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Induction of Immunological Tolerance as a Therapeutic Procedure

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ABSTRACT A major goal of immunosuppressive therapies is to harness immune tolerance mechanisms so as to minimize unwanted side effects associated with protracted immunosuppressive therapy. Antibody blockade of lymphocyte coreceptor and costimulatory pathways in mice has demonstrated the principle that both naive and primed immune systems can be reprogrammed toward immunological tolerance. Such tolerance can involve the amplification of activity of regulatory T cells, and is maintained through continuous recruitment of such cells through processes of infectious tolerance. We propose that regulatory T cells create around them microenvironments that are anti-inflammatory and endowed with enhanced protection against destructive damage. This acquired immune privilege involves the decommissioning of cells of the innate as well as adaptive immune systems. Evidence is presented that nutrient sensing by immune cells acting through the mammalian target of rapamycin (mTOR) pathway provides one route by which the immune system can be directed toward noninflammatory and regulatory behavior at the expense of destructive functions. Therapeutic control of immune cells so as to harness metabolic routes favoring dominant regulatory mechanisms has offered a new direction for immunosuppressive therapy, whereby short-term treatment may be sufficient for long-term benefit or even cure.

INTRODUCTION

A major goal of immunosuppressive therapy in management of chronic inflammatory diseases and allogeneic transplants has been to harness long-term tolerance processes from short-term treatments. This should limit morbidity from long-term undermining of immune mechanisms, which is the hallmark of current immunosuppression.

Historically, dendritic cells (DCs) have represented one arm of the innate immune system; these cells need to interact with the adaptive system to provide immunity from microbial infection. More recently, myeloid cells have also been seen as partners for acquisition of immunological tolerance in adaptive lymphocytes. Not only can they ensure appropriate antigen presentation to induce tolerance, but evidence is accumulating that they can also act as active participants to regulate or suppress the adaptive system. In this article, we examine the interplay between lymphocytes and DCs as the basis for therapeutic reprogramming of the immune system toward tolerance.

A long-standing dogma of immunology was that self-tolerance was mediated by clonal inactivation and deletion of antigen-specific lymphocytes. Much of this was thought to occur in the primary lymphoid organs, with the job completed in the so-called peripheral immune system. The identification of patients suffering from autoimmune diseases associated with the APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy) syndrome, due to mutations in the *AIRE*

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gene, provided confirmation of the importance of antigen presentation in the thymus as a key primary lymphoid organ engaged in tolerogenesis (1). Much work in the early 1990s pointed to additional regulatory mechanisms that operated to prevent autoimmune disease and gut immunopathology, as well as prevention of graft rejection. The clinical relevance of these early studies was established clearly with the identification of the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome (2, 3), in which pathology resulted from defects in expression of the *FOXP3* gene, which determines in large part the phenotype of a subset of CD4 T cells that have come to be referred to as regulatory T cells (Tregs).

The advent of monoclonal antibodies (MAbs) directed to many surface molecules of T cells not only provided magic bullets to ablate lymphocytes and their subsets but also allowed for the identification of key molecules involved in translating immunogenic signals from DCs to T lymphocytes. Nonlytic antibodies were developed to block these signals (4). Using these probes, it has been possible, in preclinical models, to induce tolerance to transplants and to restore tolerance in autoimmune disease models.

In this chapter, we will describe what we know about mechanisms that underlie such therapeutic tolerance. The harnessing of Foxp3$^+$ regulatory T cells emerges as a major feature of one form of therapeutic tolerance in these experimental systems, and orchestration of protective immunosuppressive processes in the protected tissues has a key role for innate cells. Parallels can likely be drawn between the changed microenvironment of tolerized tissues and those of tumors. We will focus largely on studies using coreceptor and costimulation blockade with CD4, CD8, and CD154 (anti-CD40L) antibodies in mouse models of allogeneic transplantation to draw mechanistic conclusions. The authors underwent major conceptual conversions in the course of this work, and we thought it would be of value to the readers of this chapter for us to give a historical overview of how that conversion took place. In particular, we wish to highlight the importance of regulatory activity operating within tolerated tissues.

Until the late 1980s immunologists favored deletional/inactivation (passive) models of tolerance and had come to regard the idea of T-cell-mediated suppression (active tolerance) as "unsafe." Against this background we were attempting to induce therapeutic tolerance to foreign proteins and to transplanted tissues, and became confronted with data that seemed incompatible with the passive interpretation. The findings, starting some 30 years ago, and summarized below, have led us to the conclusion that therapeutic tolerance can be achieved when regulatory T cells are empowered to dominate the immune response to antigen.

TOLERANCE TO FOREIGN PROTEINS CAN BE ACHIEVED WITH A SHORT PULSE OF MAbs

Our work began with the observation that rat MAbs to mouse CD4 were not, unlike other antibodies targeting lymphocytes, immunogenic (5, 6). They could, furthermore, induce tolerance to other therapeutic antibodies and to aggregated human IgG, normally a strong immunogen in mice. Particular nonlytic anti-CD4 antibodies could even induce tolerance without depleting CD4 T helper cells (7–9).

The tolerance achieved was, however, not permanent and was lost over time unless, paradoxically, animals were rechallenged with the immunogen (9). In other words, tolerance involved "memory" for the tolerizing antigen. Also, and quite surprising to us, tolerance could not be broken by transfusions of normal lymphocytes—a phenomenon for which we coined the term "resistance." At that time, it was hard to think of this form of tolerance as anything other than active, but given the then-current skepticism over suppression, we compromised on an explanation that we called the "civil service model" (10, 11). We proposed that T cells, inactivated or anergized by antigen, competed with competent T cells for antigen or activation molecules in the local microenvironment of individual "myeloid" DCs. Strong competition would result in strong suppression.

INDUCTION OF TOLERANCE TO TRANSPLANTED TISSUES

The observation that memory of tolerance could be sustained by booster doses of immunogen led us to speculate that coreceptor blockade might also enable tolerance to transplants, where the engrafted tissue would provide continuous supplies of the "booster" antigens. That indeed proved to be the case for skin grafts mismatched for multiple minor antigens (minors), as well as for major histocompatibility complex (MHC)-mismatched grafts (12).

