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Immune privilege induced by regulatory T cells in transplantation tolerance

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Summary: Immune privilege was originally believed to be associated with particular organs, such as the testes, brain, the anterior chamber of the eye, and the placenta, which need to be protected from any excessive inflammatory activity. It is now becoming clear, however, that immune privilege can be acquired locally in many different tissues in response to inflammation, but particularly due to the action of regulatory T cells (Tregs) induced by the deliberate therapeutic manipulation of the immune system toward tolerance. In this review, we consider the interplay between Tregs, dendritic cells, and the graft itself and the resulting local protective mechanisms that are coordinated to maintain the tolerant state. We discuss how both anti-inflammatory cytokines and negative costimulatory interactions can elicit a number of interrelated mechanisms to regulate both T-cell and antigen-presenting cell activity, for example, by catabolism of the amino acids tryptophan and arginine and the induction of hemoxygenase and carbon monoxide. The induction of local immune privilege has implications for the design of therapeutic regimens and the monitoring of the tolerant status of patients being weaned off immunosuppression.

Keywords: *IDO, immune privilige, tolerogenic dendritic cells, transplantation tolerance, regulatory T cells*

A graft is not simply a passive target of rejection

Our current understanding of the immunological response to an organ graft is mainly based on the premise that the immune system plays the dominant role, with the organ presenting a passive target that is recognized as foreign, therefore attacked and rejected, or alternatively is accepted as self, therefore ignored. This scenario fits well with the prevailing theory that an immune reaction led to clonal selection of antigen-specific lymphocytes, while transplantation tolerance was the converse, i.e. clonal elimination of cells with specificity for the graft. Over recent years, it has become clear that tolerance, of both self-tissues and foreign grafts, also involves non-deletional regulatory mechanisms and that these mechanisms work not only at the level of antigen-specific lymphocytes but also within the tolerated tissue itself, in terms of both the professional

antigen-presenting cells and many other interacting cells such as endothelium and epithelium. It now seems that tissue-protective mechanisms play important roles within both self-tissues and grafted organs in regulating any immune response within these.

Immune privilege associated with tolerogenic antigen presentation

It has long been considered that certain organs, such as the anterior chamber of the eye, the brain, the testes, and the placenta, represent sites of relative immune privilege, such that the administration of foreign antigens into these sites can lead to a state of tolerance rather than immunization (1). This state of 'natural' immune privilege has been associated with the presentation of antigen by immature or steady-state dendritic cells (DCs) [or an $F4/80^+$ macrophage (2)] and the expression of anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β) (3). In the case of the anterior chamber of the eye, this state has been associated with the generation of regulatory T cells (Tregs) that either produce TGF-β or are $CD8^+$ (2, 4, 5).

Tolerance maintained by Tregs that induce local immune privilege

The principal characteristic of Tregs is that they are able to regulate (6) or suppress the activation, proliferation, or function (7) of effector T cells (8), thereby damping or curtailing an immune response. Although we are only just beginning to understand the mechanisms by which Tregs work, it seems that a common theme is their ability to modulate antigen presentation and to induce a local anti-inflammatory microenvironment. In other words, Tregs act to induce a state of acquired immune privilege in the tissues with which they interact (9).

This article describes how Tregs, antigen-presenting cells, and the local tissue microenvironment interact in the process of inducing and maintaining tolerance in the context of organ grafting. We expect that these mechanisms are not unique to that context but are simply reflections of mechanisms normally operating to maintain self-tolerance.

Immune privilege and Fas ligand

There has been a considerable interest in understanding the mechanisms of natural immune privilege in the hope that they may have general application in therapeutic modulation of the immune response. While many of the details of these systems will be covered elsewhere in this volume, we concentrate here on those mechanisms that may relate to transplantation tolerance and the role of Tregs. One that was highlighted in the model of anterior-chamber-associated immune deviation (ACAID) was associated with the expression of Fas ligand (FasL) that induces apoptosis of activated T cells expressing the death receptor Fas (CD95) (10, 11). Similarly, as FasL expression correlated with the acceptance of allogeneic testes transplants, there have been a number of attempts to manipulate grafts to overexpress FasL. Surprisingly, FasL expression on cardiac allografts led to accelerated rejection due to a massive neutrophil infiltration (12), likely due to a metalloprotease cleavage to generate a chemotactic form of the FasL (13). Any role of Fas/FasL interactions as a mechanism for transplantation tolerance induced by coreceptor blockade (with anti-CD4 and anti-CD8 monoclonal antibodies) was also ruled out, as both T-cell deletion by donor bone marrow and regulatory T-cell-dependent infectious tolerance were found to be unchanged in Fas-deficient mice (14, 15).

Mouse models of monoclonal antibody-facilitated transplantation tolerance

It is now 20 years since the discovery that a brief treatment of adult mice with monoclonal antibodies against the CD4 molecule on the surface of T cells is able to induce immunological tolerance to foreign antigens given at the same time (16, 17). Although the first CD4 antibodies used were able to deplete $CD4^+$ T cells, it was soon found that overt T-cell depletion was not required (18, 19). Tolerance could also be induced directly to skin allografts by using a combination of monoclonal antibodies against CD4 and CD8 to simultaneously block the major histocompatibility complex (MHC) class-II- and class-I-directed T-cell responses (20, 21). Such tolerance clearly depended entirely on peripheral rather than on central mechanisms, as adult thymectomy had no impact on the outcome (22). This form of peripheral tolerance was not dependent on clonal elimination of donor antigen-specific T cells, as the spleen or peripheral blood cells from tolerant mice remained able to proliferate to donor antigens and generate T-helper 1 (Th1) and Th2 cytokines and cytotoxic T cells *in vitro* (22, 23).

It turned out that there was nothing unique about the epitope specificity of the particular CD4 antibodies used (24). Indeed, we now know that antibodies to CD2, leukocyte-function-associated antigen-1, CD45R, CD3, and CD40L (CD154), as well as cytotoxic T-lymphocyte antigen 4-immunoglobulin fusion protein (CTLA-4-Ig), are all capable of inducing tolerance with similar properties [reviewed by Waldmann

et al. (25) and Waldmann and Cobbold (26)]. A common theme that has emerged from all these different studies is the association between tolerance and the presence of Tregs.

Evidence for Tregs in transplantation tolerance

Experiments in neonatal tolerance models that demonstrated that T cells could suppress responses to foreign proteins or allogeneic graft rejection, after adoptive transfer into irradiated secondary recipients, were first described in the 1970s (27, 28). During the 1980s, suppressor T cells were discredited in many systems and their very existence was questioned. It became clear, however, that some form of suppressor or regulatory T-cell activity was the only viable explanation of the peripheral tolerance induced by the monoclonal antibody treatments discussed above (29). Tolerant mice were able to transfer their donor-specific tolerant state to secondary recipients, in some cases without any further manipulation of these recipients (30), and this ability was dependent on the transfer of $CD4^+$ T cells. In addition, the T cells of the secondary recipient were 'educated' in the presence of the original tolerant population and donor antigen to themselves become tolerant. This secondary population of tolerant $CD4^+$ T cells could then educate further T cells in a tertiary recipient, a process that revived the term 'infectious tolerance' to describe how this form of tolerance was passed on to the naive T cells that were continually generated by the thymus (29). Infectious tolerance provided an explanation for how tolerance, once induced, could be maintained throughout the life of the animal as long as the donor graft antigens were available (31).

