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**Review**

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# Harnessing FOXP3<sup>+</sup> regulatory T cells for transplantation tolerance

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**Early demonstrations that mice could be tolerized to transplanted tissues with short courses of immunosuppressive therapy and that with regard to tolerance to self, CD4<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells (Tregs) appeared to play a critical role, have catalyzed strategies to harness FOXP3-dependent processes to control rejection in human transplantation. This review seeks to examine the scientific underpinning for this new approach to finesse immunosuppression.**

## Introduction

The need for transplantation of histoincompatible cells and tissues provides a perpetual challenge to the field of immunology: that of overcoming rejection. From the time that Medawar and colleagues demonstrated that transplantation tolerance could be acquired, there has been the hope that tolerance mechanisms could eventually be harnessed to minimize, if not eliminate, the use of long-term immunosuppression.

The need to explain the diversity of lymphocyte receptors for foreign antigens begat the clonal selection theory and notions that self-tolerance could be fully explained by clonal deletion at an early stage of lymphocyte development. This thinking provided a high bar for ideas on translation based on targeting immature lymphocytes. The later discovery in adult rodents that tolerance to foreign proteins and tissues could be achieved following short-term immunosuppression with immunosuppressive drugs (1) and certain antilymphocyte monoclonal antibodies (2, 3) led, in turn, to the finding that such tolerance was often dominant and suppressive and largely mediated through CD4<sup>+</sup> regulatory T cells (Tregs) (4). Converging with parallel studies on self-tolerance (5–7), the major Treg responsible for maintaining allograft tolerance proved to be CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> (4, 8, 9). The longevity of tolerance seen in these experimental models was shown to be dependent on constant vigilance by FOXP3<sup>+</sup> Tregs (8) and constant recruitment of new Tregs (infectious tolerance) (8), both processes dependent on a constant supply of graft antigens (10). In addition, linked suppression meant that induced tolerance to one set of antigens could be extended to others coexisting in the same tissue, without the need for further therapeutic intervention (11, 12).

The ability to control inflammation can also be achieved with FOXP3-negative CD4 T cells that are often referred to as Tr1 or Tr1-like cells (13–16). Although offering great potential, a proper account of such cells would be outside the scope of this short review, and the reader is referred to excellent reviews on the topic (17–19).

Overall, the findings on CD4<sup>+</sup> Tregs, albeit largely derived from rodents, have provided a new optimism for the therapeutic induction of operational tolerance, because unlike strategies based on clonal deletion, one would not need to permanently inactivate all potentially destructive alloreactive T cells. The finding that self-tolerance in humans is also dependent on

FOXP3<sup>+</sup> Tregs (20–23) suggests that extrapolation from rodent studies to humans is not inappropriate.

However, harnessing Tregs, although attractive as a concept, still provides a significant challenge, as this heterogeneous cell population (24) would need to control a broad spectrum of unpredictable inflammatory responses. The challenge in transplantation is to recruit sufficient numbers of the appropriate Tregs to cover the breadth of tissue-damaging mechanisms evoked.

## Tregs and their roles in transplantation tolerance

The FOXP3<sup>+</sup> Tregs that populate the peripheral immune system comprise a set that develops in the thymus (tTregs) (25) and a minority that is induced in the periphery (pTregs) under the influence of TGF- $\beta$  and mTOR inhibition (26–32). The T cell receptor repertoires of the populations differ, with the latter showing a pattern more similar to that of conventional T cells. These features have been interpreted to indicate that tTregs may be preoccupied with ensuring tolerance to self-antigens, while pTregs operate to moderate responses to certain “foreign” antigens that might be found in the gut microbiome or in the fetus during pregnancy (33). Given the potential cross-reactivity of the T cell receptor repertoire, a proportion of tTregs would be expected to exhibit alloreactivity exploitable for tolerance induction (34, 35). The conventional repertoire of pTregs also points to a real prospect for selective tolerizing vaccinations to foreign graft antigens (36).

That tTregs can contribute to transplantation tolerance is illustrated in certain mouse strains that naturally fail to reject allografts and in which depletion of Tregs then exposes their potential to reject (37, 38). Furthermore, under conditions of lymphopenia, tTregs interfere with the homeostatic expansion of T cells that are competent to reject grafts (39). This is so even if the Tregs are derived from mice in which tolerance by clonal deletion has been enabled through hemopoietic chimerism (40). This tells us that Tregs need not exhibit allospecificity to control rejection in the context of homeostatic expansion and that they perhaps exploit their self-reactive repertoire for that purpose.

