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Mechanisms of Regulatory T cell Counter-Regulation by Innate Immunity

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The opposing roles of TGF- β and IL-6 in Treg differentiation

In 1995, a breakthrough finding identified the cells responsible for maintaining self-tolerance as CD4+CD25+ T cells (1), a population of long-lived, self-perpetuating “Tregs,” which could suppress multiple effectors in an antigen-specific manner. While the majority of peripheral Tregs originate in the thymus (natural Tregs), generation of Tregs in the periphery (adaptive Tregs) from naive CD25–Foxp3–CD4+ conventional T cells (Tconv) is also well-described. Peripheral conversion to adaptive Tregs was first recognized when CD4+CD25– T cells acquired suppressor function after transfer into immunodeficient hosts (2, 3).

Tregs are currently best defined by expression of the master transcriptional regulator of Treg development, Foxp3. Foxp3 gene transfer to non-regulatory CD4+ T cells both confers regulatory function and induces a regulatory phenotype. Foxp3 expressing cells with regulatory activity exhibit variable expression of a number of cell surface markers including: CD25, CTLA4, CD103, CD134, CD62L, GITR, GARP, CD39, CD73, surface-bound TGF- β , and CD127lo, and produce the anti-inflammatory cytokines TGF- β , IFN- γ , IL-9, and IL-10. Lack of Foxp3 is associated with absence of Tregs and development of lethal lymphoproliferation and autoimmunity (4-6).

Multiple groups have shown that *in vitro* stimulation of naive CD4+ T cells in the presence of TGF- β leads to an increase in Foxp3 expression, together with conversion to the phenotype and cytokine expression profile associated with CD4+CD25+ Tregs and acquisition of suppressive activity (7-9). Simultaneous TCR stimulation is generally required for this conversion, but reports vary as to the importance of signaling via CD25, CD28, and CTLA-4 (7, 9-11). There are contradictory reports as to how long TGF- β is required for maintenance or sustained expression of the Treg phenotype and function (9, 12).

TGF- β driven Foxp3 induction is likely the result of Smad pathway activation. Ligand binding to the TGF- β receptor complex leads to phosphorylation of Smad2 and Smad3, with

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eventual translocation of a Smad multimer to the nucleus, where they act as transcriptional activators for target genes (12, 13). An enhancer element for Foxp3 has been identified that requires Smad3 cooperation with NFAT for activity (14). The Smad proteins have also been shown to cooperate with Sp1 and AP-1 components c-fos/c-jun (15, 16) in other TGF- β -induced genes. It is therefore interesting to note that the Foxp3 gene upstream region contains both AP-1 and Sp1 binding sites as well (17), although no functional studies have been published to confirm the significance of those sequences in foxp3 expression.

Much of the *in vivo* data on the role of TGF- β in Treg differentiation comes from observations of TGF- β knockout and TGF- β overexpressing mice. Up to two-thirds of TGF- β knockout mice develop an autoimmune / lymphoproliferative disease syndrome. In 8-10 day-old neonatal mice, before the syndrome manifests, TGF- β -deficient mice have fewer CD4+CD25+ T cells circulating in the periphery. The few CD4+CD25+ T cells that do exist express lower levels of Foxp3 than those from wild-type mice. However, when these cells are transferred into lymphopenic mice with normal TGF- β 1 expression, Foxp3 expression increases to wild-type levels (12). Conversely, TGF- β over-expressing mice have higher percentages of CD4+CD25+ T cells in the peripheral blood and lymph nodes that express higher levels of Foxp3 than wild-type mice (18).

There are not yet definitive studies showing that the innate immune system is the physiologic source of TGF- β , but *in vitro* studies suggest that this may be the case. Macrophages that have ingested apoptotic fragments produce TGF- β , while down-regulating inflammatory cytokines (19). Other studies have linked the production of TGF- β by immature dendritic cells to the generation and survival of Foxp3+ Tregs (20).