Infectious tolerance and linked suppression

In addition to infectious tolerance, a second important feature of peripheral tolerance that has been observed in both transplantation (32) and autoimmune models (33) is that of linked suppression (which is strictly a form of bystander suppression where the specific tolerated antigen and third-party antigen must be linked within the same tissue or antigen-presenting cell) (32). This feature was observed in transplantation tolerance when mice tolerant of a donor allogeneic skin graft would reject third-party grafts, even if they were in the same graft bed, but would often accept the F_1 cross of (donor × third party) skin. Mice that accepted the (donor × third party)F_1 graft would then be fully tolerant of subsequent skin grafts from the same third-party strain. Linked suppression could be shown to be directly related to infectious tolerance, because it could be demonstrated in secondary recipients where the transfer of tolerance was dependent on $CD4^+$ T cells and where the original cohort of tolerant T cells had been eliminated (29, 30).

The demonstration of linked suppression was crucial to the understanding that peripheral tolerance was maintained not only by Tregs but also through some form of antigen-presenting cell acting as a regulatory 'bridge' between the donor and third-party antigens (34). Why might such a bridge be needed (*Fig. 1*)? First, the antigen-presenting cell or target tissue can play an active role, sensing the regulatory nature of the Treg and then modifying its own functions to present all antigens (both original and third party) in an obligate tolerogenic fashion to further cohorts of naive or potential effector T cells. This is effectively saying that Tregs induce an acquired immune privilege, which we discuss in detail later. Second, the antigen-presenting cell acts as a simple device to bring the regulatory and potential effector T cells together, increasing the opportunities for direct contact-mediated or indirect cytokine-mediated suppression. There has been much effort to phenotype and characterize Tregs, searching for molecules capable of exerting suppressive effects within the microenvironment around the antigen-presenting cell [reviewed by von Boehmer (35)].

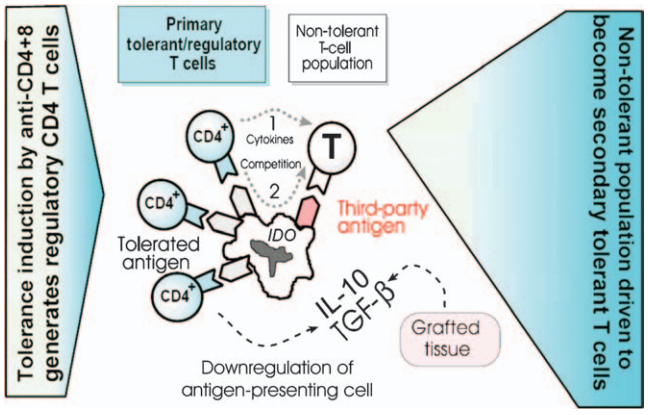

***Fig. 1.* Mechanisms of linked suppression.** Linked suppression represents a particular form of bystander suppression in which the tolerated and third-party antigens are presented by the same antigen-presenting cell or are coexpressed on the grafted tissue. It is due to the action of regulatory $CD4^+$T cells that can act in two main ways. (i) They may directly suppress other non-tolerant T cells by the secretion of anti-inflammatory cytokines, or via poorly characterized cell contact mechanisms, or by passive competition for cytokines and costimulatory ligands (i.e. the Civil Service model). (ii) They may induce changes in the antigen-presenting cell that downregulate its proinflammatory role and induce tolerance-promoting genes such as IDO. In either situation, the non-tolerant T cells perceive either the original or third-party antigens presented by the antigen-presenting cell in a non-inflammatory environment and become tolerant (and can themselves develop into secondary regulatory T cells via infectious tolerance).

Characteristics of Tregs

The best studied population of Tregs is the $CD4^+CD25^+$ 'natural' Tregs (36) produced in the thymus (37). These cells are believed to be directed to self-antigens (38), and they express the transcription factor forkhead box protein 3 (*foxp3*), essential for their differentiation (39–41). Natural Tregs have been shown to maintain self-tolerance in many autoimmune disease models (42) and are capable of enforcing acceptance of allografts after adoptive transfer into lymphopenic recipients (43). In some transplantation models, it has been suggested that preexisting natural Tregs are a requirement (44, 45), and the graft acceptance achieved by their adoptive transfer can appear to be donor antigen specific. Graca *et al.* (43, 46), however, demonstrated that $CD4^+CD25^+$ natural Tregs from either naive or tolerant mice are similar in their competence to prevent graft rejection without any particular specificity for donor alloantigens. This finding does not rule out the presence of donor-antigen-specific $CD4^+CD25^+$ Tregs, but they would have been masked by the more broadly reactive natural population. Natural $CD4^+CD25^+$ Tregs can suppress not only antigen-specific T cells but also the innate immune response, such as that seen in a *Helicobacter* infection of mice that have no T cells (47), and it has been suggested that $CD4^+CD25^+$ cells may act in lymphopenic conditions primarily by competing for homeostatic proliferation of naive T cells (48, 49). The tolerant state induced by a graft and coreceptor blockade is specific, however, to the donor antigen (20, 30) and cannot easily be explained by non-specific suppression of innate or homeostatic mechanisms. In the experiments of Graca *et al.* (46), where tolerance was induced in the absence of any lymphopenia, tolerant mice contained an additional population of Tregs within the $CD4^+CD25^-$ population of the spleen that numerically were similar in potency to the $CD4^+CD25^+$ cells. A hint that the Tregs in this model might be antigen specific was that they seemed to accumulate within the tolerated graft itself (50). In order to identify and track antigen-specific Tregs, it was necessary to develop appropriate T-cell receptor (TCR) transgenic mouse models of graft rejection and tolerance.

The A1.RAG$^{-/-}$ mouse model: a homogeneous TCR with the potential to generate heterogeneous Tregs

To closely model the induction of tolerance by coreceptor blockade in normal mice, we needed a transgenic mouse with a TCR against a minor transplantation antigen, such as the male antigen H-Y, and this antigen needed to be presented by MHC class II to stimulate the $CD4^+$ Tregs. We chose to use the CBA mouse strain because it is susceptible to tolerance induction by anti-CD4 blockade (51) and because appropriate $CD4^+$ T-cell clones were already available (52). The resulting A1(M).CBA TCR transgenic mouse behaved appropriately, in that only female mice showed a strong positive selection toward $CD4^+$ T cells with reactivity to the male DBY antigen presented by H-$2E^k$ (53). At the time, it was a surprise that such female mice were still unable to reject male skin grafts, but we now know that this was due to endogenous TCR rearrangements that allowed the escape of a natural $CD4^+CD25^+$ Treg population that suppressed the rejection response. Depletion of these Tregs with anti-CD25 antibody (44, unpublished data) or by crossing the A1(M).CBA to CBA.RAG-$1^{-/-}$ mice led to rapid rejection of male but not female skin grafts in female recipients (53).