Tolerance could even be induced this way in mice previously primed to minors (13). Tolerance across MHC barriers was somewhat more difficult to achieve and could be enhanced by costimulation blockade through the addition of an antibody to the costimulatory ligand CD40L (CD154) (14). Once again we observed "resistance," as tolerance could not be overcome by in-

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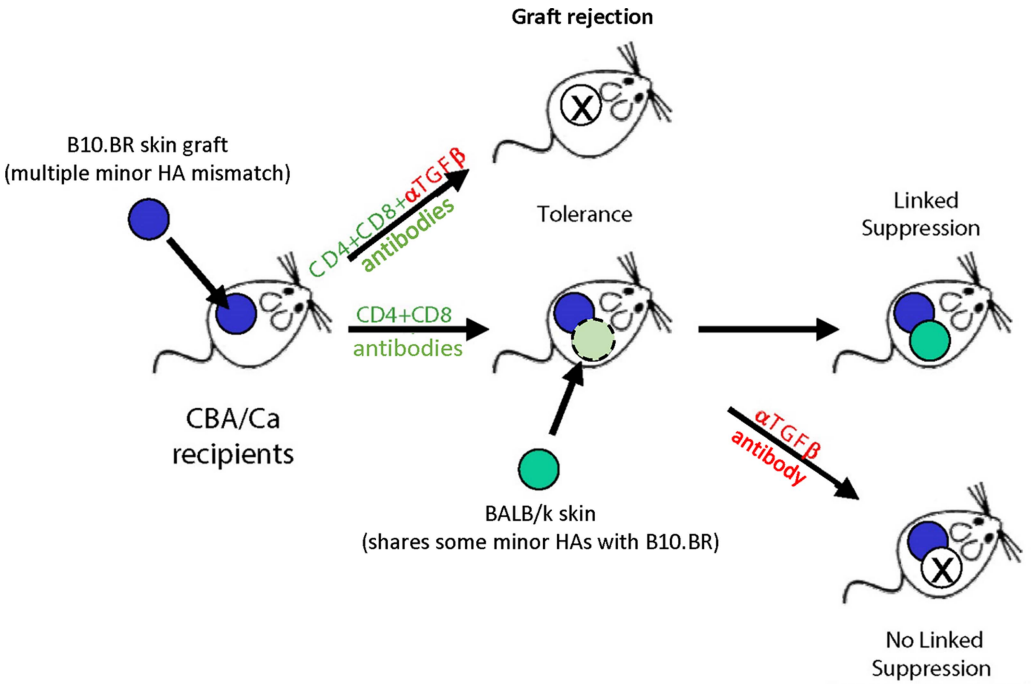

FIGURE 1 Coreceptor blockade induces transplantation tolerance and linked suppression in adult mice. CBA/Ca mice would normally rapidly reject skin grafts from either B10.BR or BALB/k mice, as they differ in minor histocompatibility antigens. If the recipients are given a brief course of nondepleting MAbs that block the CD4 and CD8 coreceptors at the time of grafting, B10.BR skin grafts are, however, permanently accepted. Remarkably, such mice made tolerant of B10.BR skin can accept third-party grafts, even from "multiple minor" different BALB/k donors, if they share sufficient antigens in common with the tolerated graft, in a process known as linked suppression. Both the induction of tolerance and the process of linked suppression are blocked by antibodies that neutralize active TGF-β.

fusion of normal lymphocytes (12). Strikingly, though, we could also demonstrate that the tolerance could spread to third-party antigens when these were presented together with the tolerated antigens in the challenge grafts (linked suppression) (15, 16) (Fig. 1). Functional studies on blood T cells and splenocytes indicated that "passive" tolerance was unlikely as mixed lymphocyte reaction (MLR), cytotoxic-T-lymphocyte generation, and interleukin-2 (IL-2), IL-4, and gamma interferon cytokine release were comparable between tolerized hosts and control mice (17, 18). This left us having to consider that a significant component of therapeutic tolerance might operate in the tolerated tissue or its draining lymph nodes. How could that be?

TRANSFERABLE SUPPRESSION BY CD4 T CELLS

Despite the universal skepticism about suppressor T cells that was rife at the time, we performed adoptive transfer studies to ask if tolerant T cells might suppress rejection by naive T cells. Not only did this turn out to be the case, but the active suppressors turned out to be $CD4^+$ T cells (13, 19, 20) and not $CD8^+$ T cells, as the discredited old literature had claimed.

We asked whether a tolerized immune system removed from its tolerated antigens would stay tolerant. The answer proved unequivocal. Tolerance was lost once lymphocytes were removed from a source of persisting antigen (12, 13, 17, 21). This definitively established that tolerance could not have arisen just through passive deletion of all antigen-specific T cells. The alternatives were that tolerance involved a reversible inactivation of T cells or dominance of an active suppressive or regulatory population, that persisting dominance being dependent on a continuous supply of antigen.

If the latter were the case, would the need for antigen be to provide boosts of the first cohort of $CD4^+$ Tregs, or might it be required to recruit further cohorts of Tregs into graft protection, or perhaps both? By using marked populations of CD4 T cells, we were able to show that new cohorts of $CD4^+$ Tregs were being continuously

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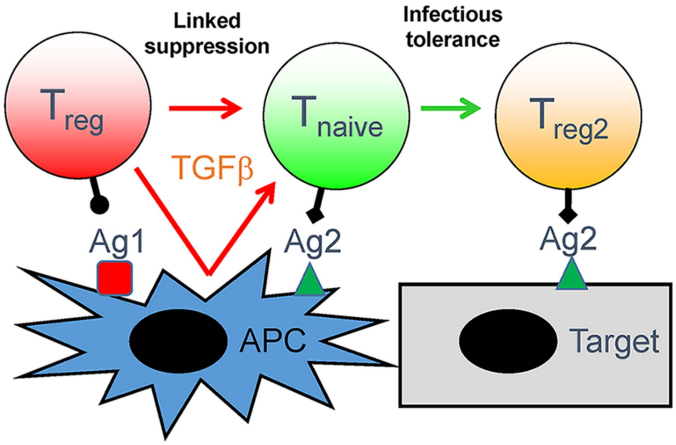
recruited within tolerant animals, under the influence of previous cohorts (19, 22). We dubbed this form of regulation "infectious tolerance." T cells mediating infectious tolerance were also responsible for linked suppression, suggesting that these two phenomena were all manifestations of the same $CD4^+$ Tregs (23) (Fig. 2).

HOW MIGHT ANTIBODY THERAPY BE FAVORING THE EMERGENCE OF Tregs?

We asked whether the tolerizing effects of antibody blockade were immediate or whether they required time to develop. Splenocytes from mice given the tolerizing antibody protocol were transferred into lymphopenic hosts (away from the therapeutic agent) at different times during the treatment regimen. In the case of tolerance to multiple minor antigens, we observed that it took some 3 weeks of antibody administration before sampled splenocytes behaved as if tolerant (21). Before then, they remained fully capable of rejecting donor grafts. In other words, tolerance could not be induced immediately but required some time to reach completion.