The best evidence that pTregs can be harnessed within the host to elicit transplantation tolerance came from studies in TCR transgenic mice using coreceptor blockade with anti-CD4 antibodies (41). Such mice carrying just one homogenous TCR generate no tTregs, yet can be tolerized to grafts carrying the nominal antigen. Tolerance is accompanied by induction of FOXP3<sup>+</sup> pTregs. However, where TGF- $\beta$  was prevented from signaling to conventional T cells or where mice lacked a functional FOXP3 gene, tolerance

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could not be achieved (9, 41, 42). Tolerance through generation of pTregs has more recently been demonstrated with antigen alone in ingenious vaccination protocols (36, 43).

The preoccupation with lineages may, however, obscure an important aspect of FOXP3 expression. The fact that CD4 T cells can transiently or “promiscuously” express FOXP3 may also be relevant to therapeutic induction of tolerance. It has long been known that ectopic FOXP3 expression can turn down inflammatory cytokine production and damaging effector functions including graft rejection (9, 44–46). On this basis, treatments that turn on FOXP3 expression and function, even transiently, may help ensure a temporary ceasefire, perhaps paving the way for stable regulation to evolve over time.

These studies teach us that both tTregs and pTregs can suppress rejection responses, even if they do not necessarily use the exact same mechanisms and TCR specificities. Consequently, where possible, our therapies should exploit both types of Tregs and even early (less stable) stages of their development.

One major gap in our knowledge of Tregs is how homeostasis of tTreg and pTreg populations is maintained, both in relation to each other and to other lymphocyte subsets (47, 48). If our ultimate goal is to exploit antigen-specific Tregs, then we need to understand the nature of the Treg “niche” and how the pTregs might be given a competitive advantage over effector cells and tTregs.

### Tregs as lineages

Much of the current thinking on the exploitation of Tregs is predicated on both types functioning as stable lineages. From a clinical perspective, such exploitation can be approached from two different angles. First, Tregs might be generated in large numbers *ex vivo* and administered as a cell product (49). Alternatively, protocols might be designed to enhance the generation of stable Treg lineages within the patient that would render these cells more amenable to standard pharmaceutical approaches. The Tregs that develop *in vivo* would, of course, be influenced and shaped in their development by graft exposure in a contextual way that might not be easily simulated by Tregs generated *ex vivo*.

### The importance of the transcription factor FOXP3

It is undeniable that FOXP3 plays a key role in ensuring immune homeostasis and tolerance. FOXP3 is essential for the development and suppressor function of Tregs, since its loss or even disruption results in overt lymphoproliferative disease, autoimmunity, and graft rejection (44, 50, 51). But clearly, there is more to Tregs than simple FOXP3 expression. They are characterized by specific epigenetic changes that define their lineage commitment. These epigenetic changes can be established independently of FOXP3, indicating that FOXP3 is a rather late-acting transcription factor in Treg lineage commitment (52, 53). Furthermore, expression of FOXP3 can occur transiently in nonregulatory cells (“promiscuous” FOXP3 expression) that fail to undergo Treg-specific epigenetic changes and lineage commitment, but can also be lost transiently in committed Tregs characterized by their epigenetic signature (“ex-Tregs”) (54–57). These findings have important implications for the way we regard the stability and plasticity of Tregs in relation to FOXP3 expression (58, 59). It suggests that FOXP3 protein, though essential for suppressor function, does not unequivocally reflect the epigenome associated with committed Tregs. From the point of view of therapy, the issues of functional stability and derivation of undesirable proinflammatory revertants need to be rigorously controlled (60).

### How is lineage stability acquired?

**TCR signaling.** Despite the fact that the epigenetic signature is the best available indicator of lineage stability to date, the signals required for its establishment remain poorly understood. The intensity and duration of TCR signaling are critical for acquisition of the Treg-specific epigenetic signature (52). This is supported by the well-known finding that CD4 T cells expressing nuclear FOXP3 following short-term TGF- $\beta$ -dependent induction *in vitro* rarely express Treg-specific epigenetic changes and, consequently, generate unstable FOXP3<sup>+</sup> cells that are able to revert to proinflammatory functions (61). In the case of tTregs, lineage-specific epigenetic marks are installed very early on in thymic development, even before FOXP3 is expressed (53, 62). Since proliferation seems of little importance, it would seem that an active mechanism is involved in DNA demethylation of the developing Treg, as has previously been suggested for the stabilization of *Il2* gene expression (62, 63).