More importantly, female A1.RAG-$1^{-/-}$ mice could be made tolerant of male skin grafts with as little as one injection of 0.5 mg of non-depleting CD4 antibody (34, 54). Evidence for tolerance via regulation, rather than immunosuppression or deletion, was that the spleens and lymph nodes of tolerant mice still contained similar numbers of male-specific T cells to control mice that had rejected a male graft. These T cells in both tolerant and rejecting mice showed similar expression of memory and activation markers both *in vitro* and *ex vivo* after a second-challenge male skin (that was also accepted only in the tolerant mice). In addition, tolerant mice, but not controls that had previously been given the anti-CD4 antibody and no graft, were able to resist the infusion of large numbers of naive anti-male T cells, demonstrating the specific presence of regulation in the tolerant recipients.

In light of the crucial role that *foxp3* plays in natural Tregs, we examined its expression in A1.RAG-$1^{-/-}$ mice during anti-CD4-antibody-mediated tolerance induction. In common with many other TCR transgenic mice crossed onto a RAG$^{-/-}$ background (55, 56), there were no detectable $CD4^+CD25^+$ or *foxp3*-expressing cells in the thymus or periphery of naive A1.RAG-$1^{-/-}$ mice (54). Exposing these naive T cells to male antigen (DBY peptide), as presented by syngeneic bone-marrow-derived DCs *in vitro*, in the presence of a blocking anti-CD4 monoclonal antibody, resulted in the *de novo* induction of *foxp3* messenger RNA (mRNA), in a dose-dependent fashion. This *foxp3* induction could be completely blocked by the neutralization of TGF-β but not interleukin-10 (IL-10) (54). A similar result was observed *in vivo*, as CD4 antibody treatment induced *foxP3*$^+$ T cells and transplantation tolerance, and this state could also be reversed by concomitant administration of neutralizing anti-TGF-β antibody but not an isotype-matched anti-IL10R (54). Recently, the induction of tolerance in normal (non-transgenic) mice to multiple mismatched skin grafts by coreceptor blockade has been found to be strictly dependent on

TGF-β, by using neutralizing antibodies at the time of tolerance induction. After challenge with a third-party (but overlapping), minor antigen-mismatched skin graft, only a proportion of the animals treated with anti-TGF-β rejected (Daley SR, Cobbold SP & Waldmann H, University of Oxford, manuscript in preparation) (*Fig.* 2), suggesting that while TGF-β may play a role in linked suppression, its contribution is less clear-cut than during tolerance induction.

TGF-β has been heavily implicated in many other *in vivo* models of tolerance that involve Tregs. In particular, the suppression of colitis obtained after adoptive transfer of $CD4^+CD45RB^{low}$ cells or $CD4^+CD25^+$ T cells is dependent on both TGF-β and IL-10 (57). Tolerance to antigens introduced via the anterior chamber of the eye (ACAID) is also dependent on both TGF-β (5) and IL-10 (58), and the treatment of autoimmune diabetes in the non-obese diabetic mouse with anti-CD3 antibodies requires TGF-β and the generation of $foxp3^+CD4^+CD62L^+$ Tregs (59, 60). In this model and in a number of other reports, it seems that Tregs express TGF-β on their surface (61) in association with latency-associated protein and latent TGF-β-binding protein (62). It has been suggested that this cell-bound TGF-β may be involved in the mechanism of suppression, as very high doses of neutralizing anti-TGF-β antibodies can block the contact-dependent suppression of naive T cells observed *in vitro* (61). Whether the source of this TGF-β is necessarily autocrine remains unclear, as are the events required to activate this latent TGF-β so that it can bind to TGF receptors on the cells that are being suppressed (3). *De novo foxp3*-expressing Tregs can also be generated *in vitro* by stimulating $CD4^+CD25^-$ naive T cells with antigen in the presence of exogenous TGF-β1 or TGF-β2 (63), and such induced Tregs are able to suppress antigen-specific immune responses *in vivo* (64, 65). We have also shown that such Tregs generated from naive A1.RAG-$1^{-/-}$ T cells *in vitro* are able to suppress male skin graft rejection after adoptive transfer into intact A1.RAG-$1^{-/-}$ recipient mice (Adams E, Cobbold SP & Waldmann H, University of Oxford, manuscript in preparation). Taken together, these data all indicate that tolerance to skin grafts induced by monoclonal antibody blockade most likely involves the TGF-β-dependent, *de novo* generation of $foxp3^+$ Tregs.

The expression of *foxp3* mRNA as a consequence of anti-CD4 treatment could be detected only transiently in the spleens of tolerized A1.RAG-$1^{-/-}$ mice and not at all in those that rejected male skin grafts (54). High levels of *foxp3* were found, however, in the tolerated grafts, and if the tolerant mice were given second-challenge male skin grafts after 100 days, both the original accepted graft and the second-challenge skin (but not rejecting grafts on non-tolerant controls) contained high levels of *foxp3*. There was no significant expression of *foxp3* in the spleens, lymph nodes, or normal skin of tolerant mice at this time, suggesting that the DBY male antigen-specific TCR transgenic regulatory cells were accumulating within the tolerated tissue and were able to rapidly home and target to a fresh challenge graft that also expressed the target antigen (54). This finding is compatible

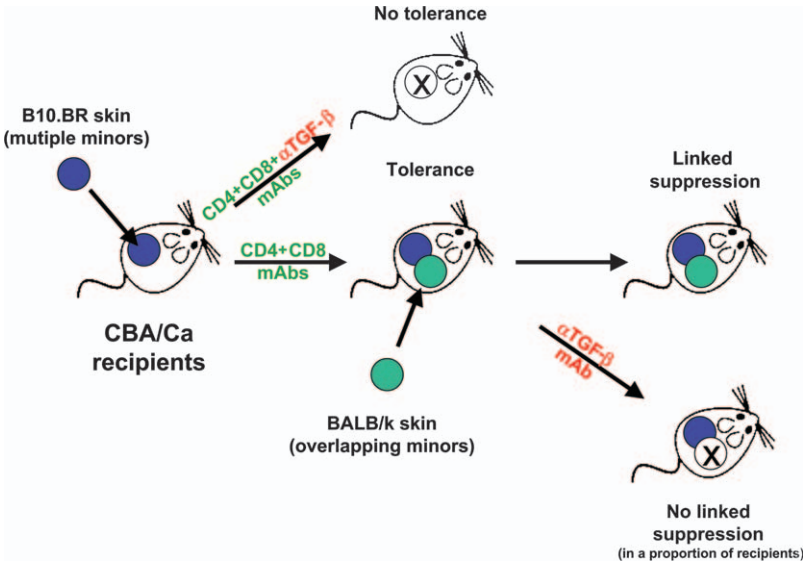

***Fig.* 2. A role for TGF-β in the induction and maintenance of skin graft tolerance.** The induction of tolerance to multiple minor mismatched skin grafts (B10.BR) by T-cell coreceptor blockade in normal CBA mice can be blocked either by coadministration of a neutralizing anti-TGF-β antibody (but not by an isotype-matched anti-IL10R antibody). In addition, the acceptance due to linked suppression of tolerant mice of a third-party graft expressing overlapping minor antigens (BALB/k) can also be partially reversed by coadministration of anti-TGF-β. This suggests that while TGF-β is strictly required for inducing tolerance in naive T cells in the presence of coreceptor blockade, its contribution during the maintenance phase of tolerance is less clear-cut. mAb, monoclonal antibody.

with data that have shown that tolerance in a mouse cardiac graft model was dependent on the homing of Tregs expressing CCR4 to the CCL22 chemokine (66).