This led us to conclude that the therapeutic antibodies likely operated by fulfilling two requirements. First, they fully controlled T-cell rejection events by blindfolding T cells for as long as it took the immune system to (secondly) establish a numerical or functional advantage for Tregs. The issue of how and where regulation operated required some way of distinguishing Tregs from other T cells, and this will be discussed later.

FIGURE 2 Linked suppression depends on an interaction between regulatory T cells and antigen-presenting cells (APCs). The copresentation of tolerated (Ag1) and third-party (Ag2) antigen by the same APCs promotes an interaction between the Tregs maintaining tolerance and naive T cells that would otherwise have the potential to develop into effector cells against tissues expressing the target antigen. This "linking" of the two antigens by the APC allows the Treg to suppress the naive T cell (i.e., linked suppression) and, through the action of TGF-β (and other additional mechanisms, not shown), to also guide the naive T cell to differentiate into a second cohort of regulatory T cells, thereby further enforcing tolerance to the target antigen (i.e., infectious tolerance).



HOW IS ANTIGEN PRESENTED TO MAINTAIN TOLERANCE AND REGULATION?

We were able to implicate host DCs as key elements in ensuring continued regulation by demonstrating that graft antigens could be processed and presented by DCs to induce tolerance and to amplify Tregs (16, 24–26) (Fig. 3). We were accustomed to the notion that DCs present antigen for immunity, but were here having to postulate that in the course of "infectious tolerance" host DCs were also presenting for tolerance and regulation.

To reconcile how this could be, we envisaged that host DCs were processing antigen from well-healed grafts that would be free of inflammatory signals (danger-free), either from modulation of DC function or simply lack of activation signals. In such an environment where inflammatory quiescence was also enforced by Tregs, the DCs might only be able to present antigen in a way that would encourage tolerance and Tregs but not provoke damaging immunity. This tolerogenic presentation of antigen by myeloid cells could then be at the root of infectious tolerance. In short, continuous antigen presentation by DCs that are compromised for activation enables them to maintain the active tolerant state (16).

DCs "DECOMMISSIONED" BY IL-10 AND TGF-β CAN PRESENT FOR TOLERANCE AND INDUCE Tregs IN THE PERIPHERY

The notion that resting DCs, or DCs decommissioned from activation, could present for tolerance was tested by asking whether IL-10- or transforming growth factor β (TGF-β)-conditioned DCs could present Dby (male) peptide for tolerance rather than for immunity in the same TCR (T-cell receptor) transgenic mice as discussed above (25). Presentation by decommissioned DCs did indeed allow tolerance, and this was associated with induction of peripheral Tregs in one TCR transgenic strain of mice but not in another. This finding in the one strain was consistent with earlier studies showing that DCs can constitutively present antigen for tolerance and regulation (27) but (on the basis of the differing result in the other strain) that outcome would depend on properties of the responding T cell.

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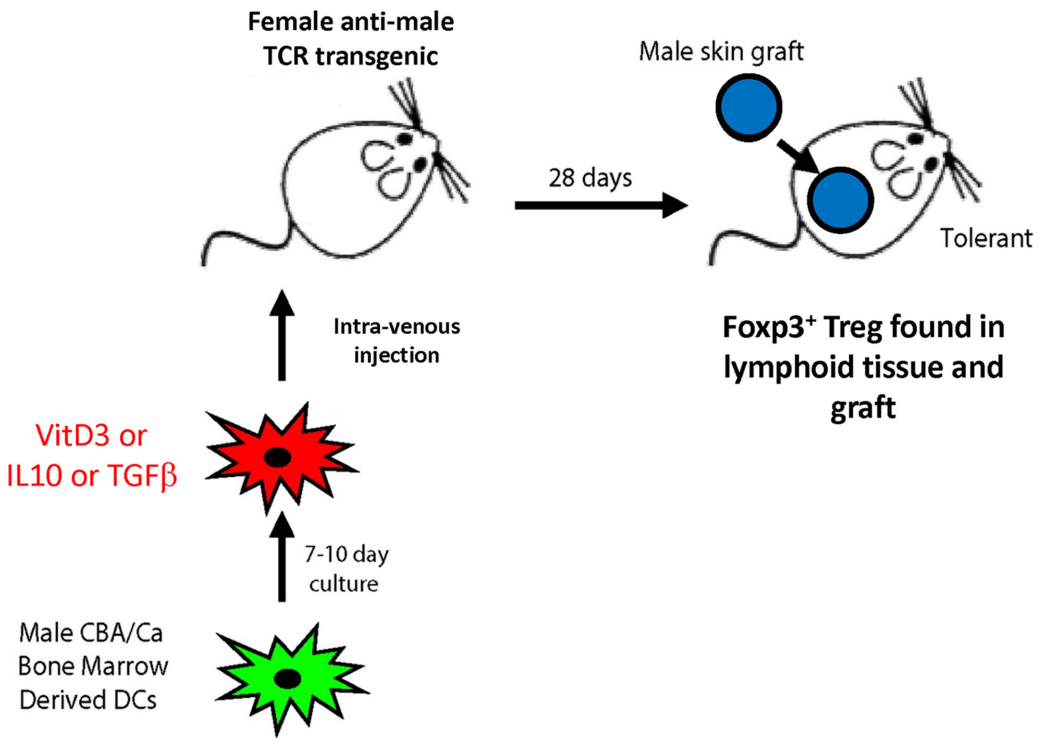

FIGURE 3 Tolerance can be induced by pharmacological modulation of DCs. Female anti-male TCR transgenic mice would normally reject male skin grafts rapidly. If such mice are administered mature (lipopolysaccharide-activated), bone marrow-derived DCs from male mice, they are further primed for graft rejection. In contrast, pretreatment of the bone marrow-derived DCs by any one of a number of modulatory agents, such as VitD3, IL-10, or TGF-β (which suppress DC maturation), induces a state of tolerance to the male antigen. Such tolerant mice now accept male skin grafts indefinitely, and while this is not associated with deletion of male-specific T cells, it does lead to the systemic induction of $Foxp3^+$ anti-male Tregs.

Foxp3 AS A MARKER OF REGULATORY T CELLS

Until some 15 years ago, it had been difficult to pursue the biology of regulatory T cells because of the lack of a surface marker by which they could be manipulated. The expression of $CD25^{hi}$ (28) or $CD45RB^{lo}$ (29) offered some opportunities, but it was only when the forkhead transcription factor Foxp3 was identified as a unique marker for Tregs (2, 30–32) that research into the mechanisms underlying transplantation tolerance could progress further.

