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Prevention of Allograft Rejection by Amplification of Foxp3⁺CD4⁺CD25⁺ Regulatory T Cells

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Immune regulation or suppression has long been proposed to play a role in transplantation. Various types of regulatory T cells have been documented since the early 1970's¹. However, the absence of specific and reliable marker(s) to discriminate regulatory T cells (T_{reg}) from effector T (T_{eff}) cells hampered their characterization. Seminal work by Sakaguchi et al revealed that CD4⁺CD25⁺ T cells were potent suppressors of autoimmunity². These CD4⁺CD25⁺ T cells were subsequently termed natural regulatory T cells, or nT_{reg} cells. Later, a transcription factor, forkhead box protein 3 (Foxp3) was identified as a master regulator for T_{reg} generation and differentiation, and as a more specific marker than CD25 (i.e., the α -chain of interleukin [IL]-2 receptor) for T_{reg} cells because the latter is also expressed on activated T_{eff} cells^{3–5}. Nonetheless, CD25 remains a reliable cell surface marker for isolation of nT_{reg} cells as staining for Foxp3 requires cell permeation leading to cell death, making Foxp3 unhelpful for cell isolation.

T_{reg} cells and transplant rejection

Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells have been increasingly documented to suppress allograft rejection even though rejection is often an inevitable outcome in non-immunosuppressed recipients due to vigorous activity of alloreactive T_{eff} cells. *In vitro*, naïve CD4⁺CD25[–] T_{eff} cells were more reactive than whole CD4⁺ T_{eff} cells against alloantigen (alloAg) stimulation⁶. Both freshly isolated and *ex vivo*-activated nT_{reg} cells potently suppressed alloAg-driven T_{eff} cell activation in mixed lymphocyte reaction (MLR) assays^{6,7}. *In vivo*, pre-transplant depletion of Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells by anti-CD25 mAbs resulted in subversion of tolerance into chronic rejection in otherwise spontaneously tolerant mouse models or conversion of chronic rejection into acute rejection in otherwise chronically rejecting rodents^{8–12}.

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T_{reg} cells and transplant tolerance

Foxp3⁺CD4⁺CD25⁺ T_{reg} cells have also been shown to mediate *in vivo* allograft tolerance. Interestingly, CD4⁺CD25⁺ cells have been shown to be the primary mediator of allograft tolerance in rats even before CD4⁺CD25⁺ cells were recognized as a population of T_{reg} cells¹³. Recently, CD4⁺CD25⁺ T_{reg} cells have been repeatedly implicated in initiation and/or maintenance of allograft tolerance induced by a variety of tolerizing treatments^{14, 15}. Under most conditions, the induced tolerance is transferable to naïve hosts through the adoptive transfer of the Foxp3⁺CD4⁺CD25⁺ population composed of expanded Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells and/or induced T_{reg} (iT_{reg}) cells from Foxp3⁺CD4⁺CD25⁺ precursors^{14, 15} from the tolerized hosts. Antigen-specific Foxp3⁺CD4⁺CD25⁺ iT_{reg} cells could also be generated *ex vivo* under special culture conditions from Foxp3⁺CD4⁺CD25⁺ precursors in the presence or absence of Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells^{16–19}. In humans, accumulating evidence suggests that a favorable allograft outcome is frequently associated with a robust population of FOXP3⁺CD4⁺CD25⁺ T_{reg} cells despite conflicting evidence emerging in part due to the confounding expression of FOXP3 by activated T_{eff} cells^{20–23}.

Here, we will review current approaches for amplification of allo-specific Foxp3⁺CD4⁺CD25⁺ T_{reg} cells for prevention of allograft rejection. Available strategies are: (i) immunotherapy with *ex vivo*-expanded Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells; (ii) immunotherapy with *ex vivo*-induced Foxp3⁺CD4⁺CD25⁺ iT_{reg} cells; and/or (iii) *in vivo* expansion and/or induction of Foxp3⁺CD4⁺CD25⁺ T_{reg} cells by tolerizing treatments.

I. Immunotherapy with *ex vivo*-expanded Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells to prevent allograft rejection

Principle

Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells have a broad T cell receptor (TCR) repertoire including clones recognizing alloAg^s^{24, 25}. Presence of such allo-suppressive clones within the fresh repertoire has been confirmed *in vitro* and *in vivo*. *In vitro*, fresh Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells from naïve rodents or humans effectively suppress alloactivation of recipient T cells against donor alloAg in MLR assays^{6, 26}. *In vivo*, sufficient quantities of freshly isolated Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells from naïve animals have been demonstrated to suppress simultaneously co-transferred T_{eff} cell-mediated allograft rejection in immunodeficient rodents²⁷. Due to the low precursor frequency, efficient *ex vivo* expansion of fresh naïve Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells is likely essential for generating sufficient numbers of cells to therapeutically target a large repertoire of alloreactive T_{eff} clones in the recipients. Additionally, *in vitro*-activated nT_{reg} cells exert stronger suppression than fresh cells against alloAg^s^{6, 28}. *Ex vivo* expansion could be achieved through alloAg-nonspecific and alloAg-specific manners. Regardless of the methods, preserving certain cell characteristics would be necessary for the effectiveness of the *ex vivo*-expanded nT_{reg} cells in targeting allograft rejection. These characteristics include: sufficient cell number, preservation of Foxp3, survival *in vivo* after infusion for some unknown period of time if not permanently, *in vivo* migration to sites of alloreactivity, and the ability to suppress alloreaction in an alloAg-specific manner.

Strategies for *ex vivo* expansion

The basic requirements to expand anergic fresh Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells include triggering of TCR (signal 1), activation of costimulation pathway(s) (signal 2), and the presence of IL-2. It is logical to achieve alloAg-specific expansion by co-culture of fresh naïve nT_{reg} cells in the presence of donor-type dendritic cells (DC). Indeed, DC-expanded nT_{reg} cells seem to be highly suppressive against same donor alloAg^s *in vitro* and *in vivo*²⁹.

