a lymphoproliferative disease similar to that in some of the autoimmune models. Similarly, CD152-deficient mice develop autoimmune pathology [59], and Fab fragments of anti-CTLA4 monoclonal antibodies can block suppression by $CD4^+CD25^+$ cells in vitro and in vivo [15, 18]. Both IL-10 and CTLA-4 have been implicated in the transferable suppression associated with tolerance to allogeneic murine cardiac grafts [60], but not to skin grafts [40]. Most recently, antibodies to GITR have also been shown to block regulatory function in vitro and in the lymphopenic autoimmune models [55, 56].

Does infectious tolerance require both contact and cytokines?

Two recent papers claim to clarify the conflicting data on the requirements for cell contact compared to soluble mediators [51, 52]. First, the secreted products such as cytokines are excluded by fixing the initial $CD4^+CD25^+$ suppressive population after CD3- and CD28-induced activation. When these cells are tested in mixture experiments in vitro, the normally responsive $CD4^+$ $CD25^-$ population is not only suppressed but also becomes anergic and able to suppress further $CD4^+$ T cells in a contact-independent manner through the production of cytokines. Although the two papers disagree on which cytokine is most important for this secondary suppression, one claims it is IL-10 and not TGF-β [51] while the other claims the opposite [52], these may be

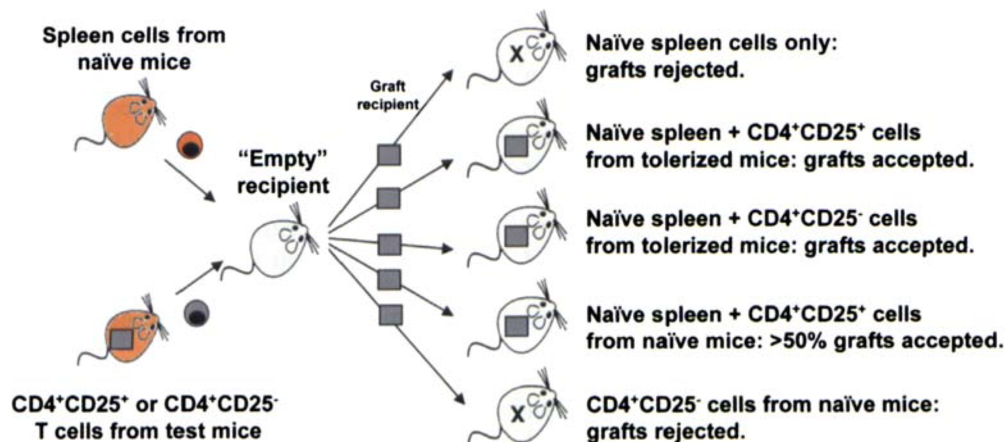
examples where multiple or sequential mechanisms can act together to increase the potency of regulatory T cells. It is also possible that similar mechanisms operate in bystander or linked suppression, and infectious tolerance in vivo. However, the in vitro data are based on the proliferation of polyclonal populations of T cells and are not antigen presenting cell dependent, while linked suppression and infectious tolerance in non-lymphopenic adult mice seem to be antigen specific, dependent on how the antigen is presented, and do not involve a defect in T cell proliferation. We therefore have to consider very carefully the evidence for any major role of $CD4^+CD25^+$ regulatory T cells in transplantation tolerance.

Are regulatory T cells in peripheral transplantation tolerance $CD4^+CD25^+$?

There are now a few published examples where $CD4^+CD25^+$ regulatory T cells would seem to transfer transplantation tolerance. Davies et al. were able to suppress the rejection of allogeneic islets by $CD4^+$ $CD45RB^{high}$ cells by co-transfer of $CD4^+$ $CD45RB^{low}$ T cells from naïve donors [61]. Wood and colleagues have claimed that antigen-specific tolerance generated to MHC-mismatched cardiac or skin grafts can be transferred with $CD4^+CD25^+$ T cells (Hara et al. [39 and Kingsley et al. 60]). It has also been shown that $CD4^+CD25^+$ T cells can be generated in vitro in a donor-versus-host mixed lymphocyte reaction under the cover of antibodies to CD40L (CD154), and that these are able to suppress graft-versus-host disease (GVHD) after bone-marrow transplantation [62]. Others have shown that simply increasing the proportion of donor $CD4^+CD25^+$ T cells in the marrow inoculum may be sufficient to suppress GVHD [63]. Such studies are interesting, particularly with regard to the potential of regulatory T cells that might be used for adoptive cell therapies as a means to induce tolerance clinically.