The need for reductionist systems to study therapeutic transplantation tolerance led us to use TCR transgenic mice whose only adaptive lymphocytes were CD4 T cells carrying a TCR directed to a single minor transplantation antigen (the male antigen Dby) (33–35). Female mice carried no T cells expressing Foxp3, as they could not generate the natural Tregs that conventional mice produce in their thymus. Females could be tolerized to male skin grafts by a short pulse of anti-CD4 treatment (18). Despite exhibiting tolerance, many TCR^+ CD4 T cells could still be detected in their lymphoid organs. $Foxp3^+$ T cells could be seen to accumulate over time in the periphery of these mice, with a particularly high frequency within the tolerated tissue itself. By definition, these must have been peripheral Tregs, induced outside of the thymus.

A precedent for extrathymic Tregs had been postulated by Wanjun Chen, who observed that CD4 T cells whose TCR was cross-linked *in vitro* in the presence of TGF-β would also convert to expression of Foxp3 (36). Consistent with our $Foxp3^+$ cells in tolerant mice, cells being programmed by TGF-β, we showed that tolerance and linked suppression were not inducible if TGF-β was neutralized by an appropriate antibody (Fig. 1), and that TCR transgenic T cells would convert to Foxp3 expression and suppressive function when exposed to antigen and TGF-β *in vitro* (14, 18, 37). In contrast,

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therapeutic tolerance could not be induced in TCR transgenic mice genetically defective in TGF-β-mediated signaling to T cells (14, 37). To establish that it was not just TGF-β but the Foxp3 induced by TGF-β that mattered, we went on to show that TCR transgenic mice lacking a functional Foxp3 gene failed to be tolerized by CD4 blockade (37).

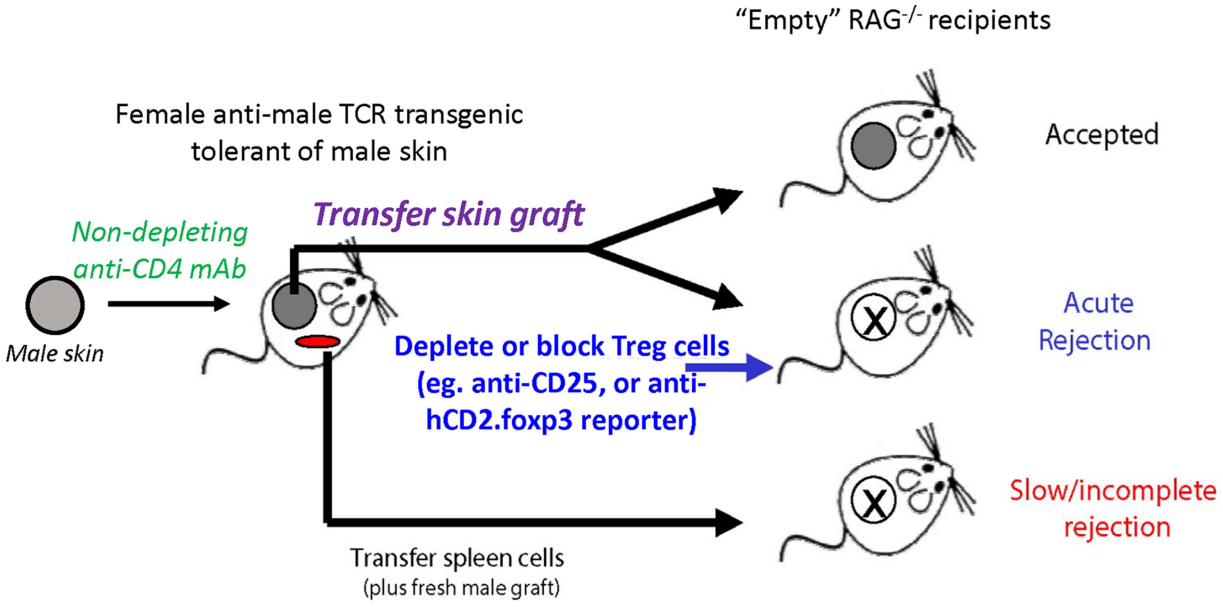
FUNCTIONING Tregs CAN BE FOUND WITHIN THE TOLERATED GRAFT

We asked whether functional Tregs could be found within a tolerated graft. To do this we retransplanted tolerated grafts onto lymphopenic mice, waited sufficient time for their immune system to reconstitute from the graft, and then tested these mice for "resistance" to breakdown of tolerance following infusion of naive lymphocytes (38). "Resistance" is what we observed. This experiment was the first to show Treg localization to tolerated grafts and was a vindication of our early thinking that active suppression must have a strong local tissue element to it.

The importance of Foxp3 expression in tolerance induction was further established when we were able to exploit a cell surface marker (human CD2; hCD2) in a novel transgenic strain in which a cDNA encoding the hCD2 was knocked in downstream of the Foxp3 locus (39). After exposure to antigen in the presence of TGF-β *in vitro*, only the T cells expressing Foxp3 were able to suppress transplant rejection *in vivo*, while their Foxp3-negative counterparts could not (37). Use of an anti-hCD2 antibody to ablate Tregs *in vivo* showed us that intragraft Tregs were active in ensuring graft protection from potential aggressors also located in the graft (40) (Fig. 4).

Finally, using the hCD2 marker and an adoptive transfer protocol, we showed that some TCR transgenic CD4 T cells that had been "suppressed" had converted to Foxp3 expression (40, 41). In other words, "infectious tolerance" could be explained, at least in part, by

FIGURE 4 Regulatory T cells actively maintain tolerance within accepted skin grafts. Female anti-male TCR transgenic mice can be made tolerant of male skin grafts by a single injection of nondepleting anti-CD4 MAb. This is associated with the induction of Foxp3$^+$ Tregs, which are particularly concentrated in the tolerated skin but are often only at a low frequency systemically. This low frequency may not be sufficient to transfer tolerance with spleen cells (unless boosted by systemic antigen), but if the tolerated graft itself is adoptively transferred to empty mice, the grafts are accepted. This is not just passive acceptance by the mice that have no adaptive immune system of their own, because the administration of antibodies that block or deplete Tregs (e.g., antibodies to CD25, the hCD2.Foxp3 reporter when hCD2. Foxp3 reporter mice were the original graft recipients, or other functional blocking antibodies such as anti-CTLA4 or anti-TGF-β) all lead to rapid graft rejection. This demonstrates that Tregs are required to actively and continuously block the action of effector T cells that are also present in the grafted and tolerated skin.



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the first cohort of Tregs enabling naive T cells to convert to peripheral Tregs under the influence of TGF-β.

THERAPEUTIC TOLERANCE REQUIRES CONSTANT VIGILANCE FROM Tregs

With this information from TCR transgenic mice, we went back to conventional mice that had been tolerized to skin grafts and, using ablative antibodies, could demonstrate that the suppression we had seen with adoptive transfer of splenic T cells some 20 years earlier was due to Foxp3$^+$ cells (40). This established clearly that suppression was a continually active process in tolerant animals maintaining constant vigilance over residual effector T cells capable of rejection.