However, it is not an efficient strategy in terms of cell yield^{6, 29}. Thus far, alloAg-nonspecific expansion using anti-CD3/CD28 mAb-coated beads plus IL-2 appears to be the most efficient method for *ex vivo* expansion of both mouse and human nT_{reg} cells^{30–32}. These alloAg-nonspecifically expanded nT_{reg} cells have been shown to suppress alloactivation *in vitro* as well as to prevent allograft rejection and graft-versus-host disease *in vivo*, although they are less efficacious than alloAg-specific nT_{reg} cells^{6, 32}. We have developed a protocol achieving a reasonable level of expansion while achieving alloAg specificity by re-stimulation of nT_{reg} cells with donor BM-derived DC (BM-DC) after initial expansion using anti-CD3/CD28 mAb-coated beads and IL-2³³. Cells expanded by this method were potent suppressors against donor but not 3rd-party alloAg *in vitro* and *in vivo*³³.

Dynamics of Foxp3 expression

Unexpectedly, a rapid decline of the percentage of intracellular Foxp3⁺ cells during alloAg-nonspecific and alloAg-specific expansion was observed in our study, but overall both Foxp3⁺ and Foxp3[−] fractions were effectively expanded^{33, 34}. By using flow-sorted Foxp3⁺ cells from Foxp3-green fluorescence protein (GFP) knock-in mice, we have recently confirmed activation-induced loss of Foxp3 in our culture system with fewer than 30% cells remaining Foxp3⁺ at day 18 of IL-2 containing culture (Xia G, et al. manuscript submitted). Decrease of Foxp3 has also been observed in other culture conditions. An example is a recent study using Foxp3⁺ T_{reg} cells isolated from Foxp3-transgenic mice which showed that Foxp3⁺ T_{reg} cells lost Foxp3 expression and became T_{eff} cells during *in vitro* Th1 and Th2 differentiation³⁵. Another recent study using Foxp3⁺ nT_{reg} cells purified from Foxp3-GFP knock-in mice has shown that 12–17% Foxp3⁺ nT_{reg} cells became Foxp3[−] cells at 24 hours after *in vitro* anti-CD3 stimulation³⁶. Decrease of Foxp3 at the gene transcript and/or protein levels has also been reported after *in vitro* activation of human CD4⁺CD25⁺ nT_{reg} cells^{37, 38}.

The exact mechanism(s) underlying the loss of Foxp3 during *in vitro* expansion are not clear. Utilizing IL-2 alone in our current protocol may be insufficient and may partially be responsible for the loss of Foxp3. Besides the crucial and dominant role of IL-2, other members of the ©-chain cytokines including IL-7 and IL-15 have also been shown to play a role in the survival of CD4⁺CD25⁺ nT_{reg} and in the maintenance of Foxp3 expression, although it needs to be further studied whether they synergize with IL-2 *in vitro* and/or *in vivo*^{39, 40}. A series of studies have shown that TGF-β is critical in generating, maintaining, and expanding the *in vivo* pool of CD4⁺CD25⁺ T_{reg} cells via Foxp3 modulation and/or induction, and is likely also important in mediating *in vivo* suppressive function^{41–44}. Indeed, adding TGF-β to our cultures has partially rescued the loss of Foxp3 and the cells retained stronger suppressive function, while similar levels of expansion were achieved³³. This is consistent with a recent study showing that addition of TGF-β to cultures helped to maintain Foxp3 in nT_{reg} cells isolated from Foxp3-GFP knock-in mice³⁶. Interestingly, in the presence of TGF-β, nT_{reg} cells not only retained higher levels of Foxp3 during *ex vivo* expansion (from 10% to 25%) but expressed much higher levels of Foxp3 *in vivo* (from 15% to 55%) after adoptive transfer³³. This is fundamentally different from *de novo in vitro*-generated Foxp3⁺CD4⁺CD25⁺ iT_{reg} cells, which generally tended to lose their Foxp3 expression *in vivo* (fewer than 10% remained Foxp3 expression in 2-wks post transfer) after adoptive transfer into allograft-bearing Rag^{−/−} mice (Xia G, et al., manuscript submitted and Luo, unpublished observations).

Addition of retinoic acid and histone deacetylase inhibitor trichostatin A into our aforementioned culture system could further improve expression of Foxp3 *in vitro* (>45% remaining Foxp3⁺) and after adoptive transfer *in vivo* (85~90% remaining Foxp3⁺) to prevent allograft rejection (Xia G, et al. manuscript submitted). Thus in our culture system,

retinoic acid and trichostatin A synergize with IL-2 and TGF- β to preserve expression of Foxp3 *in vitro*. Maintaining Foxp3 promoter CpG demethylation and protein acetylation by these agents appear to be important^{45,46,47}. Lastly, the role of chemokines in the function of T_{reg} cells has been mainly focused on chemokine-mediated recruitment of T_{reg} cells to site of inflammation^{48–51}. Interestingly, there is growing evidence to suggest that expression (or the lack of) certain chemokines is associated with altered expression levels of Foxp3^{52, 53}, adding another potential avenue for modification during T_{reg} expansion. At present time, optimizing *ex vivo* expansion protocols remains a challenge for maximally preserving Foxp3 while achieving an efficient level of expansion.