**Metabolic requirements.** Increasing evidence suggests that metabolic changes play an important role in regulating FOXP3 expression and lineage commitment, at least for pTregs. Tregs and effector T cells require different metabolic programs to function. Actively proliferating effector T cells express high levels of the cell surface glucose transporter GLUT1 and engage the relatively energy-inefficient aerobic glycolysis and glutaminolysis pathways. Despite yielding low amounts of ATP, aerobic glycolysis provides precursors for nucleotide synthesis via the pentose phosphate shunt and fatty acids via the metabolism of citrate, both of which are required for a cell to increase biomass during division. This mode of metabolism is common to tumor cells and has been termed the “Warburg effect.” In contrast to Th1, Th2, and Th17 cells, which have high plasma membrane expression of the GLUT1 glucose transporter and actively engage glycolysis, Tregs have little GLUT1 and use oxidative phosphorylation via lipid oxidation as their primary energy source (64, 65). There is an intricate link between control of the AKT/mTOR/HIF1 $\alpha$  axis and the source of ATP that a cell uses (29). At present, it is unclear whether the signals, which induce the Treg or T effector lineage, are controlled by metabolic pathway engagement, as has been described for CD8 T cells (66–68), or whether the divergent metabolism is a consequence of the lineage decision.

It is clear, however, that engagement of the mTOR pathway is linked to activation of glycolysis via HIF1 $\alpha$  and possibly NR4a1 (NUR77), transcription factors known to activate many glycolysis genes including *Glut1*, hexokinase 2, glucose-6-phosphate isomerase, and enolase 1 (65, 69, 70). Conditional HIF1 $\alpha$ -knockout mice are refractive to Th17 induction protocols, whereas FOXP3 expression is increased in the same cells. mTOR coordinates and processes multiple environmental signals including stress, oxygen levels, nutrient and amino acid concentrations, energy status of the cell, and, in T cells, strength of the TCR stimulus (71). Partial inhibition of mTOR activity via nutrient starvation, inadequate TCR triggering, or rapamycin treatment favors the induction of Tregs, along with a skewing of the metabolic profile toward fatty acid oxidation–fueled oxidative phosphorylation. Inhibition of the activity of both subcomplexes of mTOR, mTORC1, and mTORC2 in naive T cells results in the inability to differentiate into T effector lineages, but the ability to differentiate into Tregs is not inhibited (31). Recently, the role of mTOR in Treg function has been further dissected using raptor-deficient (mTORC1-deficient) Tregs. TORC1 activity was shown to be essential for Treg suppressive

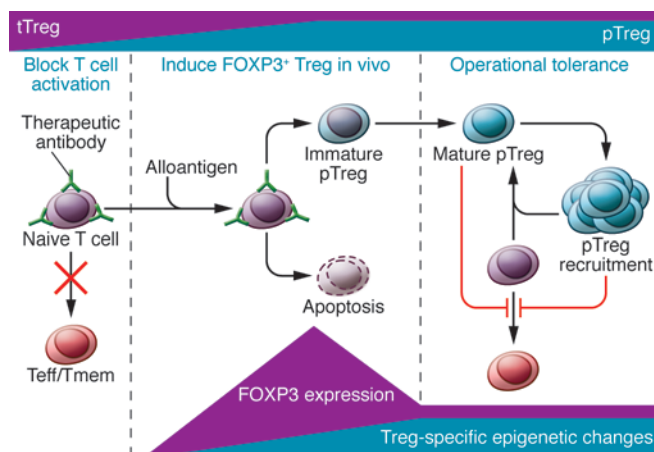
function and survival by enhancing lipid metabolism and inhibition of TORC2 signaling (29). It is still unclear, though, whether the engagement of glycolysis downstream of HIF1a activation or the activation of lipid metabolism by inhibition of TORC2 is a prerequisite for these lineage decisions or merely a result of different fuel demands. The implications of these studies are that there may be a number of metabolic targets for skewing T cells toward Treg development.

### Tregs and their role in tolerated tissues

The demonstrations of linked suppression (11, 12) and FOXP3<sup>+</sup> Tregs in tolerated grafts suggested (72, 73) that Tregs operate within tissues to protect them against immune attack. Direct evidence for this has been provided by retransplantation of tolerated grafts into recipients with no adaptive immune system, in which ablation of CD25<sup>+</sup> (73) or FOXP3<sup>+</sup> T cells (8) resulted in graft rejection by residual lymphocytes within those grafts. This tells us that even though the tolerated tissue contains T cells capable of rejecting it, they are constrained from doing so by Tregs within them. It seems unlikely that Tregs fulfill this role as the sole arbiters of suppression. Rather, we imagine that they initiate a cascade of antiinflammatory behavior to which many different tissue components contribute.