AIMING FOR AN OVERALL SCHEME TO EXPLAIN THERAPEUTIC TOLERANCE

In studies of gene expression between DCs interacting with Tregs, and between tolerated and rejecting skin grafts, we observed an upregulation of enzymes that can catabolize essential amino acids (EAAs) (42). We wondered whether local depletion of EAAs by these enzymes might control the immune responses through a nutrient-sensing mechanism within the mTOR (mammalian target of rapamycin) pathway. A precedent for such thinking comes from the pioneering work of Mellor on the role of indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan catabolism in the control of maternal alloreactivity to the fetus (43, 44). The suggestion had been that tryptophan depletion was sensed by general control nonrepressed 2 (GCN2) through the integrated stress response. We had noticed, however, that the induction of Foxp3 in naive CD4$^+$ T cells in the presence of low doses of TGF-β *in vitro* was not affected by activating the GCN2 pathway, whereas inhibition of the mTOR pathway using rapamycin enhanced Foxp3 expression. This observation offered us one attractive route by which tolerance and regulation might be maintained: through depletion of essential nutrients at sites of antigen encounter (Fig. 5).

DEPLETION OF EAAs PROVIDES ONE ROUTE TO MAINTAINING A TOLEROGENIC MICROENVIRONMENT IN LYMPHOID ORGANS AND WITHIN TOLERATED TISSUES

The identification of IDO as a player in acquired tolerance has offered the clearest example of immune regulation due to amino acid catabolism, perhaps because tryptophan is normally at the lowest concentration in body fluids when compared to other EAAs. This might explain why various routes to catabolizing tryptophan have been associated with graft protection in models of transplantation tolerance. Arginase (ARG1) expression has also been implicated in regulating maternal alloreactivity during pregnancy (45, 46), and is also described as upregulated in type 2 macrophages (47) within tissues (48). When arginine is limiting, arginase and also inducible nitric oxide synthase can reduce levels of this EAA further, to the point of causing mTOR inhibition and reduced T-cell effector function (49). IL4-induced 1 (IL4i1), known to be induced in myeloid cells under Th2 conditions and able to deplete EAAs such as phenylalanine, is also upregulated in DCs cocultured with Tregs (49).

We reported that many of these EAA-consuming enzymes could be induced in DCs *in vitro* by a cognate interaction with Tregs, through cytokines such as gamma interferon, IL-4, or TGF-β or via coinhibitory molecules such as cytotoxic-T-lymphocyte-associated antigen 4 (CTLA4) (49). In addition, among these induced enzymes, threonine-catabolizing enzymes such as threonine dehydrogenase and Bcat1 (branched-chain amino acid aminotransferase) appeared upregulated soon after skin grafting, even in grafts placed onto recipients with no adaptive immune system. It may be then that many tissues carry innate nutrient-sensing mechanisms to protect them against inflammatory damage, with that mechanism contributing to the induction and maintenance of tolerance.

One such cell source might be mast cells, which seem to be needed for induction of therapeutic tolerance (34, 50, 51), and also to allow transplantation of syngeneic tumor cell lines (52). Tryptophan hydroxylase within murine mast cells may be responsible for this "innate" tolerance (53).

We propose, therefore, that tolerance is maintained, at least in part, by Tregs that perpetuate tolerogenic microenvironments within lymphoid organs and tissues, where induction of diverse enzymes deplete local EAAs. The resulting nutrient deficiency is then being sensed by T cells through the mTOR pathway (49, 54). This would result in the inhibition of effector T-cell priming and function, while Foxp3$^+$ Tregs would be selectively amplified. This pathway based on "infectious" nutrient deficiency may offer one route to "infectious tolerance" within the host.

If the above were an avenue by which tolerance could be induced and maintained, how might mTOR inhibition bring about the necessary controlling effects on the immune system?

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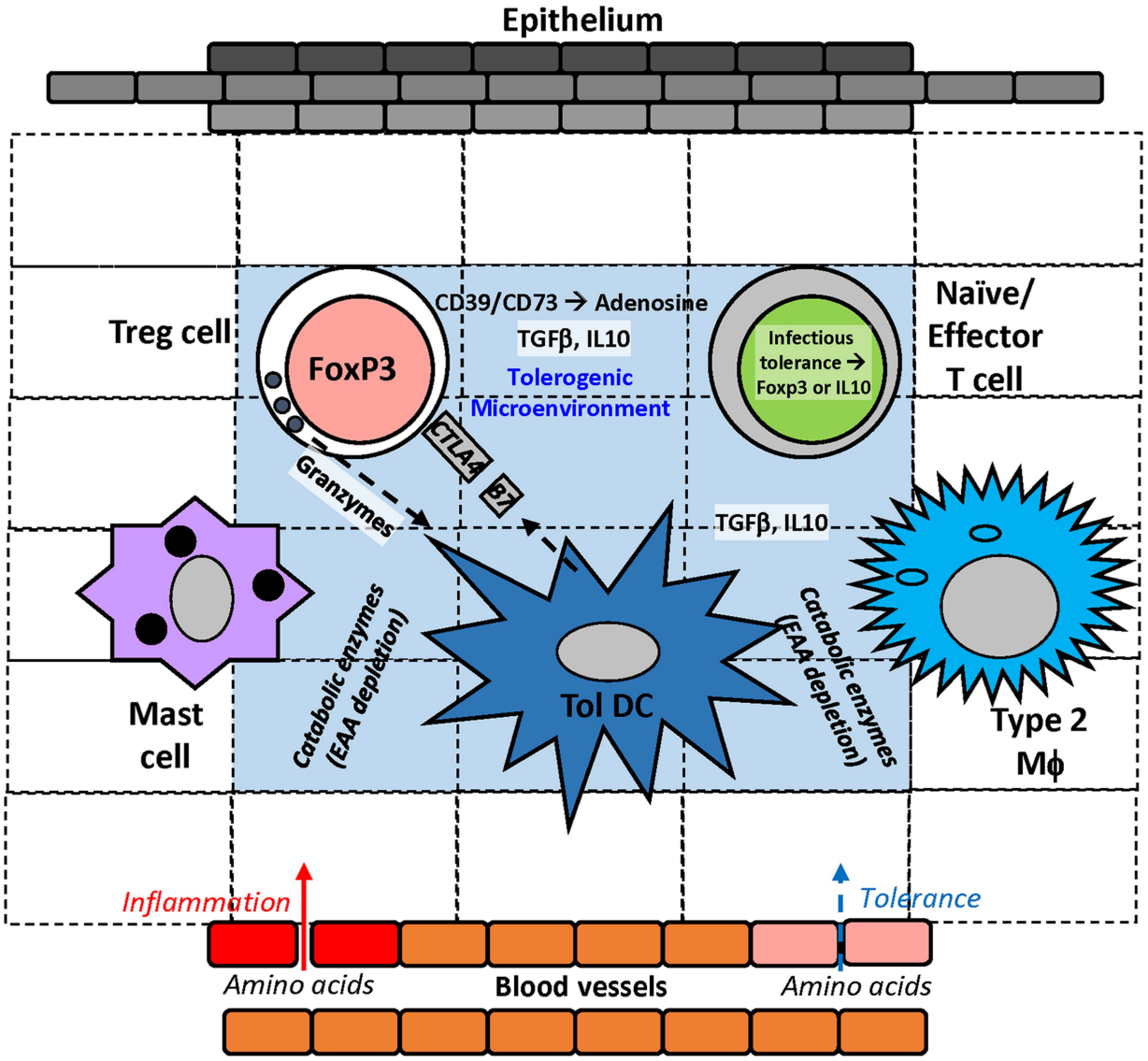