Engraftment, trafficking and homing

After adoptive transfer, *ex vivo*-expanded nT_{reg} cells survived, expanded long-term and trafficked through the peripheral blood and lymphoid tissues in allograft-bearing mice, suggesting that similar to fresh T_{reg} cells, their maintenance could be driven by self- or allo-Ags and that their homing capability was not apparently impaired after *ex vivo*-expansion^{6,32,33}. Moreover, nT_{reg} cells migrated to and resided within allografts after adoptive transfer suggesting their intra-graft presence may be essential for exerting graft protection^{14,32,33}. We have observed that various degrees of loss of Foxp3 expression occurs after adoptive transfer of the *in vitro* expanded or generated T_{reg} cells (ref.32 and Luo, unpublished observations), although others have shown stable maintenance of such Foxp3⁺ cells *in vivo*⁵⁴. Emerging data now suggest that cross-differentiation of Foxp3⁺ cells to other Th-type cells such as IFN- γ -secreting Th1 cells or IL-17-secreting Th17 cells with or without concurrent Foxp3 expression is possible *in vitro* and *in vivo* under appropriate stimulation or inflammation micro-environment^{35,55,56}. Single cell cloning of FOXP3⁺ cells in humans further revealed significant heterogeneity of this cell population⁵⁷. These data suggest that further identification of better-defined starting populations as well as optimal control of *in vivo* cytokine milieu will assist optimal survival and sustained expression of Foxp3 of the adoptively transferred T_{reg} cells generated *ex vivo*.

Development of allospecific suppression

The exact mechanism of allosuppression mediated by adoptively transferred nT_{reg} cells is unclear, but multiple mechanisms may be involved. *In vitro* data suggest that once nT_{reg} cells become activated, their suppression is Ag non-specific²⁸. However, recent data have suggested that the repertoire of nT_{reg} cells could be shaped *in vivo* to suppress T-cell reactivity in an Ag-specific manner^{58–60}. In our system, alloAg-specific clones were first selected *ex vivo* by co-culture with donor BM-DC and subsequently maintained *in vivo* via cognate interactions with alloAg expressed on heart allografts, likely via both direct and indirect alloAg presentation pathways^{33, 58}. Indeed, some recent studies have suggested that CD4⁺CD25⁺ nT_{reg} are particularly potent at recognizing alloAg via the indirect pathway, and that *ex vivo* priming through the indirect pathway using recipient DCs is necessary for overcoming chronic rejection *in vivo* after adoptive transfer in mice^{61,62}. Furthermore, adoptively transferred T_{reg} cells may facilitate development and/or expansion of endogenous T_{reg} cells specific for donor alloAg, thereby prompting a cascade of T_{reg} cell-amplification resulting in a robust status of allograft tolerance, a process known as “infectious tolerance”⁶³. This possibility could be mediated through direct T cell-T cell interaction or through induction of and interactions with “tolerogenic” dendritic cells^{64–68}. Thus, a potential state of pan-immunosuppression is avoided and alloAg-specific immunotherapy with Foxp3⁺CD4⁺CD25⁺ nT_{reg} cells may be used as a non-toxic modality for allograft rejection.

Challenges in clinical application

In reality, tolerance does exist after liver and kidney transplantation in humans, albeit a rare event⁶⁹. Amplification of host CD4⁺CD25⁺ T_{reg} cells would be of critical value in promoting transplantation tolerance in humans. The basic principles and expansion strategy successful in rodents seem to be generally applicable to humans^{70–72}. In the organ transplantation setting, nT_{reg} cells would be of recipient origin. For application in clinical practices, all procedures including nT_{reg} cell isolation and expansion, facility, equipments, reagents, and quality control measures should be in compliance with good manufacturing practice requirements⁷³. Besides stringent regulatory, logistic, and financial challenges, there are unique technical challenges for the success of human T_{reg} cell immunotherapy. First, purification of *bona fide* FOXP3⁺CD4⁺CD25⁺ nT_{reg} cells is problematic due to contamination of activated T_{eff} cells if purification is simply based on the expression of CD25^{high}. Recent studies have progressed to using a combination of surface markers such as CD127, CD49d, CD45RA and CD25 to purify nT_{reg} cells^{74–76}. However, continuing search for better membrane markers is still needed to identify *bona fide* nT_{reg} cells. Second, expression of FOXP3 is not necessarily an indicator of suppressive capacity, as activated human T_{eff} cells also express FOXP3, albeit transient and/or at a lower level, without suppressive function⁷⁷. Third, a likely expansion strategy is a combination of alloAg-nonspecific (e.g., by anti-CD3/CD28-coated artificial antigen presenting cells, or aAPC) and alloAg-specific (alloAg-bearing APC-mediated) expansion⁷⁸. However, the optimal form of APCs has yet to be identified⁷⁹. Fourth, in experimental studies using adoptive transfer model, we and others have shown that the ratio of T_{reg}:T_{eff} determines the efficacy of T_{reg} cells in controlling allograft rejection; usually at least 1:1 T_{reg}:T_{eff} ratio is required to achieve significant graft-prolonging benefits^{27,32,80}. The number of cells necessary for achieving clinical benefits in humans is yet to be defined. Fifth, pre- and/or peri-transplant induction therapy (to deplete or functionally inactivate T_{eff} cells) is probably important for maximizing the benefits of nT_{reg} cell immunotherapy³³. Induction therapies that do not interfere with the survival and function of adoptively transferred *ex vivo*-expanded, recipient-origin nT_{reg} cells will need to be designed. Likewise, choice of maintenance immunosuppressants is likely to be critical as well, as they may interfere with the adoptively transferred nT_{reg} cells⁸¹. Lastly, further challenges exist in human nT_{reg} cell therapy in tracking, monitoring and functional assessment of these cells *in vivo* once they are delivered to the recipients.

II. Immunotherapy with *ex vivo*-induced Foxp3⁺CD4⁺CD25⁺ iT_{reg} cells to prevent allograft rejection

Theoretical advantages

As discussed earlier, one of the main challenges for expanding existing nT_{reg} cells is the lack of reliable cell surface markers that would allow sorting out nT_{reg} cells of high purity, thus risking contamination with non-T_{reg} CD4⁺ cells and subsequent expansion of the latter as an effector population. Therefore, if culture conditions exist that would convert non-T_{reg} cells into functionally suppressive Foxp3-expressing T_{reg} cells (iT_{reg}), this would not only alleviate concerns over contamination of the expanding nT_{reg} cell population with non-T_{reg} cells, but would also allow exploration of the much more abundant Foxp3[−]CD4⁺CD25[−] T cell population for conversion to Foxp3⁺CD4⁺CD25⁺ suppressor cells.