One should remain cautious, however, because a careful study that directly compared the potency of $CD4^+CD25^+$ T cells with $CD4^+CD25^-$ cells from either tolerant or na mice came to rather different conclusions [40]. It was found that $CD4^+CD25^+$ T cells from either naïve or tolerant mice could suppress rejection and induce tolerance in lymphopenic recipients reconstituted with limiting numbers of normal $CD4^+$ T cells (Fig. 2). The ability of na $CD4^+CD25^+$ T cells to induce such tolerance is surprising, as they have never seen the graft antigens before the transfer. There are two possible hypotheses to explain these findings. First, that $CD4^+CD25^+$ T cells have specificity for self or environmental antigens (e.g. gut flora), and that a significant proportion is able to cross-react with donor transplantation antigens, perhaps expand, and induce suppression and tolerance. Alternatively, it may be that $CD4^+$ $CD25^+$ T cells have no antigen specificity relevant to the grafted tissue, but act non-specifically to suppress the rejection response and allow transplantation tolerance to develop. They would do this in a fashion analogous to the induction of tolerance with many different monoclonal antibodies that block T cells 'non-specifically' [5]. If the latter is the case, it may be that $CD4^+CD25^+$ cells normally operate to control lymphocyte homeostasis

Fig. 2 Regulatory T cells from tolerant mice are both $CD25^+$ and $CD25^-$. Regulatory activity within $CD4^+$ T cells subsets (MACS purified) of tolerised or naïve mice was assayed by adoptive transfer into 'empty' T cell-deficient recipients given a limiting number (10^7) of normal (*naïve*) spleen cells plus a donor skin graft. Such mice, given naïve spleen cells only, rejected all their grafts acutely. The co-transfer of as few as 10^6 $CD4^+CD25^+$ T cells from tolerised mice was sufficient to suppress rejection in all the recipients completely, demonstrating the presence of regulatory T cells. The co-transfer of 10^7 $CD4^+CD25^-$ T cells from tolerised mice was also able to suppress rejection completely, although the equivalent population from naïve mice was not effective. Surprisingly, co-transfer of 10^6 $CD4^+CD25^+$ T cells from naïve donors was still able to delay the rejection in all recipients, and more than 50% of these mice accepted their grafts indefinitely



[64] and have no specific role in peripheral transplantation tolerance.

We therefore have to consider if there is evidence for any new population of regulatory T cells that can be observed in tolerant but not naïve mice. In their experiments, Graca et al. did indeed observe a new population of $CD4^+CD25^-$ cells with regulatory activity only from tolerant mice [40]. Although these were less potent 'per cell' than $CD4^+CD25^+$ cells when titrated, we do not know the frequency of graft reactive cells within the two populations, and there are ten times as many $CD4^+CD25^-$ cells in the tolerant animals. This raises the possibility that this $CD4^+CD25^-$ population contains the induced, antigen-specific regulatory T cells that are responsible for infectious transplantation tolerance and linked suppression. Interestingly, these observations are compatible with some recent data from TCR transgenic mice made tolerant of their cognate peptide antigen [65]. In these mice it was found that $CD4^+CD25^+$ regulatory T cells were derived from the thymus, while $CD4^+CD25^-$ regulatory T cells were generated by peripheral presentation of antigen for tolerance.

The experiments by Gavin et al. [66] may shed some light on the relationship between the two subsets of regulatory T cells. It was found that if CFSE-labelled $CD4^+CD25^+$ T cells were transferred into lymphopenic recipients alone, then they proliferated (either homeostatically or to self- or environmental antigens) but in the process became $CD4^+CD25^-$. Such expanded, now $CD4^+CD25^-$, T cells seemed even more potent (on a per-cell basis) at suppressing proliferation of na T cells in vitro. Similarly, in the transplantation tolerance models, treatment with non-depleting CD4 or other monoclonal antibodies may allow the expansion of a $CD4^+CD25^+$, graft cross-reactive population to generate a more potent $CD4^+CD25^-$ antigen-specific regulatory T cell.

Gene expression analyses of regulatory T cells

One of the major limitations to both our understanding of the mechanisms in the experimental models and our ability to induce tolerance in the clinic is that we still have no good markers for tolerance or regulatory T cells. All the molecules that have been discussed, such as CD25, CTLA4, GITR, and cytokines, are also expressed by recently activated effector T cells. We really need to identify molecules that are uniquely expressed by regulatory T cells, both to identify them for diagnostic purposes and to focus on their mechanism(s) of action.

DNA microarrays or 'gene chips' [67] and serial analysis of gene expression (SAGE) [68] are similar in that they simultaneously measure the expression pattern of many thousands of gene mRNA transcripts and try to identify how this changes from one cell or tissue sample to another, preferably using as many relevant samples as possible. In principle, this allows the identification of specific clusters of genes that are up or down regulated in just the cells of interest, such as the regulatory T cells.