FIGURE 5 Interactions between regulatory T cells and myeloid cells maintain a tolerogenic microenvironment within tolerated tissues. Myeloid cells, including certain dendritic cells (Tol DC), type II macrophages (Mϕ), and mast cells can all express a range of enzymes, either intracellularly or secreted, that catabolize or utilize EAAs. In the context of an intact vasculature, this leads to the local depletion of amino acids, which represents one component of an immune-privileged or tolerogenic microenvironment. In addition, CD39 and CD73 coexpression on Tregs and other cell types, when enhanced by the local secretion of TGF-β, generates the anti-inflammatory mediator adenosine. Some Tregs also secrete IL-10, which further inhibits the ability of dendritic cells and Mϕ to present antigens for effector cell differentiation. Costimulatory ligands are also depleted from myeloid APCs by transendocytosis after capture by CTLA4 at the surface of Tregs. All these components of the tolerogenic microenvironment cooperate to limit the activity of naive and effector T cells while promoting Foxp3 expression and infectious tolerance. Graft rejection, on the other hand, is associated with leaking blood vessels and edema, which disrupts the tolerogenic niche, overwhelms the catabolic enzymes, and provides essential nutrients for T-cell activation and effector function.

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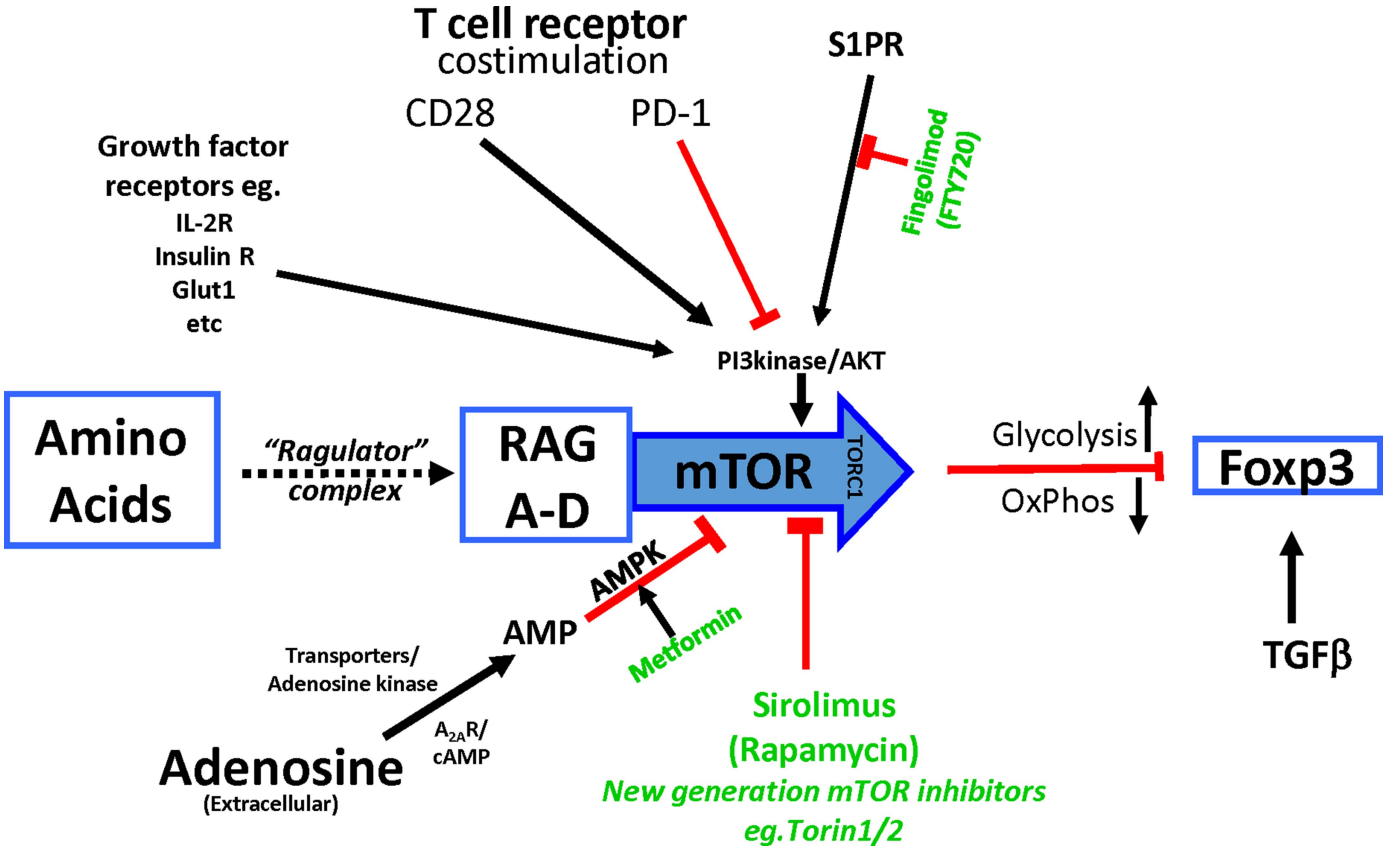
mTOR INTEGRATES NUTRIENT SENSING AND ACTIVATION OF T CELLS