We also need to consider the likely source of TGF-β that is required to induce and/or maintain (67) Tregs, as there seems to be a lot of conflicting data. Although TGF-β1$^{-/-}$ mice develop an autoimmune-like pathology, they are still capable of producing T cells with a regulatory phenotype that are capable of suppressing *in vitro* and *in vivo* (68, 69). This ability suggests that Tregs are not required to make their own source of TGF-β1, either to develop or to function. T cells expressing transgenic dominant-negative TGF-βR2, however, are unable to respond to TGF-β and are unable to be suppressed by natural Tregs in a colitis model (70). This finding suggests that there is likely an important source of TGF-β to maintain tolerance that does not come directly from the Tregs. Interestingly, quantitative reverse transcriptase polymerase chain reaction (RT-PCR) of tolerated versus rejecting male skin grafts in the A1.RAG-1$^{-/-}$ CD4 treatment model showed that TGF-β2 but not TGF-β1 was upregulated in tolerated grafts (71). TGF-β1 is the isoform normally expressed by T cells, while TGF-β2 is found in a wide range of non-lymphoid tissues, including normal skin, and is probably important in wound healing after grafting (72). This finding again suggests that the tolerated graft is itself contributing to the tolerant state, a theme that we return to later.

When we consider these data with the other observations that Tregs in non-transgenic, tolerant mice are also concentrated within the graft (50) and that in some circumstances, tolerant mice can distinguish between genetically identical skin grafts at different sites (73), accepting a fresh challenge donor-type graft while acutely rejecting a graft that had been tolerated for more than 100 days, it seems possible that tolerance maintained by Tregs is predominantly a local phenomenon that acts within and with the cooperation of the grafted tissue itself.

Peripheral tolerance and the local action of Tregs

Further evidence for the predominance of local immune regulation came from experiments that retransplanted the tolerated male skin grafts from the A1.RAG-1$^{-/-}$ mice onto secondary RAG$^{-/-}$ recipients. Such grafts contained *foxp3* and CD4^{+}CD25^{+} T cells coexpressing glucocorticoid-induced tumor necrosis factor receptor (GITR), as would be expected for Tregs, but this population represented only about half of the T cells that can be extracted from the tolerated grafts (54). The retransplanted grafts were normally accepted, as were the control fresh grafts, by RAG$^{-/-}$ recipients that have no T cells of their own to cause rejection, but treatment of the recipients with an antibody to CD25 at the time of graft transfer led to a rapid and acute rejection of the grafts from the tolerant donors (*Fig.* 3). This study demonstrates that the tolerated grafts contained T cells with the capacity to reject the grafts, but they were being held in check by the CD25^{+} Tregs. That this regulation was primarily acting within the local environment of the tolerated graft is confirmed, in that the spleen cells from the same tolerant mice were still able to cause rejection (albeit slowly) after adoptive transfer to RAG$^{-/-}$ recipients given a male graft. The question therefore arises as to how Tregs can act locally and how they can elicit cooperation from the graft itself. To approach this problem, we need to understand more about the unique properties of Tregs and the antigen-presenting cells with which they interact.

Common features of different Treg populations

It is now becoming clear that there are many different populations of lymphocytes with demonstrable regulatory properties [reviewed by Waldmann *et al.* (74)]. We have already discussed natural and induced CD4^{+} Tregs expressing *foxp3*, but there are other generally less well-characterized regulatory cells, including CD8^{+} T cells expressing *foxp3* (75), natural killer T cells (76, 77), CD4^{-}CD8^{-} T cells (78, 79), Th3 cells secreting TGF-β (80, 81), anergic CD4^{+} T cells (20, 82, 83), and T regulatory 1 (Tr1) cells principally expressing IL-10 (84–86). One approach to identify unifying molecular mechanisms of regulation is to examine the genes and proteins expressed by the different regulatory populations and to identify those expressed in common when compared with non-Tregs. To achieve this identification, we need to know in a single defined system which pure populations of T cells behave as effectors or regulators of graft rejection. Using the A1.RAG$^{-/-}$ mouse, we were able to generate a range of DBY male antigen-specific T-cell clones and lines from naive, tolerant, or primed mice with a range of defined properties and test their *in vivo* function after adoptive transfer. First, it was clearly demonstrated, contrary to dogma at the time, that both Th1 and Th2 cell clones were equally effective at causing skin graft rejection in T-cell-depleted or RAG$^{-/-}$ recipients (71) and that this rejection was independent of interferon-γ (IFN-γ) or IL-4, respectively [using antibody neutralization (unpublished data)]. In contrast, Tr1-like clones were unable to reject male skin grafts in RAG$^{-/-}$ recipients, despite evidence that they were able to home to the graft [by demonstrating expression of the Tr1-associated repressor of gata (*rog*) gene by quantitative RT-PCR in the grafts] (71). Mice that had failed to reject their grafts after transfer of the clone Tr1D1 were able to resist large numbers of