A direct comparison, by SAGE, between murine Th1, Th2 and Tr1 cells with identical T-cell receptors against the male transplantation antigen (HY) presented by MHC-II, has been published [41, 69]. Perhaps surprisingly, there were very few, if any, transcripts that were uniquely expressed by the regulatory Tr1 clone, although a number of genes expressed in Th2 cells were up-regulated. More significantly, Tr1 cells had lost many transcripts associated with, either directly or as transcription factors for, effector functions of Th1 and Th2 cells. Examples included the loss, by Tr1 cells, of Th2 transcription factors GATA-3 and Egr-1, and Th1 effector molecules RANTES and Ly6C. It is possible that the most important characteristic of regulatory T cells is this loss of effector functions, while retaining an ability to recognise antigen and compete with na or memory T cells for antigen, APC or cytokines (the 'civil-service model' [5]). Additionally, they may retain molecules that normally intrinsically limit the clonal expansion or aggressive functions of effector Th1 or Th2 cells, such as CTLA4.

Microarray analyses of both activated $CD4^+CD25^+$ T cells [56, 66] and SAGE analyses of Tr1 clones [41, 69] have identified a number of genes in common that are increased on regulatory T cells. These include GITR (mentioned above), OX40, preproenkephalin, $\alpha E/\beta 7$ (CD103), and granzyme A. However, all of these are expressed by Th2 cells as well [41]. We therefore still have no specific molecular marker for regulatory T cells, and it may be for diagnostic purposes that we will have to devise assays based on a differential loss of effector molecules.

The role of the antigen-presenting cell

One hypothesis that may explain linked suppression is that anergic or regulatory T cells are able to down-regulate co-stimulatory ligands on antigen-presenting cells [5, 70, 71]. In order for CD80 and CD86 to be up-regulated during an immune response, the APC must first be given a signal to mature, predominantly through the CD40 interaction with CD40 ligand on activated $CD4^+$ T cells. However, the expression of CD40 is itself tightly controlled in the APC at both the transcriptional level and in the production of specific splice variants of the CD40 message [72], depending on whether inflammatory signals have also been received through the Toll family of receptors for various pathogen products such as LPS or CpG [73].

As we begin to understand more about the relationship between T cells and antigen-presenting cells, there is a growing interest in the possibility that there might be

natural lineages or subsets of dendritic cells specialised in presenting antigen to naïve T cells (a unique feature of dendritic cells) for tolerance. Indeed, immature dendritic cells may themselves be inherently tolerogenic in the absence of inflammatory and maturation stimuli [74]. Many groups now attempt to identify agents that can modify or lock the dendritic cell activity into this putative tolerogenic phenotype. Probably the two most advanced candidates are IL-10 and 1α 25-dihydroxy vitamin D3. We have already considered IL-10 as a product of anergic or regulatory T cells, but it also has profound effects on dendritic cell maturation. IL-10 treated, immature bone-marrow derived dendritic cells fail to mature normally in response to inflammatory stimuli and are unable to stimulate a mixed lymphocyte reaction in vitro [75]. Vitamin D3- (or various metabolite- and analogue-) modulated dendritic cells are also able to induce tolerance and evidence of regulatory T cells to allogenenic islet transplants given under the cover of mycophenolate mofetil [76]. Dendritic cells can be further modulated by treatment with proteasome inhibitors to preferentially present antigen for regulatory T cells [77].

There have also been some interesting developments in the genetic manipulation of antigen-presenting cells. The evolutionary conserved Notch1 cell surface receptor plays an important role in a wide range of developmental decisions [78], including T-cell development in the thymus. Over-expression of Serrate1 (a ligand for Notch1) in antigen-pulsed APCs is able to generate tolerance [79], regulatory cells, and linked suppression in murine models of house-dust-mite allergy [80]. Interestingly, the regulatory T cells also seem to up-regulate additional Notch ligands that may play a role in contact-mediated signalling to naïve T cells for infectious tolerance, and similar changes can be observed after activation of $CD4^+CD25^+$ regulatory T cells [28]. The data implicating Notch in tolerance are, at present, indirect, and have not yet been supported by any direct evidence of a physiological role for Notch family molecules in tolerance.

The local graft environment

It is, therefore, clear that there is a synergistic interaction between the regulatory T cells and modulated APCs in the generation and maintenance of peripheral tolerance. The recent demonstration that regulatory T cells are concentrated within a tolerated graft [81] (see Fig. 3) suggests that the graft micro-environment may be very important for the maintenance of tolerance. It is possible that regulatory T cells function predominantly within the graft itself, perhaps explaining why we have been unable to detect any differences in proliferation or cytokine secretion between tolerant and rejecting recipients, either in vitro or ex vivo, using spleen or draining lymph node cells as a source of 'tolerant' T cells [8].