The mTOR pathway (Fig. 6) coordinates cell growth and metabolism. mTOR comprises two different signaling complexes (TORC1 and TORC2) (55, 56). The former contributes the nutrient-sensing complex and comprises the serine/threonine kinase mTOR itself, raptor, FKB12, deptor, mLST8, and the regulatory subunit PRAS40, a target of AKT downstream of phosphatidylinositol 3-kinase (PI3K) signaling. Most of the signals that lead to TORC1 activation do so via PI3K signaling. This results in phosphorylation of mTORC1 via the tuberous sclerosis (TSC) 1/2 complex and the Ras homolog expressed in brain (Rheb). Rheb is contained within a lysosomal compartment, and its interaction with TORC1 depends on sensing of amino acids. Downstream signaling requires the four Ras-related GTP-binding (or RAG GTPase-RRAG) proteins (A to D) in conjunction with the ragulator complex (57, 58). Through this route, amino acid deprivation acts to in-

FIGURE 6 Nutrient and environmental sensing via mTOR regulates metabolism and Foxp3 expression. mTOR acts as an integrator of signals that arrive from a range of cell surface receptors and nutrient-sensing pathways and is critical in regulating cell metabolism and Foxp3 expression. The majority of these signals, including the TCR, growth factors, positive and negative costimulation (CD28 and PD-1), and the sphingosine 1-phosphate receptor (S1PR), converge on mTOR via the PI3K/AKT pathway, and are all dependent on recruiting phosphorylated Rheb to form the active TORC1 complex. The TORC1 complex can only be formed if there are sufficient EAAs to activate the regulator complex and the RAG proteins A to D. This means that a lack of amino acids effectively trumps all other signals via mTOR, inhibiting TORC1 activation, leading to enhanced oxidative phosphorylation (OxPhos), and allowing the Foxp3 gene to respond to TGF-β-mediated induction. Adenosine may also act indirectly on mTOR via cell surface receptors and/or adenosine transporters via AMP kinase (AMPK) signaling. It is interesting to note that at least three different classes of licensed drugs with immunomodulatory or metabolic activity (rapamycin, fingolimod, and metformin) target components of the mTOR signaling network.



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hibit TORC1 activity. Rapamycin, an immunosuppressive drug when bound to FKB12, also inhibits formation and function of mTORC1 (59).

TORC1 is important for the initiation of mRNA translation and the upregulation of amino acid transporters at the cell surface. It also activates lipid oxidation and cell proliferation and inhibits the expression of *FOXP3* and Treg differentiation while favoring Th1 and Th17 cells (60). The TORC2 complex can sense reactive oxygen and also glucose availability via a cyclic AMP/protein kinase A pathway. TORC2 controls spatial aspects of cell growth, such as cell polarity and responses to chemotactic signals (61).

mTOR SIGNALING INHIBITS Foxp3 EXPRESSION

mTOR inhibition might enhance Foxp3 expression through a number of different pathways. These could be in part through effects on Foxp3 translation via inhibition of S6K1 and reduced phosphorylation of the ribosomal protein S6. In addition, mTOR could act indirectly through suppressor of cytokine signaling 3 (SOCS3) or directly on signal transducer and activator of transcription 3 (STAT3). In addition, FOXO3a and SMAD3, which promote FOXP3 expression, are themselves inhibited by AKT, which is downstream of TORC2 (62–64). Evidence from knockout mice with T-cell-targeted deficiencies in TORC1 or TORC2 suggests that TORC1 activation promotes Th1 differentiation (65), while TORC2 may have a bias toward Th2 (66). Inhibition of both complexes seems to be required for the optimal induction of Foxp3$^+$ Tregs.

Foxp3 EXPRESSION REQUIRES mTOR INHIBITION, WHILE mTOR ACTIVATION IS REQUIRED FOR REGULATORY FUNCTION

Mice with T-cell-specific mTOR inactivation show an enhanced capacity to generate Foxp3$^+$ Treg cells over other effector cells (65). However, the genetic inactivation of TORC1 in Foxp3$^+$ Treg cells resulted in a scurfy/IPEX-like syndrome (67). Inactivation of TORC1 activity in all T cells did not produce disease, suggesting that effector T cells were also compromised. This raises the possibility that the optimal induction and expansion of Foxp3$^+$ Tregs takes place in mTOR-inhibited microenvironments but that Tregs can exert their suppressive function only when there is a trigger of inflammation involving mTOR activation. It has been suggested that optimal induction of Treg cells requires alternate cycles of mTOR inhibition to promote induction and mTOR activation to promote proliferation (68).

Tregs, TGF-β, AND ADENOSINE GENERATION

TGF-β is able to induce the coexpression of two surface membrane ectoenzymes, CD39 and CD73, in many cell types (69, 70). These two enzymes are constitutively expressed at high levels in murine Tregs (71, 72). They act to convert extracellular sources of ATP into the anti-inflammatory adenosine. Extracellular adenosine can, in turn, act on specific G protein-coupled receptors on diverse immune cells to exert these inhibitory effects (73). It may be that generation of adenosine offers an additional element to the creation of a tolerogenic microenvironment by Tregs (Fig. 7). The other well-defined contributors would be mTOR inhibition (as discussed above) and inhibitory ligands such as CTLA4 interacting with costimulatory receptors on DCs. Not only may CTLA4 on Tregs suppress immune function by competing with costimulatory molecules for binding to their receptors on DCs (74), but it may also induce IDO induction by a subset of DCs (75) and effectively strip costimulatory receptors off the DC membrane (76), three routes to decommissioning the immune effects of DC.

CLINICAL RELEVANCE

For the past 25 years we have endeavored to take infectious tolerance to the clinic, searching for long-term benefit derived from a short-term treatment. We accumulated substantive preclinical data that tolerance can be induced not only in mouse models of transplantation and autoimmune disease, but also in prevention and correction of unwanted immunity to therapeutic proteins. To this end we humanized antibodies to human CD4 and CD8 as potential therapeutic candidates. Tolerance induced to foreign proteins with anti-CD4 therapy has already been demonstrated in nonhuman primates (77). Sadly, coreceptor blockade has not yet attracted pharmaceutical partners committed to short-term therapy, and this is probably due to a number of logistical and commercial reasons. A humanized antibody to the costimulatory molecule CD40L did enter the clinic, but development was curtailed due to risks of thromboembolism (78).