Strategies and mechanisms for *in vitro* induction of Foxp3⁺CD4⁺CD25⁺ iT_{reg} cells from Foxp3[−]CD4⁺CD25[−] T cells

While lack of functional Foxp3 does not seem to affect the intrathymic development of T_{reg} cells⁸², maintenance of T_{reg} cell function has been shown to be critically dependent on the

continued expression of functional Foxp3⁸³. Consequently, much effort has been focused on the induction of Foxp3 for generation of iT_{reg} cells.

Chen et al demonstrated that when naïve Foxp3⁻CD4⁺CD25⁻ T cells were stimulated by anti-CD3 and anti-CD28 in the presence of TGF- β 1 and IL-2, both CD25 and Foxp3 were up-regulated, and consequently these cells phenotypically became Foxp3⁺CD4⁺CD25⁺ T cells¹⁶. Functionally, these cells were suppressive toward naïve cell proliferation stimulated by anti-CD3 and anti-CD28. This phenomenon was later reproduced by many. In some cases, IL-2 appeared to be obligatory^{84,85}, whereas in others it appeared to be dispensable^{86,87}, although the possibility remained that the activated T cells were the source of IL-2 in the process of conversion.

To delineate the role of APCs in the process of TGF- β -dependent induction of T_{reg} cells, we have taken advantage of transgenic T cells with a TCR that recognizes a single diabetogenic epitope represented by the mimotope BDC peptide, and tested dendritic cell sub-populations in this process⁸⁶. We found that compared to granulocyte macrophage colony stimulation factor (GM-CSF)-differentiated BM-DCs that express high levels of costimulation molecules, splenic CD11c⁺ DCs expressing much lower levels of costimulation molecules are far more conducive to TGF- β -dependent induction of Foxp3 expression. In a more recent study, splenic DCs were further fractionated and the CD8 α ⁺ DCs were shown to be the main subset within the splenic DCs that determined inducibility of Foxp3 by TGF- β *in vitro*⁸⁸. While coinhibitory signals through programmed death ligand 1 was obligatory for conversion, costimulatory signals through glucocorticoid-induced TNFR-related protein (GITR) suppressed conversion, implicating a balance of signal strengths being an important determinant for *de novo* T_{reg} induction. *In vivo*, this subset of DCs likely also plays a role in differentiation of peripheral Foxp3⁺ T_{reg} cells, and this ability is at least in part due to their ability to produce TGF- β ⁸⁹. Another recent series of reports identified that DCs derived from gut-associated lymphoid tissue, particularly the lamina propria, are highly efficient at *de novo* generation of Foxp3⁺ T_{reg} cells from Foxp3⁻ precursors, and that this ability can be attributed to their capacity to produce retinoic acid (RA) and the gut-homing receptor CD103^{90,91}. This is an important finding in iT_{reg} biology, because TGF- β has also been found to promote the differentiation of naïve T cells into proinflammatory IL-17 producing cells in the presence of IL-6⁹². It appears that TGF- β -dependent T cell differentiation is reciprocally controlled by RA and IL-6. In the presence of RA, IL-17 differentiation is inhibited and iT_{reg} cell differentiation is enhanced; whereas in the presence of IL-6, the opposite occurs⁹³.

At a molecular level, it has recently been shown that the promoter of the Foxp3 gene is highly de-methylated in nT_{reg} cells, correlating with high levels of Foxp3 expression; whereas in non-T_{reg} cells, this region is highly methylated correlating with minimal Foxp3 expression^{45, 94}. When TGF- β is present in the process of naïve T cell activation, this region can be induced to become *partially* de-methylated, leading to induction of Foxp3 expression^{45,95}. We have recently shown that the activation state of the extracellular-regulated kinase (ERK) is implicated in the TGF- β -induced demethylation of the Foxp3 gene promoter⁴⁶. It appears that ERK phosphorylation during T cell activation can be significantly inhibited by the presence of TGF- β . Inhibition of ERK phosphorylation in turn leads to down-regulation of DNA methyl-transferases, which could account for the partial de-methylation of the Foxp3 promoter. This hypothesis was corroborated by demonstrating that Foxp3 expression could be induced by direct ERK inhibition using a small molecule inhibitor of ERK, UO126, during T cell activation. Another recent report also implicated other signaling network involving phosphatidylinositol 3-kinase, protein kinase B, and the mammalian target of rapamycin in the epigenetic regulation of Foxp3 gene expression⁹⁶.

Careful promoter studies of the *Foxp3* gene revealed that in mouse T cells, TGF- β -induced promoter hypo-methylation involves only selected CpG islands, and that the temporarily hypo-methylated sites could be readily re-methylated upon retrieval of TGF- β , suggesting that the induced expression of *Foxp3* may not be stable⁴⁵. Furthermore, in human T cells, it appears that TGF- β -induced FOXP3 expression was not associated with FOXP3 promoter de-methylation at all⁹⁷. A recent study showed that in human cells TGF- β -induced FOXP3⁺ T cells were neither anergic nor suppressive⁷⁷. These studies raise further questions with respect to the utility of TGF- β -induced T_{reg} cells in humans.

The role of iT_{reg} cells in models of allogeneic transplant rejection

As described earlier, MLR has been traditionally used as an *in vitro* measurement for allogeneic responses in which recipient T cells are cultured with donor APCs. Interestingly, in combination with TGF- β and IL-2, naïve recipient T cells stimulated in MLRs have been shown to convert to CD4⁺CD25⁺ regulatory T cells^{66, 98}. These early studies utilized irradiated donor whole splenocytes with or without T cell depletion as allogeneic stimulators. Such *in vitro* MLR-generated CD4⁺CD25⁺ regulatory T cells were capable of prolonging donor-specific cardiac graft survival. Furthermore, in such MLRs containing both CD4⁺ and CD8⁺ recipient T cells, TGF- β appeared to be able to induce *Foxp3*-expressing suppressive (regulatory) cells in both T cell subsets, a phenomenon described by several other groups as well^{99–101}.