Furthermore, it appears that TGF- β sits at the intersection of Tregs and Th17 cells, a subset of CD4+ T cells that form part of the defense against fungi and extracellular bacteria and contribute to autoimmune disease (21). In the absence of additional inflammatory cytokines, TGF- β stimulates Foxp3 expression, which may actively inhibit T_H17 differentiation by antagonizing the transcription factor ROR- γ t. TGF- β is required for both Treg and Th17 commitment, but the addition of IL-6 promotes Th17 development. When naive CD4+ T cells are transferred to IL-6-overexpressing SCID mice, fewer Tregs develop than when the T cells are transferred to non-IL-6-overexpressing SCID mice (22). IL-6 deficient mice, although having the same number and percentage of CD4+CD25+Foxp3+ T cells at baseline as wild-type mice (23), fail to develop a Th17 response following stimulation and instead become skewed towards Foxp3+ Tregs (24). Some groups even report that co-culture with IL-6 can convert natural Tregs into Th17 cells (25), although this has not been replicated by other groups (22).

IL-6 is produced by fibroblasts, keratinocytes, and endothelial cells in response to injury, but also by cells of the innate immune system (24, 26). In the setting of inflammation, monocytes and macrophages produce IL-1, IL-6, and TNF- α , creating an environment that prevents Treg differentiation and encourages Th17 predominance (27, 28). The inhibition of natural Tregs by innate signals may foster the development of Th17 cells (29). Dendritic cells secrete IL-6 in response to activation of Toll-like receptor 4 (TLR-4) and TLR-9 (26), two members of a large family of receptors that recognize pathogen-associated molecular patterns, which allow the innate immune system to detect microbial and viral infection. The defect in lupus prone mice has been traced to IL-6 over-production by dendritic cells (30).

Bettelli *et al.* showed that IL-6 completely inhibits TGF- β -induced Foxp3 upregulation (31), using a reporter gene in the Foxp3 locus. This probably occurs through IL-6-induced expression of SMAD7, an endogenous inhibitor of TGF-beta signaling, which is normally

down-regulated in Tregs, but is upregulated when IL-6 is added to Treg-producing cultures (32). IL-6 also downregulates Foxp3 binding to chromatin (33).

Another indication of the counter-regulatory effect of IL-6 on Tregs comes from gp130-STAT3 defective mice. IL-6 transmits signals through association with gp130, resulting in activation of transcription factors STAT1 and STAT3 (21). Naive T cells from gp-130-STAT3 signaling-defective mice cultured with IL-6 and TGF- β fail to develop into Th17 cells and instead become Foxp3⁺ (23). In humans, it is possible that Th17 cells originate from natural Tregs rather than from conventional naive CD4⁺CD25⁻ T cell precursors (34-36). Regulatory T cells require sustained expression of Foxp3 expression to maintain Treg suppressor function and phenotype (37, 38), and reduced expression of Foxp3 results in expression of cytokines not normally expressed by Tregs such as IL-2, IL-4, IL-17, and IFN- γ (39, 40). This plasticity brings into question the stability of T cell differentiation and raises uncertainty about the likely efficacy of Treg transfer approaches seeking to achieve stable transplant tolerance; for example, *in vivo*, will the Tregs further differentiate into another lineage and continue to express Th1 and Th17 cytokines. This leads us to hypothesize that in the setting of an acutely transplanted graft, not only are Tregs compromised, but Th17 differentiation and expansion is promoted.

Both cytokines and cell surface molecules modulate Treg function

Even with successful Treg commitment, IL-6 production by innate immune cells has a counter-regulatory effect on Treg function. Tregs isolated from IL-6-overexpressing mice have normal suppressive function *in vitro* (22), but when cultured in the presence of IL-6 overproducing dendritic cells, they lose their ability suppress wild-type CD4⁺CD25⁻ T cell proliferation (30). IL-6, along with TNF- α , has also been demonstrated to be important in the ability of TLR-activated antigen presenting cells to abrogate Treg function following (41). This may be partly due to a direct proliferative effect of IL-6 on naive T cells (42) that is not necessarily mediated through the Treg.