We have previously referred to the tolerogenic microenvironment that is maintained by Tregs within a tolerated tissue as a form of “induced immune privilege” (73). A classic example of tissue-restricted immune privilege is the maintenance of the semi-allogeneic fetus in pregnancy, in which a role for Tregs has also been implicated (74, 75). One critical component in maintaining tolerance to the murine fetus is the expression in the placenta of the tryptophan catabolizing enzyme indoleamine 2,3 dioxygenase (IDO). Tryptophan is an amino acid essential for T cell proliferation and effector cell differentiation. Blocking IDO-mediated tryptophan depletion by administration of the inhibitor 1-MT induced rejection of allogeneic, but not syngeneic, fetuses (76). Redundancies in pathways of amino acid catabolism may explain why a role for IDO in pregnancy has not been a universal finding (77, 78). T cells can sense a lack of tryptophan via GCN2 and the integrated stress response pathway, which suppresses their proliferation, and this has been claimed to enhance their differentiation into FOXP3-expressing pTregs (79). Mast cells can also deplete tryptophan by expressing tryptophan hydroxylase (TPH1), and thus also contribute to the tolerogenic milieu (80).

Tryptophan is the least abundant of the essential amino acids and presumably the easiest to deplete, yet IDO- and GCN2-knockout mice were fully permissive for the induction of tolerance to allogeneic skin grafts by coreceptor blockade (ref. 81 and our unpublished observations). We hypothesized that the principle of nutrient depletion maintaining tolerance might also extend to other essential amino acids and pathways of nutrient sensing, perhaps in a redundant fashion. We observed in a number of *in vitro* and *in vivo* systems that Tregs were associated with an increased expression of a number of enzymes that could catabolize or consume each of the nine different essential amino acids (77, 82). Depletion of any one of the essential amino acids was found to block T cell proliferation and to synergize with TGF- $\beta$  for the further induction of FOXP3-expressing Tregs. Both of these features were dependent on amino acid sensing via the RAGulator/mTORC1 pathway (our unpublished observations) rather than on GCN2 (77).



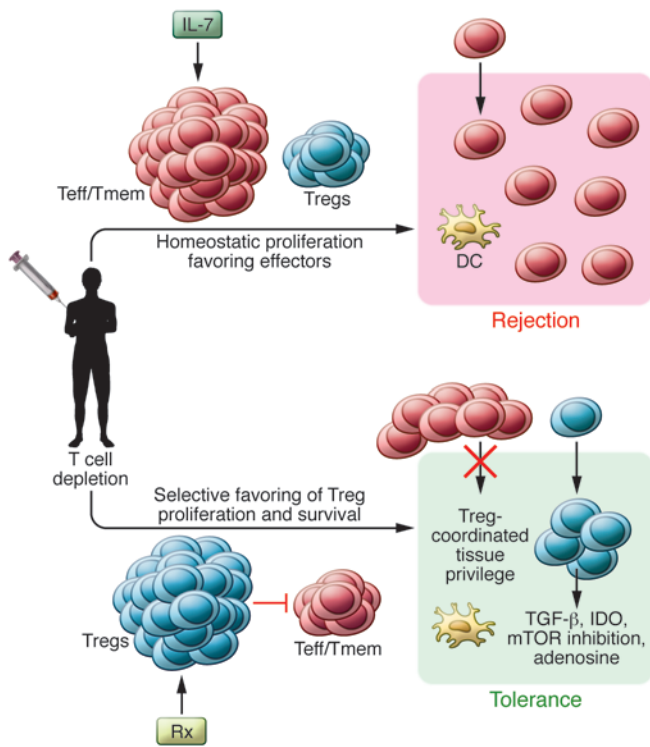
**Figure 1**

Therapeutic intervention aimed at inducing transplantation tolerance can be thought of in three stages. The first (left) is concerned with creating a ceasefire, in which inflammation and danger signals are minimized. In the example given, short-term inhibition of T cell activation is achieved with antibodies that block T cell coreceptors and costimulatory molecules such as CD4, CD8, and CD40L. Under these nondepleting conditions, in the second stage (middle), both self-reactive and alloreactive FOXP3<sup>+</sup> natural Tregs (tTreg) are recruited and expanded. Alloantigen would also induce anergy and apoptosis of some naive T cells and induce others to express FOXP3. A proportion of these FOXP3-expressing cells will continue differentiation toward mature pTregs with a well-established Treg-specific epigenome. Meanwhile, even those T cells transiently expressing FOXP3 will be restrained from participation in the inflammatory (rejection) process (9, 44–46). As the therapeutic agents clear from the system, the favorable balance of regulation (based on expanded antigen-specific tTregs and pTregs) creates the third stage of “operational tolerance” (right). Tregs maintain that operational tolerance as a result of continued exposure to the engrafted donor antigens and recruitment of new pTregs by “infectious tolerance” (4).