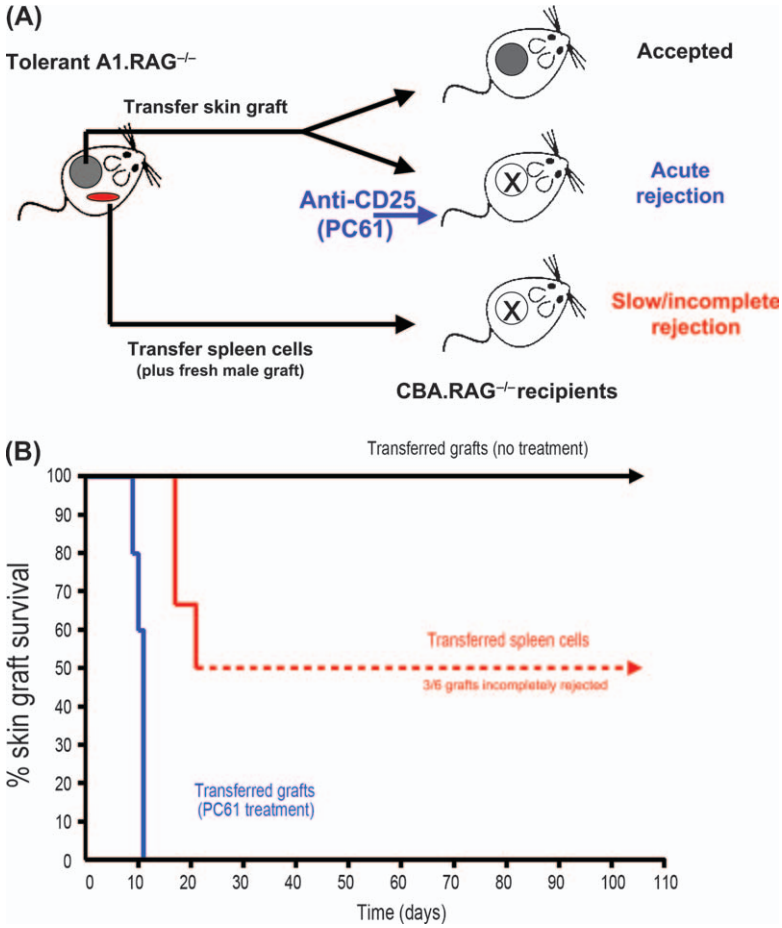

***Fig. 3.* Regulatory T cells act locally within a tolerated skin graft.** (A) Tolerance to male skin grafts can be induced in A1.RAG$^{-/-}$anti-male TCR transgenic recipients by a single injection of non-depleting CD4 at the time of grafting. This tolerance is associated with CD4^{+}CD25^{+}GITR^{+}T cells and high levels of *foxp3*within the graft (but not in the spleen or draining lymph nodes). Only about half of the T cells within the graft, however, express CD25 and GITR (54). These grafts, which had been accepted for more than 80 days, were then transferred to secondary, syngeneic RAG$^{-/-}$female recipients (that have no T cells and do not reject male or allogeneic skin), and half were treated with anti-CD25 (1 mg PC61 antibody on the day of graft transfer) to deplete (or starve of IL-2) any CD25^{+}regulatory T cells carried over in the graft. (B) Only those recipients treated with anti-CD25 rapidly rejected their grafts ($n = 5$), demonstrating that there were sufficient effector T cells within the transferred graft to cause acute rejection, whereas the untreated recipients maintained their grafts indefinitely ($n = 6$), showing that CD25^{+}regulatory T cells within the graft were actively maintaining the tolerant state ($P \leq 0.001$). In addition, the spleen cells from tolerant mice were able to reject (albeit incompletely) male skin grafts after adoptive transfer to female RAG$^{-/-}$recipients ($n = 6$; $P \leq 0.06$ compared with untreated skin transfer group), suggesting that tolerance in the original host was contained primarily within the tolerated graft itself.

either the Th1 or Th2 clones that were able to reject in empty RAG$^{-/-}$ recipients, showing that Tr1 cells are capable of acting as Tregs in this system. At no time have these Tr1 cells expressed *foxp3*, even under conditions that induce *foxp3* in naive T cells, nor was there any *foxp3* found in grafts that were being maintained after adoptive transfer of the Tr1 cells, showing they are a quite distinct cell population. This was confirmed by a detailed gene expression comparison using serial analysis of gene expression (SAGE) of CD4^{+}CD25^{+} T cells and Tr1 clones (87) and more recently by microarray comparisons of TGF-β-induced Tregs and Tr1 cells (unpublished data).

There were, however, a few genes that were coexpressed on both CD4^{+}CD25^{+} and Tr1 Tregs, including CD103, GITR, and CTLA-4 (34, 87). While CD103 may be mainly indicative of TGF-β exposure (Tr1 cells secrete both IL-10 and some TGF-β), GITR (88, 89) and CTLA-4 (35, 90) both seem to have potentially important roles in T-cell regulation. Interestingly, both genes were also expressed on the anergic and suppressive T cells generated after transfer of female A1.RAG$^{-/-}$ male-specific T cells into male (but not female) RAG$^{-/-}$ recipients, even though these cells were not expressing *foxp3*, *rog*, or IL-10 (83), distinguishing them from CD25^{+} Tregs or Tr1 cells. Agonistic antibodies to GITR have been shown to reverse suppression both *in vitro* and *in vivo* (88), but cloning of the mouse GITR ligand demonstrated that GITR behaves primarily as a costimulatory molecule in all T cells, giving a signal through

nuclear factor-κB that is able to override some of the effects of suppression (91). CTLA-4, however, was known to be a negative costimulator for T cells involved in the termination of responses (92–94), possibly by competing with its activatory partner CD28 for the CD80 and CD86 costimulatory ligands on antigen-presenting cells (95). A chimeric molecule known as CTLA-4-Ig was developed as a potentially immunosuppressive agent able to deliberately compete with CD28 and block costimulation (96). It was only recently discovered however, that CTLA-4-Ig could itself have profound effects on antigen presentation by signaling to $CD8^+$ DCs to induce indoleamine 2, 3-dioxygenase (IDO), an enzyme that catabolizes tryptophan (97, 98).

CTLA-4 expression by Tregs induces IDO and tryptophan catabolism

Tryptophan is nutritionally an essential amino acid, and it also seems to be required for normal T-cell proliferation (99). This finding means that any *in vivo* microenvironment that has been depleted of tryptophan is likely to be immunocompromised (100). The catabolism of tryptophan by IDO expression in tissues or antigen-presenting cells would locally starve T cells of this amino acid, limiting their proliferation in response to antigen. In addition, the kynurenine products of tryptophan catabolism can enhance the apoptosis of activated T cells (101). The physiological importance of IDO activity was first demonstrated in the context of a natural semiallogeneic graft: the fetal mouse (100). It had been a long-standing mystery how the immune system was able to avoid rejecting a fetus that expressed foreign histocompatibility antigens from the father when the mother's systemic immune system showed no overall signs of immune suppression. The essential role of IDO was demonstrated by giving pregnant mice the specific inhibitor 1-methyl tryptophan (1-MT) that was able to induce spontaneous rejection of semiallogeneic but not syngeneic concepti (100). Subsequently, the survival of semiallogeneic concepti was also found to be dependent on the presence, in pregnant mice, of $CD4^+CD25^+$ Tregs (102). As it is known that Tregs constitutively express CTLA-4 (103), it is tempting to speculate that they may act, in part, by inducing IDO in the fetomaternal environment, but this mechanism has not been directly demonstrated.

IDO and tryptophan depletion as one mechanism for linked suppression

As previously indicated, CTLA-4 is commonly expressed by different types of Tregs, including $CD4^+CD25^+$ Tregs, anergic suppressive cells, and Tr1 clones. The availability of Tr1 clones that overexpress CTLA-4 and are specific for the DBY antigen peptide presented by $H\text{-}2E^k$ (Tr1D1) (86) allowed us to test whether CTLA-4 induction of IDO on DCs may be a plausible mechanism for linked suppression. It had been shown that CTLA-4-Ig induced IDO on a particular population of splenic DCs that express B220 and CD8 (97), and we found that the Tr1D1 clone was also able to induce IDO protein (by immunohistochemistry) and IDO activity (tryptophan catabolism) in these DCs presenting the male peptide *in vitro*. This IDO activity was sufficient to inhibit the response of TCR transgenic $CD8^+$ T cells against $H\text{-}2K^b$ expressed on the same DCs as a demonstration of linked suppression (98). Suppression of $CD8^+$ T cells by the Tr1 clone in this system was entirely IDO dependent, in that it could be more than reversed by the IDO inhibitor 1-MT, or by adding back tryptophan, and was not observed if $IDO^{-/-}$ splenic DCs were used (98).