The innate immune system also modulates Treg function by way of cell surface molecules. GITRL, is expressed at low levels by macrophages, dendritic cells, and B cells (43, 44). Expression increases dramatically after stimulation by IFN-gamma, LPS, or IgM, but drops to below pre-stimulation levels within 24-48 hours (43, 44). Increased expression has also been observed in antigen presenting cells of draining lymph nodes after viral infection (45). GITRL expression seems to be controlled by transcription factor NF- κ B (44), and could be one way in which the innate immune system controls the function of GITR high-expression Tregs during the early immune response.

In vitro studies have found that signaling through glucocorticoid inhibitory TNF receptor (GITR), a molecule expressed on the surface of Tregs, inhibits their regulatory function (46, 47). It was first discovered in a mouse hybridoma T cell line and subsequently found to be up-regulated during T cell activation and to confer resistance to anti-CD3-mAb induced apoptosis (48). There have been multiple reports that GITR stimulation increases T cell activation and proliferation (44, 47, 49, 50) although others have reported no salutary effect on T cells in the absence of CD4⁺CD25⁺ T cells (46, 51). It is likely that GITR acts as a co-stimulatory signal, whose effect depends on the strength of the T cell stimulus and the presence of additional co-stimulatory molecules such as CD28 (47).

Unlike the upregulation seen after activation of CD4⁺CD25⁻ T cells, GITR is expressed constitutively at high levels in CD4⁺CD25⁺ Tregs (45). Many groups have observed *in vitro* that GITR stimulation abrogates Treg suppressive activity (46, 47), which may be partially explained by the direct effects on CD4⁺CD25⁻ T cells described above, but experiments using mouse-specific anti-GITR antibodies in combined rat-mouse proliferation assays

suggest that GITR signaling on both CD4+CD25+ and CD4+CD25- T cells is involved (47). In some *in vivo* models of transplant tolerance, it appears that GITR exerts its pro-inflammatory effects through CD4+CD25+ Tregs and not CD4+CD25- T cells (51), while other infectious, auto-immune, and transplant models implicate both (45, 47, 50, 52, 53).

GITR stimulation results in increased activation of NF-kappaB (44, 50, 54), which could explain its activating effects on CD4+CD25- T cells, but it is not so clear how this disrupts Treg function and very little work has been done to address this question. GITR signaling may play a role in the development of induced Tregs (51), but again, there is little data to support or contradict this hypothesis. It has been suggested that reverse signaling in allergic airway disease, where GITR also transmits a signal through GITRL during binding, decreases the pro-inflammatory activity of plasmacytoid dendritic cells (55). This may serve to limit T cell activation, but the significance of this finding is not established.

Strategies for minimizing the counter-regulatory activity of the innate immune system

Dominant among the receptors that activate the innate immune response is the Toll-like receptor family, which serves to recognize microbial and viral associated molecules and heavily influences regulatory T cell function. CpG, a TLR9 ligand, prevents TGFβ/IL-2-mediated differentiation of adaptive Tregs *in vitro* (56). *In vivo*, Pasare and Medzhitov found that mice deficient in MyD88, the central adaptor of TLR signaling, fail to mount a response to subsequent antigen challenge. Depletion of Tregs allows the mice to respond normally. MyD88 control of Treg suppressive activity appears to be mediated through dendritic cells and B lymphocytes (26, 57, 58).

Thornley and colleagues have demonstrated that engagement of several TLRs (TLR 2, 3, 4, or 9) deactivates regulatory T cells and overrides tolerance induced by co-stimulatory blockade in a model of skin transplantation (59). Cardiac and islet transplants show a similar dependence on TLR signaling for prompt rejection (60-62). Neither co-stimulatory blockade alone nor MyD88-deficiency alone results in long-term acceptance of skin grafts across major MHC incompatibilities, but co-stimulatory blockade in MyD88 -/- mice does result in tolerance. Depletion of CD4+CD25+ Tregs restores prompt rejection (63). It has been proposed that tolerance is difficult to achieve in skin, lung, and intestine grafts because they are constantly exposed to pathogens that initiate persistent engagement of TLR (64). Tregs which provide graft protection in the absence of inflammation lose their suppressor function in an acute, inflamed setting (37). Interfering with TLR signaling may represent one way of preventing the innate immune system from abrogating Treg mediated allograft tolerance.