The mTORC1 complex acts as a major integrator of nutrient sensing and growth factor signaling in all eukaryotic cells, which in turn leads to coordination of protein translation, cell proliferation, and metabolism. It is also involved in the response to hypoxia, principally by the sensing of intracellular AMP/ADP to ATP ratios via AMP kinase. It seems that this pathway can also be used to sense extracellular adenosine (83). Inflammation and cell death are associated with the release of ATP, which can signal via the P2X family of receptors to activate both T cells and APCs, but this can be antagonized by the expression of the cell surface ectoenzymes CD39 and CD73 that convert ATP into adenosine (84). TGF- $\beta$ , operating in the local microenvironment, is a powerful stimulator of CD39 and CD73 expression (85). Adenosine can signal via the adenosine G protein-coupled family of receptors or be taken up directly via adenosine transporters, both of which lead to an increase in intracellular AMP, the activation of AMPK, and, as with amino acid depletion, the inhibition of mTOR, favoring FOXP3 expression (86).

We propose, therefore, that the tolerogenic microenvironment is one in which the availability of nutrients is tightly controlled, requiring any T cell that enters a tolerated tissue to adapt its metabolism to use either those external nutrients available or to rely on autophagy and salvage pathways to recycle intracellular





**Figure 2**

In the absence of clinically available non-T cell-depleting coreceptor and costimulator blockade, T cell depletion may be exploited as an alternative approach to achieve operational tolerance. Lymphocyte depletion is typically followed by homeostatic proliferation, resulting in a rebound of effector T (Teff) and memory T (Tmem) cells (upper panel). This process is driven by cytokines such as IL-7 and is not matched by an adequate repopulation of Tregs, thereby impeding the mechanisms required for induction of operational tolerance. If, however, the reconstitution phase can be guided to favor early emergence of Tregs, then operational tolerance might be possible (lower panel). Recent advances in understanding the molecular and epigenetic mechanisms of FOXP3 expression and Treg differentiation will enable such new associated treatments (Rx) that selectively favor Treg reconstitution. These “favored” Tregs will, among other activities, orchestrate the development of immune privilege at the tissue site.

components that provide sufficient energy to function and survive. Evidence is growing that T cell differentiation and metabolism are inherently coupled in determining the development of memory, effector, or regulatory T cells (87, 88).

**Toward therapeutic applications**

*Administration of Tregs from without.* The discovery of Tregs has spawned great interest in the use of in vitro-expanded, personalized Treg therapy (19, 89–97). Undoubtedly, this will provide important proof-of-principle discoveries, but routine clinical application, even with evidence of efficacy, may still be a significant challenge. The constraints on the application of Treg therapy are scientific, logistical, and commercial. We still know little about the mechanisms of suppression, the factors affecting stability of their antiinflammatory functions (98), or about Treg heterogeneity and context-related requirements, and we lack well-validated biomarker handles to control the quality of the therapeutic prod-

ucts. The availability of humanized mouse models to test efficacy is certainly a significant step in this field (93, 99, 100). With regard to logistics, the need to generate purified cells to GMP standards is itself no small challenge. Finally, as for all personalized cell therapies, the numerous barriers to commercialization certainly need to be overcome.

*Enhancing Tregs from within.* Given these considerations, what principles can be exploited to favor the regulation and dominant tolerance generated within the host?

There are relatively few strategies proposed for selectively expanding endogenous Tregs. One based on selective vaccination has already been reported (36, 43). The other, based on the known ability of IL-2 to stabilize and expand Tregs, uses IL-2 or IL-2-anti-IL-2 antibody complexes to act on endogenous Tregs (101–103). The latter would be clinically attractive if one could generate a druggable form of IL-2 that would signal only to Tregs and not to proinflammatory effector T cells.

Murine models have provided a number of antibody-based protocols based on short-term treatments that appear to favor Tregs.