Mast cells and even Tr1 cells themselves within tolerated tissues may also be able to locally deplete tryptophan due to the expression of the enzyme tryptophan hydroxylase (TPH) that converts the amino acid into 5-hydroxytryptamine (serotonin) (71). It is only very recently that mast cells have been implicated in immune regulation (104), as opposed to their more generally accepted role in allergy and anaphylaxis. We have previously found that Tr1 cells express high levels of mRNA (87) and secrete the protein for IL-9 (measured by enzyme-linked immunosorbent assay) (unpublished data), particularly after activation, and that they tend to encourage mast cell growth and survival in tissue culture (71). Quantitative RT-PCR of tolerated skin grafts further demonstrated an excess of mast-cell-related genes, such as TPH, mast cell protease 5, and FcεR1α, when compared with rejecting allogeneic or syngenic skin grafts (71), suggesting that the presence of mast cells in some way correlates with the induction or maintenance of tolerance and the possibility that they may themselves play a role.

IDO and tryptophan transporters

Tryptophan depletion can also be achieved by non-IDO-dependent mechanisms. Lung epithelium expresses different forms of the tryptophan receptors (105, 106), consisting of CD98 in combination with either L-amino acid transporter 1 or 2 (LAT1 or LAT2), to give a low- or high-affinity receptor on the respective apical or basolateral surfaces (107). This mechanism ensures that tryptophan transport is polarized to deplete the amino acid from the luminal spaces and presumably acts to limit the response to environmental antigens.

IDO is also expressed constitutively on the interstitial DCs in the lung (108), such that the transported tryptophan is further depleted. Under conditions of inflammation, IFN-γ can also induce IDO on the epithelium directly (107), suggesting that the lungs have multiple levels at which they can limit the immune response, presumably because excess inflammation in the lungs is life threatening, as in the case of anaphylactic reactions. Appropriate expression of tryptophan transporters is also required in antigen-presenting cells, so that the tryptophan outside the cell can be depleted by the internally expressed IDO enzyme (105). Inhibitors of the tryptophan transporter system are able to block the ability of antigen-presenting cells to deplete tryptophan and suppress T-cell proliferation (105). This may explain why IDO protein expression has not always been seen to correlate with tryptophan-depleting activity.

Non-optimal signaling by antigen-presenting cells induces and maintains tolerance

IDO-mediated tryptophan depletion is only one example of how antigen-presenting cells may be able to drive the immune response toward tolerance. We have previously suggested that any environment where T cells receive incomplete or low and chronic antigen presentation is likely to initiate a default tolerogenic response (26). The minimalist Civil Service model (26) was an example of this response, where tolerant or anergic T cells were proposed to passively compete at sites of antigen presentation (acting like civil service bureaucrats), simply getting in the way of the efficient activation of non-tolerant naive T cells that would then default to a tolerant and anergic state, thereby propagating the process as an explanation of infectious tolerance. Evidence that incomplete antigen presentation can indeed generate tolerance and Tregs has been obtained in many systems, but we again focus on the A1.RAG$^{-/-}$ male skin transplantation system (53). Two examples already discussed, the blockade of CD4 (or other relevant surface molecules such as CD40L) with monoclonal antibodies *in vivo* and the transfer of the male-specific TCR transgenic T cells into unmanipulated RAG$^{-/-}$ male recipients, might be considered situations where T cells are interacting with antigen under non-optimal conditions for activation. Both situations generate tolerance, although the phenotype of Tregs that are induced appears to differ, with anti-CD4 inducing *foxp3*$^{+}$CD25^{+} Tregs (54), while exposure of male-specific T cells to a complete male mouse seemed to generate only *foxp3*$^{-}$CD25^{-} anergic T cells (83). These findings suggests that the mechanism is more complex than the minimalist Civil Service model would imply. A more deliberate means to ensure incomplete signaling to the TCR is to generate artificially altered peptide ligands (APLs) that are equivalent to the native antigen peptide in binding to the MHC class II on the antigen-presenting cell but which have a conservative amino acid substitution at a TCR contact residue, such that they behave as only partial agonists (109). A partial agonist APL of the DBY male peptide was found to only weakly activate A1.RAG$^{-/-}$ T cells *in vitro* with a predominant IL-10 cytokine response reminiscent of Tr1 cells (110). When administered *in vivo* in advance of a male skin graft, tolerance was induced that was correlated with some T-cell deletion as well as with the generation of CD25^{+}*foxp3*$^{+}$ Tregs that were again found particularly in the graft (110). This study shows that compromised TCR signals (signal 1) can lead to tolerance and the generation of Tregs, but it would seem likely that that the antigen-presenting cell is a strong candidate for the identification of further mechanisms to downmodulate signals and enforce tolerance in T cells.

Tolerogenic DCs

There is now considerable evidence that DCs are not only important sentinels for alerting the immune system to infection (111) but also are able to circulate through tissues in the steady state and acquire self-antigens to continually maintain self-tolerance in the periphery (112, 113). It remains a matter of some debate whether there are specialized subsets of naturally occurring DCs that present only for tolerance or whether the immature phenotype of the steady-state DC distinguishes its presentation for tolerance from that of the inflammatory state of mature DCs. The discovery that large numbers of DCs can be generated by growing either bone marrow or peripheral blood monocytes in appropriate cytokines *in vitro* has stimulated much interest in their possible uses in cell therapies, either to improve vaccination (114) or to induce tolerance after appropriate manipulation to 'freeze' them in an anti-inflammatory or tolerogenic state (115, 116). It seems that a number of pharmacological manipulations are indeed able to modulate bone-marrow-derived DCs toward a potentially tolerogenic phenotype, expressing lower levels of MHC class II and costimulatory ligands, such as CD40, CD80, and CD86 on their surface, together with a reduced secretion of certain proinflammatory cytokines, such as IL-12, even after subsequent exposure to Toll-like receptor maturation signals, such as lipopolysaccharide (LPS) (34, 117). These agents include vitamin D3 (116) and its analogues, TGF-β (118), IL-10 (117, 119), vasoactive intestinal peptide (120), aspirin (121), and dexamethasone (122, 123).

We have taken an approach similar to that taken with Tregs for DCs. To identify the most important molecular mechanisms, we looked for a gene expression signature that is shared by a range of different DCs with a proven ability to induce tolerance in a single *in vivo* system.

Gene expression in tolerogenic DCs

Bone-marrow-derived DCs from male CBA mice were treated with each of the pharmacological agents vitamin D3, IL-10, or TGF-β, either alone or in combination with the maturation stimulus of LPS. Each population of modulated DCs was injected into A1.RAG$^{-/-}$ TCR transgenic mice that were challenged 1 month later with male skin grafts (*Fig.* 4). While control A1.RAG$^{-/-}$ mice and those that received DCs matured with LPS alone rapidly rejected their male grafts, all the pharmacologically treated groups held their grafts indefinitely, even if the DCs had been exposed to LPS after their modulatory treatment (Yates *et al.*, manuscript in preparation, Paterson *et al.*, manuscript in preparation). In addition, mice given untreated immature DCs also became tolerant. All tolerant mice showed evidence of *de novo foxp3* expression in their spleen cells and a high level of *foxp3* in their tolerated grafts (as measured by quantitative RT-PCR), indicating that tolerance had been generated via the induction of Tregs. This study validates all three pharmacological treatments as being tolerogenic in this model and further demonstrates that such modulated DCs are resistant to Toll-like receptor maturation signals, such as LPS, *in vitro* and do not revert to an immunogenic phenotype after adoptive transfer *in vivo*.