Immediately downstream of MyD88 are the important IL-1-receptor associated kinases (IRAKs), in particular IRAK and IRAK-2. Induction of IRAK-M, an inhibitor of IRAK activity, by previous TLR activation leads to a state known as "TLR tolerance" or "endotoxin tolerance." In this state, cells that have been previously exposed to a TLR ligand are refractory to further activation. Other inhibitory factors such as A20 are also upregulated. A20 overexpression leads to alterations in ubiquitination that favor proteasomal degradation of IRAK. Natural inhibitory pathways represent potential strategies for attenuating TLR signaling in the quest for transplant tolerance. Indeed, it has been previously demonstrated that physiologic inhibitors can be replenished intracellularly by coupling of the protein to a membrane translocating motif.

On the other hand, Caramalho *et al.* reported that CD4+CD25+ Tregs express TLR-4 and treatment of Tregs directly with TLR-4 ligand (lipopolysaccharide), in the absence of antigen presenting cells, increases CD4+CD25+ cell suppressor efficiency *in vitro*. *In vivo*,

these LPS-activated Tregs controlled naive CD4 T cell-dependent wasting disease. Paradoxical augmentation of Treg function with TLR agonists occurs through innate immune cells as well. High doses of TLR-9 ligand, CpG, result in potent suppressor activity that is dependent on blocking IL-6 production by plasmacytoid dendritic cells (65).

Another receptor that promotes allograft rejection is CD40, a co-stimulatory molecule found on dendritic cells and macrophages that is necessary for their activation. CD40L is upregulated on activated T cells. Blockade of CD40-CD40L interaction prolongs survival in multiple rodent and nonhuman primate transplantation models (66-69). In an adoptive transfer model into RAG-deficient mice alone or in combination with donor-specific transfusion, CD154 blockade (with CD40L-deficient cells or by antibody) on CD4+CD25+ regulatory T cells enhanced their suppressor function and prolonged allo-skin transplant (70). A single dose of anti-CD40L results in decreased responder CD8+ T cells and increased graft infiltration of CD4+CD25+ Tregs (71).

Tregs also possess intrinsic pathways that utilize inflammatory signals from the innate immune system to augment their suppressor function. For example, IFN- γ is produced by activated natural killer cells (72) and dendritic cells (73), and normally drives Th1 differentiation in inflammatory states (74), through what was previously considered a Th1 specific transcription factor T-bet (75). A recent study showed that T-bet is also up-regulated in foxp3+ Tregs in response to IFN- γ , where it directs the expression of CXCR3. CXCR3 is a chemokine receptor that allows Tregs to home to inflammatory sites, where they continue to display suppressive activity and acquire a competitive survival advantage (76, 77). In fact, when T-bet deficient foxp3+ Tregs are transferred to foxp3- *scurfy* mice, they fail to suppress auto-immunity that normally develops in the *scurfy* mice, even though T-bet wild-type foxp3+ Tregs efficiently prevent auto-immunity in those mice (76). Thus the ability to suppress a Th1 response requires Treg expression of a Th1 transcription factor. Likewise, Treg suppression of Th2 response requires expression of Irf4, a Th2 transcription factor (77, 78). This model is complicated by data which demonstrates that Irf4-deficient Tregs may also be required for control of Th1 or Th17 responses. However, enhancing such pathways is another potential method for promoting Treg-dependent tolerance in the setting of transplantation.

Summary

Tregs represent a promising strategy for promoting allograft tolerance. However, activation of the innate immune system by tissue injury associated with the transplant procedure initiates multiple pathways of Treg counter-regulation, which interfere with the effectiveness of Treg therapy. Although these pathways originally evolved to protect against microbial infection and stimulate tissue repair, it may be possible to promote long-term, allograft-specific Treg function by temporarily interfering with innate immune activity at the time of transplant, without increasing general immunosuppression.

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List of abbreviations

Tregs	regulatory T cells
Tconv	conventional T cells

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