First, let us consider the situation of tolerogenic protocols that block coreceptor and/or costimulatory signals. Regulation seems to be favored when the following three (somewhat overlapping) stages are completed (Figure 1).

First, a “ceasefire” from aggression must be established for long enough to ensure no proinflammatory “sniper” activity while tolerance mechanisms are induced. As a consequence of the ceasefire, some effectors undergo apoptosis, and this, together with tissue-healing events, encourages the production of active TGF-β, inhibition of mTOR and proinflammatory cytokines, and the extinction of danger signals. Many of the situations in which mTOR is inhibited can locally antagonize destructive immune responses.

The second stage is based on the conversion of naive CD4<sup>+</sup> T cells into pTregs. It is here that the molecular mechanisms involved in FOXP3 expression, outlined above, are integrated. Although transient expression of FOXP3 may be insufficient to establish stable Tregs, it may contribute to the restraint (9, 45, 46), and even apoptosis (104), of potential effector cells and thus support the drug-initiated ceasefire. This buys the necessary time (105) for some FOXP3-converted cells to undergo the epigenetic changes that eventually stabilize them (52). Tregs with specificity for antigen are equipped with metabolic characteristics that provide them with the capacity to gain relative benefit from available antigen stimulation and nutrients, in which conventional cells may be compromised by the therapeutic agents or the metabolic environment (i.e., EAA depletion and mTOR inhibition), thereby allowing preferential expansion of Tregs and consolidation of the immune-privileged microenvironment they impose in the tissues.

Finally, we propose that tolerance enters an autonomous phase not requiring further maintenance drug therapy. As the graft heals, its immunogenic power will diminish, as will the impact of direct allorecognition. The “vaccination” of pTregs, which see donor peptides indirectly presented on host MHCs, will on the contrary increase, so that by the time therapeutic agent levels disappear, regulation and infectious tolerance have merged as the dominant processes (12). Of the many induced CD4<sup>+</sup> T cells that express FOXP3 en route to tolerance, only a minority will have become committed Treg lineage cells, but a combination of their numbers and strategic placement (draining lymphoid tissue and the graft itself) should allow them to have long-term dominant (antigen-



specific) control on residual potential effectors of immunity. There is no doubt that donor antigens continuously supplied by the engrafted tissue are critical to maintain active Treg-mediated tolerance (10). Such antigens can be considered the “booster” doses often needed in conventional vaccines. This, together with the finding that Treg depletion after tolerance induction reverses the tolerant state (8), provides compelling evidence that continued vigilance by Tregs as well as infectious tolerance are both sufficient and essential for long-term graft survival.

Of course, in patients, a single set of generic principles may not always be appropriate for generating tolerance, but they may still be conducive to drug minimization strategies. Prior priming need not preclude amplification of tolerance mechanisms if sufficient regulation can be generated (106, 107).

The above is clearly an oversimplified scheme, but it is consistent with much of the data derived from experimental tolerance studies in rodents using CD4 (plus CD8), CD40L, and CD3 antibodies as agents for tolerance induction. The ceasefire created by these treatments relies on signaling blockade rather than T cell depletion and subsequent lymphopenia.

Unfortunately, many of the agents that have proven most effective in generating dominant tolerance discussed above have not yet emerged as licensed drugs available for clinical trials. This is partly because some have exhibited undesirable side effects, partly because there has not been any easy route to establishing definitive trials to enable drug approval, and, perhaps most poignant, because there is uncertainty about tolerance-promoting agents as commercial products. The harsh reality may be that we need to build on those drugs that are currently commercially

available (e.g., ATG, alemtuzumab, abatacept) to determine how to incorporate them into tolerogenic protocols.

Given the limited repertoire of licensed drugs, induction strategies based on lymphocyte depletion provide immediate opportunities. The major disadvantage of lymphocyte depletion is the homeostatic expansion of host lymphocytes and immune reconstitution favoring memory and effector T cells that provide a barrier to tolerance (108). With that in mind, a modification of the tolerance-inducing principles espoused above should aim to enable Tregs to emerge as a dominant repopulating element, able to override any expanding effector and memory T cells. Treatments might also take advantage of the range of transiently expressed factors that are known to contribute to induction of pTregs (Figure 2). We are confident that the rapidly expanding knowledge of the molecular mechanisms orchestrating Treg development will facilitate the early application of such protocols, which we coin with the acronym PARIS (p<sub>h</sub>ysician-a<sub>i</sub>ded r<sub>e</sub>constitution of the immune system).

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