A gene expression signature in common to all three types of modulated DCs, both before and after LPS exposure, was searched for using a combination of a wide range of SAGE libraries (34) and microarray experiments. Data handling and pattern searching algorithms were developed in-house (Cobbold *et al.*, manuscript in preparation) to identify the most consistent cluster of genes that were positively or negatively associated with the tolerogenic DCs. Two overall patterns of gene expression emerged from these studies that both confirmed and extended the observations already published for the effects of IL-10 treatment on bone marrow DC gene expression (34, 117). First, it was clear that the LPS response of all the modulated DC populations was somewhat blunted, as had been expected from their reduced expression of MHC class II, CD80, CD86, CD40, and IL-12, although the different populations each failed to upregulate different sets of

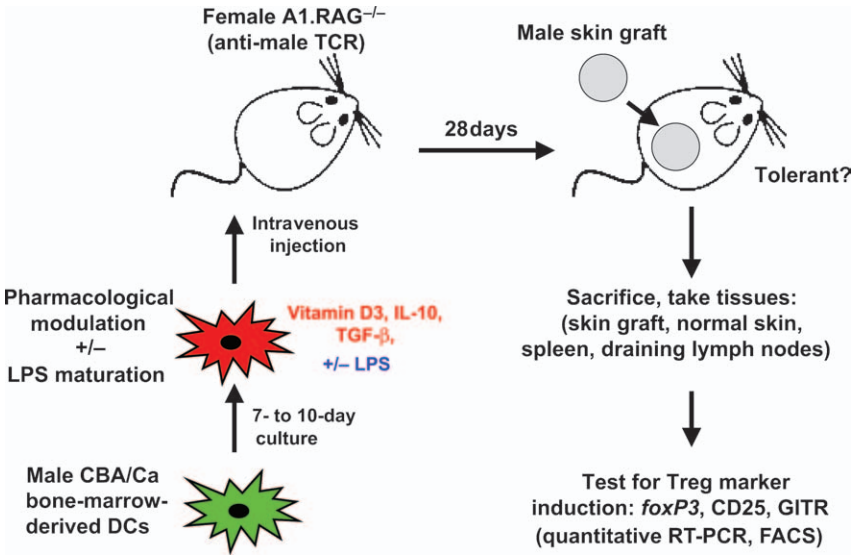

Fig. 4. Pharmacologically modulated DCs induce tolerance *in vivo*. Male CBA/Ca bone-marrow-derived DCs were generated *in vitro* by culture in the presence of exogenous granulocyte macrophage colony stimulating factor (GM-CSF). Some of these DC cultures were further exposed to one of the pharmacological agents at appropriate concentrations: 1,25-dihydroxy vitamin D3, IL-10, or TGF-β. Each type of modulated or control DC culture was also split, and half were exposed to a dose of bacterial LPS sufficient to mature untreated DC cultures. These cells were then injected intravenously into A1.RAG$^{-/-}$TCR transgenic female mice, and 28 days later they were challenged with male skin grafts. Control recipients that did not receive any DCs rejected their male grafts rapidly, as did those that received LPS-matured, untreated DCs. All groups that received modulated DCs, regardless of exposure to LPS, and those that received immature DCs kept their male skin grafts indefinitely. After more than 1 month, the spleens and healthy skin grafts were then analyzed by immunofluorescence and quantitative RT-PCR. Tolerant mice all had higher levels of *foxp3*in the spleen and especially within the tolerated grafts. In addition, there was an increased proportion of T cells in the spleen that coexpressed CD4, CD25, and GITR, indicating the presence of regulatory T cells induced by the tolerogenic DCs.

LPS-responsive genes. Second, it was possible to define a core set of signature genes that were maintained in the majority of tolerogenic populations, particularly after their exposure to LPS (Paterson *et al.*, manuscript in preparation). This definition suggests that there is a balance of activatory and inhibitory signals that normally give a measured inflammatory response to pathogens, but that tolerogenicity reflects a change in this balance toward inhibition. Many of the genes we found maintained in tolerogenic DCs are currently being investigated further, including hemoxygenase-1 (HO-1), programmed cell death ligand-1 (PD-L1), and three immunoreceptor tyrosine-based inhibitory motif-bearing receptors, gp49B, PILRα, and Fcγr2b. Both gp49B and PILRα are members of the immunoglobulin-like transcript family that includes, in humans, ILT3 and ILT4, which are upregulated in DCs that have been exposed to Tregs *in vitro* (75, 124). Although ILT3 is upregulated in human monocyte-derived DCs treated with vitamin D3, it has been shown that this is not necessary for their ability to induce Treg cells *in vitro* (125). The mouse equivalents of ILT3 and ILT4 remain unclear, although they appear to have close homology to the mouse gp49B gene (126). Members of the paired immunoglobulin-like receptor family (127, 128) are believed to exist as mutually antagonistic pairs, such that an excess expression of the inhibitory partner would generate an overall negative signal. Such negative signals may be important to maintain the DCs in their 'semimature' state in the face of maturation signals via the Toll-like receptors or T-cell costimulation.

Negative costimulation

In addition to signal 1 provided by the TCR recognition of MHC-presented antigen peptides, the activation of a T cell is modulated by a series of costimulatory signals (together known as signal 2) that are principally provided by the interaction of cell surface receptors of the CD28 (129) and tumor necrosis factor receptor (TNFR) families with their ligands on the antigen-presenting cell. The overall level of costimulation given to the T cell seems to depend on an integration of both positive and negative signals given through different members of each family. For example, CD28 binding to the ligands CD80 and CD86 on the antigen-presenting cell represents the classical positive costimulatory stimulus for T-cell activation, while the closely related molecule CTLA-4 binds to and probably competes for the same antigen-presenting cell ligands and gives a strong negative signal that inhibits T-cell activation. Because CTLA-4 is normally expressed only after the activation of naive or effector T cells, while CD28 is constitutive, it is believed that the positive CD28 costimulation dominates the initial antigen recognition events while CTLA-4 is later involved in limiting overexcessive activation and proliferation of activated T-cell clones (130). As discussed above, it has also recently been found that the CTLA-4 interaction with either CD80 or CD86 can also have functional consequences for the antigen-presenting cell as well, inducing a tolerance-promoting activity dependent on IDO induction (97).

Another member of the CD28 family, programmed cell death 1 (PD-1), was identified recently as a negative costimulatory molecule particularly associated with anergic T cells (131) that was able to induce their apoptosis (132). This molecule has two known ligands (PD-L1 and PD-L2) (133) that, like CD80 and CD86, are members of the B7 family. PD-1 has been shown to regulate the alloimmune-specific delayed-type hypersensitivity response *in vivo* (134), and it can suppress $CD4^+$ T-cell-mediated graft rejection (135). In certain chronic viral infections, cytotoxic T lymphocyte 'exhaustion' can also be reversed by blocking PD-1/ligand interactions (136). PD-L1 is expressed at high levels on the placental deciduas (137) and plays an important role in maintaining the maternal tolerance of the fetus, as PD-$L1^{-/-}$ mice or mice given a blocking anti-PD-L1 (but not anti-PD-L2) antibody reject a semiallogeneic but not a syngeneic pregnancy. This effect was apparently independent of IDO expression (138). It is therefore quite possible that the expression of PD-L1 within the signature for a tolerogenic DC is of functional relevance.