As with the induction of autoantigen-specific iT_{reg} cells, the role of APCs in efficient *de novo* induction of alloantigen-specific iT_{reg} cells remains to be defined. In our experience, immature spleen CD11c⁺ DCs isolated from the donor strain are poor inducers for conversion of recipient bulk CD4⁺ T cells to *Foxp3*⁺CD4⁺CD25⁺ iT_{reg} cells in primary cultures, even in the presence of IL-2 (Luo, unpublished observation). On the other hand, BM-DCs that are “alternatively activated” have been shown to be “toleragenic” and are able to induce T_{reg} cells *in vivo* and *in vitro*. BM-DCs differentiated in the presence of IL-10 and TGF- β have been shown to be poor stimulators of allogeneic T cell proliferation, but they strongly induce IL-10 production and expansion of *Foxp3*⁺CD4⁺CD25⁺ cells in cultures¹⁰². Likewise, BM-DCs differentiated in pharmacological agents such as the mTOR inhibitor sirolimus or LF 15-0915, a chemical analogue of 15-deoxyspergualin, have also been shown to selectively increase the *Foxp3*⁺CD4⁺CD25⁺ population^{103–105}. In these systems, the fact that the starting population was comprised of naïve whole CD4⁺ cells made it difficult to discern whether an increase in the *Foxp3*⁺CD4⁺CD25⁺ T cell population came from expansion of existing nT_{reg} cells or *de novo* conversion of previous non-T_{reg} cells. In addition, whether the direct (by using donor APCs) or indirect (by using recipient APCs) presentation is more efficient in iT_{reg} cell generation *in vitro* remains to be defined.

III. Tolerance strategies for *in vivo* expansion and/or induction of *Foxp3*⁺CD4⁺CD25⁺ T_{reg} cells

Role of *Foxp3*⁺CD4⁺CD25⁺ T_{reg} cells in existing tolerance protocols

Several well-studied tolerance protocols in transplant models have shown *in vivo* expansion and/or induction of *Foxp3*⁺CD4⁺CD25⁺ T_{reg} cells as one of the mechanisms for tolerance induction. These protocols utilized mitogenic or non-mitogenic anti-CD3 monoclonal antibodies, non-depleting anti-CD4 and anti-CD8 antibodies, anti-CD154 (anti-CD40L) antibody, CTLA4-Ig, and more recently toleragenic DCs. Anti-CD3 mAbs have been widely studied, particularly in autoimmunity (reviewed in ref.106). Contradicting the initial assumption that these were T cell depleting agents, it is now known that depletion of T cells by these antibodies was rather modest (20% to 50%, varying depending on FcR-binding capacity)¹⁰⁷. Rather, T cell anergy, as well as a significant increase in the

Foxp3⁺CD4⁺CD25⁺ T_{reg} cell population, can be induced^{108,109}. The capacity of anti-CD3 mAb to induce operational tolerance has been demonstrated in an allogeneic cardiac transplant model¹¹⁰, as well as in the spontaneous autoimmune diabetes NOD model¹⁰⁸. The induced tolerance was exquisitely dependent on TGF- β in the NOD model. Subsequent studies demonstrated that treatment with anti-CD3 results in two sources of increased TGF- β production: first, by phagocytic macrophages and immature DCs triggered by the apoptotic T cells induced by anti-CD3¹¹¹; and second, by the CD4⁺CD25⁺ T cells themselves^{108,112}. Therefore, it is conceivable that a positive feedback loop may be established involving initial iT_{reg} cell induction by phagocyte-produced TGF- β which is later augmented by TGF- β production by T_{reg} cells themselves. Further expansion of nT_{reg} and/or iT_{reg} cells through such an “infectious tolerance” process leads to a durable state of immunoregulation by the anti-CD3 treatment.

Another robust tolerance regimen for allogeneic transplantation in mice and in nonhuman primates is the i.v. infusion of donor cells (donor-specific transfusion (DST)) with concomitant administration of anti-CD154. Depletional studies demonstrated a critical role of CD4⁺CD25⁺ T_{reg} cells in the tolerance induced by this regimen^{113–115}. Interestingly, CD154 plays a direct role in dampening the suppressive function of CD4⁺CD25⁺ T_{reg} cells. Blocking the effects of CD154 on CD4⁺CD25⁺ T_{reg} cells by anti-CD154 or genetic deletion of CD154 enhanced the suppressive function of CD4⁺CD25⁺ T_{reg} cells to an adequate level such that there was no additional need to block CD154 on effector CD25[−] T cells in order to establish graft protection¹¹⁶. More recently, it has been shown that in this regimen plasmacytoid DCs carrying alloantigens were preferentially directed towards the lymph nodes, rather than the spleen, where they efficiently induced the development of alloAg-specific Foxp3⁺CD4⁺CD25⁺ T_{reg} cells from Foxp3[−]CD4⁺CD25[−] T cells⁶⁵.

In recent years, direct manipulation of DCs has been exploited for generation of “tolerogenic DCs” for induction of peripheral tolerance to alloAg (reviewed in ref.67). The methods for DC manipulation can be categorized into: 1) *in vitro* generation of tolerogenic DCs from bone marrow-derived precursors; and 2) *in vivo* targeting of steady-state DCs. The principle of DC manipulation is to endow DCs with the ability to resist maturation and to subsequently deliver negative signals to the interacting T cells, down-regulate effector T cell responses and possibly expand T_{reg} cells.

Among *in vitro*-generated DCs, BM-DCs cultured in the presence of rapamycin¹⁰³, LF15-0195 (an analogue of deoxyspergualin)¹⁰⁴, and vitamin D3 metabolites¹¹⁷, have all been shown to prolong allogeneic graft survival upon transfer *in vivo*, and do so at least in part through expansion and/or induction of CD4⁺CD25⁺Foxp3⁺ T_{reg} cells. These agents have also been shown to inhibit DC maturation *in vivo* when directly administered to the host. Among *in vivo* existing DC subsets, both CD8 α ⁺ DCs and B220⁺ DCs have been implicated in tolerogenicity of specific tolerance protocols; it therefore appears that tolerogenicity is not restricted to any particular DC subsets, rather all DCs have the plasticity to promote either immunity or tolerance under the appropriate signal combinations.