We have, however, observed long-term benefit from short-term therapy following lymphocyte depletion with our humanized anti-CD52 antibody CAMPATH-1H (alemtuzumab) (79). In light of our ideas on therapeutic tolerance requiring the harnessing of regulatory T cells,

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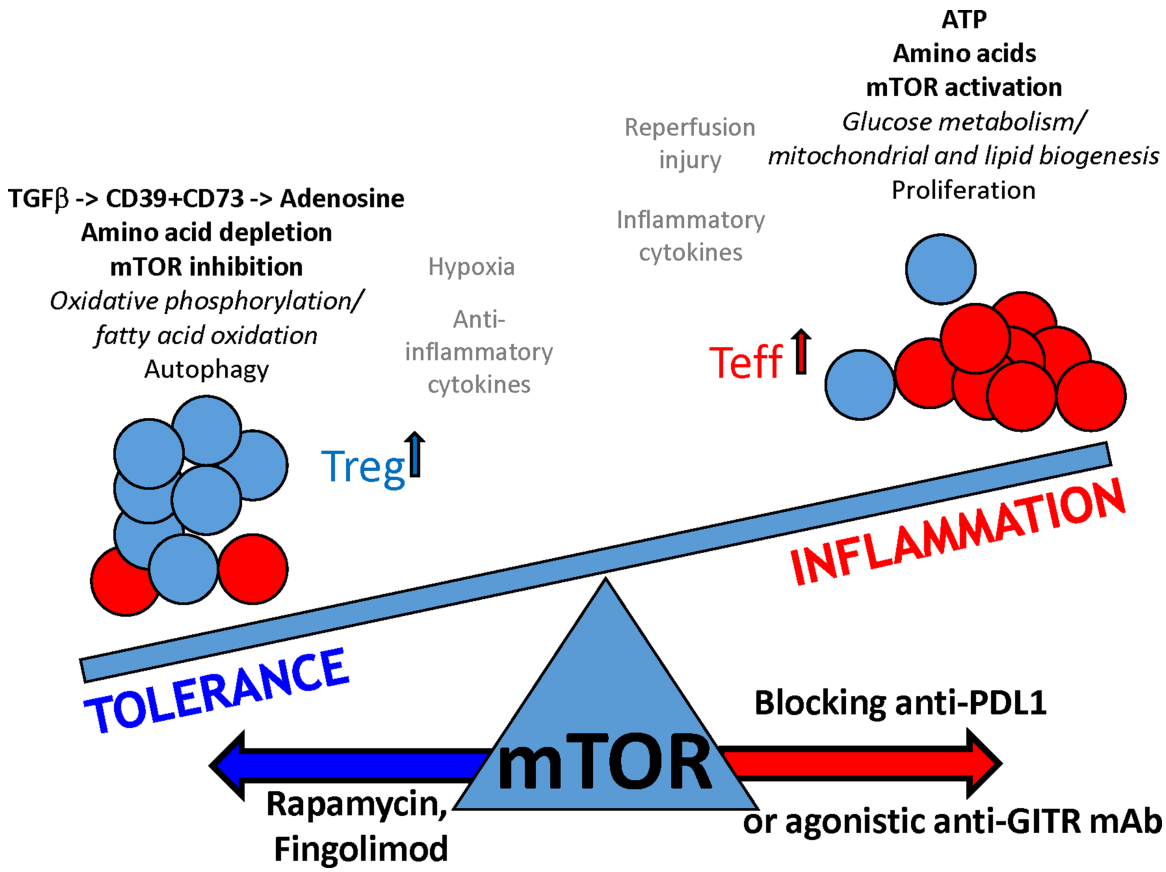

FIGURE 7 Tolerance or inflammation depends on the balance of Treg to T effector cell (Teff) and the response of mTOR to a range of microenvironmental factors. Whether the outcome of an immunological response is one of tolerance or inflammation is increasingly being considered as a balance between the number of Tregs and Teffs. This needs to be tempered by the influence of a number of other factors, particularly within the local microenvironment of the tolerated tissue, including the balance of adenosine to ATP, the effectiveness of amino acid depletion, and probably many other factors that may be associated with particular tissues and their state of health, such as inflammatory or anti-inflammatory cytokines, hypoxia, and reperfusion injury. Drugs that inhibit or activate mTOR might be able to adjust the balance point in favor of tolerance (for autoimmune disease and transplantation) or inflammation (for treatment of cancer) and provide a long-lasting therapeutic outcome from a short course of treatment.

we sought evidence of whether long-term tolerance could be harnessed following T-cell depletion in our rodent models of transplantation.

LYMPHOCYTE DEPLETION, HOMEOSTATIC EXPANSION, AND "PHYSICIAN-AIDED RECONSTITUTION OF THE IMMUNE SYSTEM"

Using such a triple short-term combination, we were able to generate tolerance in CBA/Ca mice to very immunogenic MHC-incompatible skin grafts. To model the clinical situation, we created mice transgenic for human CD52 in their T cells and then gave short pulses of the depleting anti-CD52 antibody CAMPATH-1 to produce an ~2-log reduction in their peripheral T cells (80). Despite such depletion, all mice rejected MHC-mismatched skin grafts, albeit at a reduced tempo when compared to controls. Rejection could only have been mediated by T cells spared by the therapy or those that had derived by expansion from those residual cells. Based on our concepts emerging from coreceptor blockade, we speculated that the reconstitution of T cells after depletion (homeostatic expansion) may not have provided Tregs with a numerical advantage over T effectors. To test that idea, we attempted to guide the

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reconstitution phase by addition of short-term therapy with the mTOR inhibitor rapamycin, and also MAb blockade of the IL-7 receptor (80). The latter was based on the observation that resting Tregs carry very little IL-7 receptor compared to other lymphocytes, and that the cytokine IL-7 is important in maintaining the normal balance of B cells and T cells in the immune system.

One week after the initiation of treatment we observed a strong numerical bias of detectable regulatory T cells in the spleens, that bias disappearing after 1 month (80). Tolerant mice also exhibited linked suppression whereby they could not reject grafts carrying a mix of the tolerated antigens with additional third-party antigens. This is strong evidence of active, dominant tolerance mediated by regulatory T cells, just like that operating after coreceptor blockade.

These data suggest that enhancing Treg numbers and function early in the process of reconstitution may give Tregs the protracted advantage needed to establish a long-term tolerant state.

We also speculate that some of the unwanted autoimmune phenomena that follow lymphocyte depletion and homeostatic expansion in the lymphocyte reconstitution phase (80, 81) can be prevented by such guided antilymphocyte therapy early in the inductive process.

CONCLUSION

On the basis of these studies, we feel that there are opportunities to therapeutically reprogram the immune system based on dominant tolerance by influencing initial lymphocyte perceptions of antigen so as to favor regulation. Thus far, MAbs have figured strongly in our protocols, but as we achieve a greater understanding of the metabolic processes underlying infectious tolerance, and the key elements contributing to tolerogenic microenvironments, hopefully new combinations of "rational" drugs will emerge to enable reprogramming to be safe and more predictable. Understanding of tolerance mechanisms that operate may also permit the development of "smart" biomarker sets that guide the physician to better enhance particular regulatory systems. Importantly, we should also recognize that this is not just a question of controlling adaptive immune responses, but that innate tolerance mechanisms are essential elements in ensuring the creation of protective tissue microenvironments.

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