Most tissues have a variety of protective responses to various types of stress, including immune attack. For example, there are pathways to protect cells from reactive oxygen species generated as by-products of oxidative metabolism that are also used as cytocidal agents by

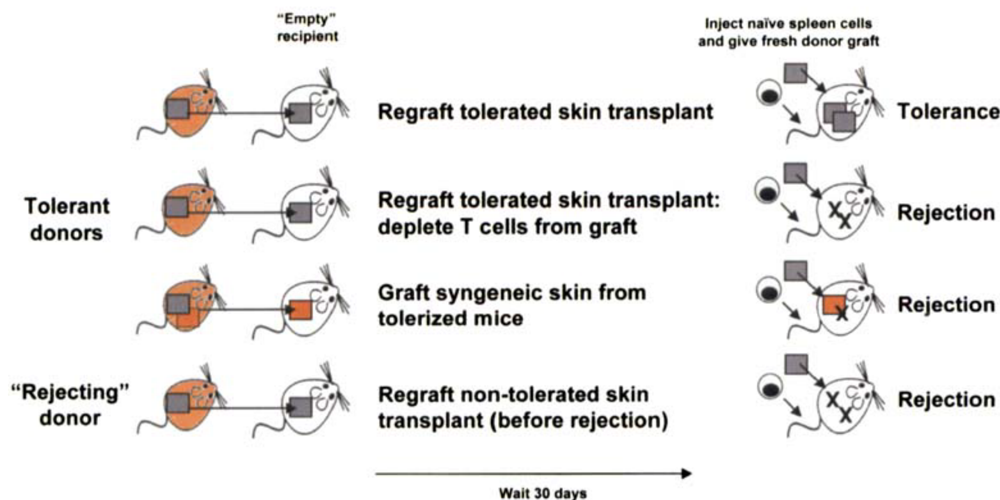


Fig. 3 Regulatory T cells are found within tolerated skin grafts. The presence of regulatory T cells within tolerated skin grafts was demonstrated by transferring the grafts onto 'empty' T cell deficient mice and waiting 30 days to allow any T cells carried over in the graft to repopulate the recipient. Any repopulating T cells in these recipients were then tested for their regulatory capacity by giving a fresh donor skin graft together with limiting numbers of naïve spleen cells. It was found that tolerated grafts could indeed carry over regulatory cells that could induce donor-specific tolerance in the recipient, and that these were clearly T cells (as T cell depletion at the time of graft transfer eliminated the suppression). By additional controls, it was shown that any T cells carried over by normal syngeneic skin from a tolerised donor, or grafts that would have proceeded to rejection on non-tolerant donors, were unable to suppress rejection by the naïve spleen cells

activated macrophages [82]. Indeed, it has been suggested that the up-regulation of one of these protective proteins, haemoxygenase, may be associated with tolerance to cardiac grafts in CD4-treated mice [83]. Similarly, the ligand for CD95 (FasL) is expressed in immuno-privileged sites [84], such as the testes, although no role for Fas–FasL interactions has been found in the transplantation tolerance or suppression induced by anti-CD4 treatment [85].

Summary and conclusions

Peripheral tolerance depends on regulatory $CD4^+$ T cells that interact with and modulate both the antigen-presenting cells and the graft local environment. We do not yet have any good markers or in vitro assays for regulatory T cells, nor do we really understand how they are induced or how they function. If we look at the range of monoclonal antibodies that have been claimed to induce peripheral tolerance or regulatory T cells [5], they include TCR and co-receptor specificities (non-activating CD3, CD4, CD8), co-stimulatory blockade (CD40L, CTLA4-Ig, CD80 and CD86), adhesion molecules (LFA-1, CD2) and cytokine receptors (CD25 or IL-2 receptor). One possibility is that the specificity is largely irrelevant, as long as the immune system is blocked from acute rejection and allowed to generate tolerance for itself. Indeed, this may be the way that some of the experiments that achieve tolerance through the transfer of (non-specific) $CD4^+CD25^+$ cells are operating, and further effort may identify safe ways to use adoptive regulatory cell therapies clinically. In general, we need to identify those agents, either among the currently available immunosuppressants, or newer agents such as monoclonal antibodies, that work together to block rejection but promote the generation of regulatory T cells. Most important of all, we still need to identify surrogate markers for regulatory T cells and tolerance that allow effective monitoring of the patient after a transplant, and these are most likely to come from detailed gene-expression studies of appropriate T-cell populations.

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