Direct targeting of recipient DCs *in vivo* represents a more recent advance in DC-based tolerance therapy. The principal of *in vivo* DC targeting is to deliver the necessary pool of alloantigens to host DCs in such a way that the DCs remain un-activated while maintaining their ability to induce T cell tolerance. One such method is to deliver donor cells that are undergoing apoptosis¹¹⁸ or to deliver donor APC-derived exosomes¹¹⁹, both of which provide the entire spectrum of donor antigens to recipient DCs without altering the quiescent status of the recipient DCs. It is interesting to note that phagocytes of different classes, including macrophages, lymphoid DCs and myeloid DCs, may all be involved in recognizing and uptaking apoptotic cells/bodies, but may do so in a temporarily staggered

fashion, and may subsequently influence each other. For example, macrophages engulfing apoptotic cells may direct subsequent apoptotic cells to be up-taken by the more tolerogenic $8\alpha^+$ DCs, rather than $8\alpha^-$ DCs¹²⁰. In addition, $8\alpha^-$ DCs may acquire 8α positivity upon taking up apoptotic cells¹²¹. Interactions between apoptotic cells with host phagocytes may also induce robust generation of T_{reg} cells, likely via a TGF- β -dependent mechanism¹¹¹. Such *in vivo* DC targeting strategies in combination with transient immunosuppression have been shown to induce indefinite allograft survival^{118,119}.

Role of $Foxp3^+CD4^+CD25^+$ T_{reg} cells in a novel donor-specific tolerance protocol using ECDI-treated donor cell infusion

We have developed a novel and robust tolerance regimen that utilizes infusions of donor cells pre-treated with a chemical cross-linker called 1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide, or ECDI, without further immunosuppressive therapy, for tolerance induction in allogeneic islet cell transplantation. Previous studies have shown that i.v. injection of antigen-pulsed splenic APCs chemically fixed with ECDI was a powerful and safe method for inducing antigen-specific T cell tolerance *in vivo*^{122,123}. Specifically, myelin peptide-coupled, ECDI-fixed syngeneic APCs could effectively ablate induction and progression of experimental autoimmune encephalomyelitis (EAE), a murine Th1/17-mediated model of multiple sclerosis (MS)¹²⁴. This protocol is also effective in preventing and treating autoimmune diabetes in the non-obese diabetic (NOD) mice¹²⁵.

We found that i.v. infusions of ECDI-treated donor splenocytes induced indefinite donor-specific tolerance in allogeneic islet cell transplantation. Here, the antigens of interest are mainly donor MHC class I and II molecules that are an integral surface component of donor lymphocytes, and ECDI treatment presumably interferes with costimulatory signals, thereby inducing tolerance to the membrane-bound allogeneic MHC antigens¹²⁶. Graft protection is donor-specific and is associated with markedly diminished donor-specific allo-responses measured by delayed type hypersensitivity, MLRs, IFN- γ production, as well as production of anti-donor antibodies, thus suggesting effective down-regulation of both donor-specific T and B cell responses¹²⁷.

Interestingly, treatment with the ECDI-fixed donor splenocytes induced a significant increase in the number of $CD4^+CD25^+Foxp3^+$ T_{reg} cells in the spleen of tolerized mice vs. controls. The importance of the $CD4^+CD25^+$ T_{reg} cells in this protocol was further supported by the fact that depleted/inactivated T_{reg} cells by anti-CD25 mAb treatment around the time of initial tolerance induction completely blocked tolerance. Conversely, anti-CD25 treatment in long-term tolerized recipients (120 days post-transplant) did not break the established tolerance. These results indicate that $CD4^+CD25^+$ T_{reg} cells are critical for the initial establishment of donor-specific tolerance, however once tolerance has been established, active regulation is less important. Thus, other mechanisms, such as anergy, may be required for long-term maintenance of donor-specific unresponsiveness in this model. The nature of the increased $CD4^+CD25^+Foxp3^+$ population (are they expanded n T_{reg} cells versus *de novo* induced iT $_{reg}$ cells?) is currently under intense investigation.

The critical difference between this tolerance protocol and others using infusions of donor cells, *e.g.*, DST, is that efficient tolerance induction is achieved in the complete absence of immunosuppression, including transient cell-depletion, antibody-mediated blockade of costimulatory signals, and peri-transplant application of immunosuppressive drugs. The exact mechanism by which ECDI-treated cells induce donor-specific tolerance is not completely understood. Cell tracking indicates that ECDI-treated cells distribute widely, but intact cells disappear within 48 hours. Therefore, while direct presentation may play a role, this mechanism is likely transient. In contrast, indirect presentation of alloAg by host regulatory APCs is likely the predominant tolerance mechanism. Recent data in the EAE

model suggest that host plasmacytoid DCs are crucial in the tolerogenic cross-presentation in this protocol¹²⁸, and that the spleen is likely where the tolerogenic interactions occur.

Future perspectives

Considerable insight has been gained into the biology of T_{reg} cells and their role in mediating transplant tolerance. Both *ex vivo* expansion/induction of alloAg-specific T_{reg} cells in tissue cultures and *in vivo* expansion/induction of alloAg-specific T_{reg} cells via tolerizing regimens have shown promises in promoting durable allo-specific tolerance. It is possible that a favorable T_{reg}:T_{eff} ratio set forth by conditioning tolerance regimens through targeting of multiple mechanisms could be further enhanced by infusion of *ex vivo*-expanded or induced T_{reg} cells. Therefore, combination cell-based therapies could be theoretically advantageous. Linked suppression may further allow effective *in vivo* suppression of allo-responses in the absence of specificities toward the entire spectrum of alloAg. A major challenge in this area of transplant immunobiology is translating the accumulated knowledge into human clinical application, with important limitations arising from heterogeneous immunity of the hosts, the need for reliable tools for measuring host tolerance, and regulatory issues.