Blocking costimulation through TNF/TNFR interactions, in particular with antibodies to CD154 to block the activation of the antigen-presenting cell via CD40, tends to bias the immune response towards tolerance (139–141). While this is mainly believed to be due to an inhibition of DC maturation and activation, there is some suggestion that this may involve the induction of the cytoprotective molecule HO-1 in either the antigen-presenting cell or the grafted tissue (142). Induction of HO-1 (but not HO-2) by cobalt protoporphyrin injection or the induction of Th2 cytokines blocked chronic cardiac graft rejection mediated by alloantibodies (143).

HO and carbon monoxide in T cells, antigen-presenting cells, and tissues

We have observed that HO-1 expression is found in a wide range of T cells, including both $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets and also Th1 and Tr1 clones (as measured by SAGE) (34). It does seem, however, to be specifically upregulated mainly in the LPS-treated tolerogenic but not the immunogenic DC populations. HO-1 is an enzyme that catalyzes the rate-limiting

step in the breakdown of heme, resulting in the generation of iron, carbon monoxide (CO), and biliverdin (144). Biliverdin is further converted to bilirubin by biliverdin reductase. HO-1-deficient mice develop an autoimmune-like chronic inflammation with splenomegaly, hepatic inflammation, and occasional glomerulonephritis and exaggerated Th1 responses (145). HO-1 deficiency in humans is also associated with lymphadenopathy and an increased sensitivity to oxidant injury (146). Expression of HO-1 in tolerated tissues could therefore protect from immune attack involving the oxidative burst of activated neutrophils and macrophages associated with a delayed-type hypersensitivity reaction and has been postulated as a mechanism of action for natural $CD4^+CD25^+$ Tregs (147). In addition, the CO generated may itself have important regulatory functions, as it has been shown to have anti-proliferative effects on both immune and non-immune cells. Although the mechanism for the anti-proliferative effects of CO is not well understood (148), it has been suggested to act by increasing the levels of cyclic guanosine-3′,5′-monophosphate, enhancing the mitogen-activated protein kinase signaling pathway, while inhibiting extracellular signal-regulated kinase activation (149). In T cells, CO has been shown to block IL-2 production in naive T cells (150), suggesting a possible mechanism for the induction of anergy. CO itself can be administered to mice to downregulate inflammation and treat autoimmune colitis (151). Furthermore, it has been found that CO can block the upregulation of inducible nitric oxide synthase (iNOS) (152) and therefore the production of the Th1-related toxic NO effector molecules.

It may also be relevant that the NO generated by iNOS is a product of the catabolism of the amino acid L-arginine. iNOS is upregulated by both Th1 cells and macrophages, and the NO generated can act not only as a toxic effector molecule, killing target cells, but also to limit the size of the Th1 response (153), inducing apoptosis in Th1 and cytotoxic T cells (154, 155). An alternative pathway of L-arginine metabolism is the urea cycle via the enzyme arginase (that exists in two isoforms) (156). We have found arginase I to be highly expressed in tolerogenic DCs (especially IL-10-treated DCs +/−LPS) (34, 117), and this enzyme could theoretically act in a manner analogous to IDO in catabolizing arginine and reducing the potential for NO production by limiting the substrate. Others have also observed arginase upregulation in alternatively activated antigen-presenting cells (157). Transport of L-arginine across the cell membrane is also mediated by the same y+/LAT/CD98 transporters as tryptophan (158); therefore, it is likely that the same mechanisms that deplete tryptophan from the lumen of the lungs, for example, and transport it into cells for catabolism by IDO (107) also apply to arginine, especially if cells also express arginase for catabolic activity. It has been suggested that arginine levels are detected by T cells through the same stress response kinase (GCN2) that is believed to be required for effective IDO suppression and that there is cross-talk between the IDO and iNOS pathways (159).

The healing graft itself may be a major stimulus for this upregulation of arginase, as it can be a major source of both IL-10 and TGF-β, which are produced by both tissue macrophages and keratinocytes (160), and these may act to both maintain local DCs in their more tolerogenic state, including the expression of arginase, and induce or maintain local Tregs (161). All the above mechanisms acting together within the graft in response to local Tregs are proposed to maintain tolerance by the active participation of both the antigen-presenting cell and the grafted tissue itself in providing protection through a form of acquired immune privilege (*Fig.* 5).

Conclusions and looking forward

While the focus on mechanisms of transplantation tolerance over the last 10 years has been primarily on the generation of

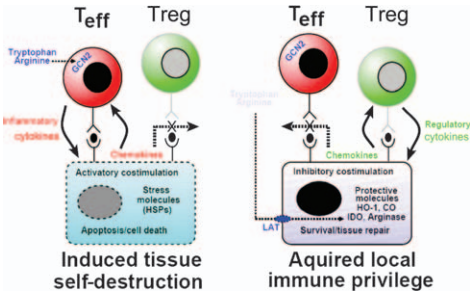

***Fig.* 5. The interaction of regulatory T cells and target cells in induced immune privilege.** Antigen-presenting cells and the target tissue cells actively participate in both rejection of and tolerance to a graft. In the case of rejection, effector T cells (T_{eff}) are preferentially attracted by proinflammatory chemokines and cytokines, where the presentation of antigen with costimulatory ligands induces their activation and proliferation, as long as sufficient tryptophan and probably also arginine are available for metabolism. The death of the target cell is also an active response to both stress signals and apoptotic signals through the activation of caspases by death receptors and granzymes. Alternatively, in the case of tolerance, anti-inflammatory cytokines and chemokines that attract regulatory T cells predominate. The interaction of negative costimulatory ligands with their receptors on both T cells and APCs ensures that the tolerogenic environment is maintained and further induces protective genes in the APCs and target cells, including HO-1 that generates CO that has additional anti-inflammatory properties, and IDO and arginase that together with the common LAT can deplete tryptophan and arginine from the local environment. The levels of these two amino acids are sensed in T cells by the kinase GCN2 that may be responsible for limiting their activation and proliferation. HSP, heat shock protein.

Tregs and how they may directly suppress the immune response, it is becoming clear that the role of the graft in protecting itself from immune attack may be of equal importance. This understanding has many implications both for the requirements to achieve therapeutic tolerance and for surrogate assays to determine the status of a transplanted patient before attempting to reduce or wean him/her off immunosuppression. We already know that certain immunosuppressive drugs may be countertolerogenic (162) because they block T-cell activation signals required to generate effective Tregs, but we should also consider whether any of the standard immunosuppressive agents may also block the upregulation of protective mechanisms within the graft itself (163). Similarly, we have tended to concentrate on biomarker assays that may indicate the presence or activity of Tregs, for example, it has been shown that *foxp3* transcripts in urine are a useful predictor of renal allograft survival (164), and it may be necessary or more sensitive to measure indicators of gene expression that are associated with tissue self-protection in order to reliably predict whether a patient will keep his/her graft if immunosuppression is tapered off. Finally, in the future, we may be able to design drugs that complement the induction of Tregs and tolerance by inducing protective genes within the organ graft directly.

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