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REFERENCES

1. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol.* 2003; 3(3):199–210. [PubMed: 12658268]
2. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* 1995; 155(3):1151–1164. [PubMed: 7636184]
3. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol.* 2003; 4(4):330–336. [PubMed: 12612578]
4. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003; 299(5609):1057–1061. [PubMed: 12522256]
5. Ramsdell F. Foxp3 and natural regulatory T cells: key to a cell lineage? *Immunity.* 2003; 19(2):165–168. [PubMed: 12932350]
6. Xia G, Kovochich M, Truitt RL, Johnson BD. Tracking *ex vivo*-expanded CD4+CD25+ and CD8+CD25+ regulatory T cells after infusion to prevent donor lymphocyte infusion-induced lethal acute graft-versus-host disease. *Biol Blood Marrow Transplant.* 2004; 10(11):748–760. [PubMed: 15505606]
7. Xia G, Truitt RL, Johnson BD. Graft-versus-leukemia and graft-versus-host reactions after donor lymphocyte infusion are initiated by host-type antigen-presenting cells and regulated by regulatory T cells in early and long-term chimeras. *Biol Blood Marrow Transplant.* 2006; 12(4):397–407. [PubMed: 16545723]
8. Benghiet FS, Graca L, Braun MY, et al. Critical influence of natural regulatory CD25+ T cells on the fate of allografts in the absence of immunosuppression. *Transplantation.* 2005; 79(6):648–654. [PubMed: 15785370]
9. Schenk S, Kish DD, He C, et al. Alloreactive T cell responses and acute rejection of single class II MHC-disparate heart allografts are under strict regulation by CD4+ CD25+ T cells. *J Immunol.* 2005; 174(6):3741–3748. [PubMed: 15749914]
10. Fucs R, Jesus JT, Souza Junior PH, et al. Frequency of natural regulatory CD4+CD25+ T lymphocytes determines the outcome of tolerance across fully mismatched MHC barrier through

- linked recognition of self and allogeneic stimuli. *J Immunol.* 2006; 176(4):2324–2329. [PubMed: 16455989]
11. Sanchez-Fueyo A, Sandner S, Habicht A, et al. Specificity of CD4+CD25+ regulatory T cell function in alloimmunity. *J Immunol.* 2006; 176(1):329–334. [PubMed: 16365425]
 12. Li W, Kuhr CS, Zheng XX, et al. New insights into mechanisms of spontaneous liver transplant tolerance: the role of Foxp3-expressing CD25+CD4+ regulatory T cells. *Am J Transplant.* 2008; 8(8):1639–1651. [PubMed: 18557727]
 13. Hall BM, Pearce NW, Gurley KE, Dorsch SE. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. *J Exp Med.* 1990; 171(1):141–157. [PubMed: 2136906]
 14. Graca L, Thompson S, Lin CY, Adams E, Cobbold SP, Waldmann H. Both CD4(+)CD25(+) and CD4(+)CD25(–) regulatory cells mediate dominant transplantation tolerance. *J Immunol.* 2002; 168(11):5558–5565. [PubMed: 12023351]
 15. Bushell A, Karim M, Kingsley CI, Wood KJ. Pretransplant blood transfusion without additional immunotherapy generates CD25+CD4+ regulatory T cells: a potential explanation for the blood-transfusion effect. *Transplantation.* 2003; 76(3):449–455. [PubMed: 12923427]
 16. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med.* 2003; 198(12):1875–1886. [PubMed: 14676299]
 17. Fu S, Zhang N, Yopp AC, et al. TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 – precursors. *Am J Transplant.* 2004; 4(10):1614–1627. [PubMed: 15367216]
 18. Karim M, Kingsley CI, Bushell AR, Sawitzki BS, Wood KJ. Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25–CD4+ precursors in a thymus-independent process. *J Immunol.* 2004; 172(2):923–928. [PubMed: 14707064]
 19. Walker MR, Carson BD, Nepom GT, Ziegler SF, Buckner JH. De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+CD25– cells. *Proc Natl Acad Sci U S A.* 2005; 102(11):4103–4108. [PubMed: 15753318]
 20. Salama AD, Najafian N, Clarkson MR, Harmon WE, Sayegh MH. Regulatory CD25+ T cells in human kidney transplant recipients. *J Am Soc Nephrol.* 2003; 14(6):1643–1651. [PubMed: 12761267]
 21. Meloni F, Vitulo P, Bianco AM, et al. Regulatory CD4+CD25+ T cells in the peripheral blood of lung transplant recipients: correlation with transplant outcome. *Transplantation.* 2004; 77(5):762–766. [PubMed: 15021844]
 22. Louis S, Braudeau C, Giral M, et al. Contrasting CD25hiCD4+T cells/FOXP3 patterns in chronic rejection and operational drug-free tolerance. *Transplantation.* 2006; 81(3):398–407. [PubMed: 16477227]
 23. Muthukumar T, Dadhania D, Ding R, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med.* 2005; 353(22):2342–2351. [PubMed: 16319383]
 24. Pacholczyk R, Ignatowicz H, Kraj P, Ignatowicz L. Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity.* 2006; 25(2):249–259. [PubMed: 16879995]
 25. Fazilleau N, Bachelez H, Gougeon ML, Viguier M. Cutting edge: size and diversity of CD4+CD25high Foxp3+ regulatory T cell repertoire in humans: evidence for similarities and partial overlapping with CD4+CD25– T cells. *J Immunol.* 2007; 179(6):3412–3416. [PubMed: 17785774]
 26. Jiang S, Tsang J, Lechler RI. Adoptive cell therapy using in vitro generated human CD4+ CD25+ regulatory t cells with indirect allospecificity to promote donor-specific transplantation tolerance. *Transplant Proc.* 2006; 38(10):3199–3201. [PubMed: 17175221]
 27. Nishimura E, Sakihama T, Setoguchi R, Tanaka K, Sakaguchi S. Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ regulatory T cells. *Int Immunol.* 2004; 16(8):1189–1201. [PubMed: 15237110]
 28. Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol.* 2000; 164(1):183–190. [PubMed: 10605010]

29. Yamazaki S, Patel M, Harper A, et al. Effective expansion of alloantigen-specific Foxp3⁺ CD25⁺ CD4⁺ regulatory T cells by dendritic cells during the mixed leukocyte reaction. *Proc Natl Acad Sci U S A*. 2006; 103(8):2758–2763. [PubMed: 16473944]
30. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood*. 2002; 99(10):3493–3499. [PubMed: 11986199]
31. Godfrey WR, Ge YG, Spoden DJ, et al. In vitro-expanded human CD4(+)CD25(+) T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures. *Blood*. 2004; 104(2): 453–461. [PubMed: 15031211]
32. Xia G, He J, Zhang Z, Leventhal JR. Targeting acute allograft rejection by immunotherapy with ex vivo-expanded natural CD4⁺ CD25⁺ regulatory T cells. *Transplantation*. 2006; 82(12):1749–1755. [PubMed: 17198271]
33. Xia G, He J, Leventhal JR. Ex vivo-expanded natural CD4⁺CD25⁺ regulatory T cells synergize with host T-cell depletion to promote long-term survival of allografts. *Am J Transplant*. 2008; 8(2):298–306. [PubMed: 18190656]
34. Chai JG, Coe D, Chen D, Simpson E, Dyson J, Scott D. In vitro expansion improves in vivo regulation by CD4⁺CD25⁺ regulatory T cells. *J Immunol*. 2008; 180(2):858–869. [PubMed: 18178825]
35. Kasprowicz DJ, Droin N, Soper DM, Ramsdell F, Green DR, Ziegler SF. Dynamic regulation of FoxP3 expression controls the balance between CD4⁺ T cell activation and cell death. *Eur J Immunol*. 2005; 35(12):3424–3432. [PubMed: 16285010]
36. Pyzik M, Piccirillo CA. TGF-beta1 modulates Foxp3 expression and regulatory activity in distinct CD4⁺ T cell subsets. *J Leukoc Biol*. 2007; 82(2):335–446. [PubMed: 17475784]
37. Yagi H, Nomura T, Nakamura K, et al. Crucial role of FOXP3 in the development and function of human CD25⁺CD4⁺ regulatory T cells. *Int Immunol*. 2004; 16(11):1643–1656. [PubMed: 15466453]
38. Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human CD4⁺CD25⁺Foxp3⁺ regulatory T cells cultured with rapamycin. *J Immunol*. 2007; 178(1):320–329. [PubMed: 17182569]
39. Vang KB, Yang J, Mahmud SA, Burchill MA, Vegoe AL, Farrar MA. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4⁺Foxp3⁺ regulatory T cell development. *J Immunol*. 2008; 181(5):3285–3290. [PubMed: 18714000]
40. Wuest TY, Willette-Brown J, Durum SK, Hurwitz AA. The influence of IL-2 family cytokines on activation and function of naturally occurring regulatory T cells. *J Leukoc Biol*. 2008; 84(4):973–980. [PubMed: 18653463]
41. Huber S, Schramm C, Lehr HA, et al. Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4⁺CD25⁺ T cells. *J Immunol*. 2004; 173(11):6526–6531. [PubMed: 15557141]
42. Peng Y, Laouar Y, Li MO, Green EA, Flavell RA. TGF-beta regulates in vivo expansion of Foxp3-expressing CD4⁺CD25⁺ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A*. 2004; 101(13):4572–4577. [PubMed: 15070759]
43. Schramm C, Huber S, Protschka M, et al. TGFbeta regulates the CD4⁺CD25⁺ T-cell pool and the expression of Foxp3 in vivo. *Int Immunol*. 2004; 16(9):1241–1249. [PubMed: 15249539]
44. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. A critical function for TGF-beta signaling in the development of natural CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *Nat Immunol*. 2008; 9(6):632–640. [PubMed: 18438410]
45. Floess S, Freyer J, Siewert C, et al. Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*. 2007; 5(2):e38. [PubMed: 17298177]
46. Luo X, Zhang Q, Liu V, Xia Z, Pothoven KL, Lee C. Cutting Edge: TGF-β-Induced Expression of Foxp3 in T cells Is Mediated through Inactivation of ERK. *J Immunol*. 2008; 180(5):2757–2761. [PubMed: 18292494]
47. Samanta A, Li B, Song X, et al. TGF-beta and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. *Proc Natl Acad Sci U S A*. 2008; 105(37):14023–14027. [PubMed: 18779564]

48. Lee I, Wang L, Wells AD, Dorf ME, Ozkaynak E, Hancock WW. Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. *J Exp Med.* 2005; 201(7):1037–1044. [PubMed: 15809349]
49. Moreira AP, Cavassani KA, Massafra Tristao FS, et al. CCR5-dependent regulatory T cell migration mediates fungal survival and severe immunosuppression. *J Immunol.* 2008; 180(5):3049–3056. [PubMed: 18292527]
50. Hasegawa H, Inoue A, Kohno M, et al. Therapeutic effect of CXCR3-expressing regulatory T cells on liver, lung and intestinal damages in a murine acute GVHD model. *Gene Ther.* 2008; 15(3):171–182. [PubMed: 17989707]
51. Sather BD, Treuting P, Perdue N, et al. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J Exp Med.* 2007; 204(6):1335–1347. [PubMed: 17548521]
52. Qin S, Sui Y, Soloff AC, et al. Chemokine and cytokine mediated loss of regulatory T cells in lymph nodes during pathogenic simian immunodeficiency virus infection. *J Immunol.* 2008; 180(8):5530–5536. [PubMed: 18390737]
53. Ensminger SM, Helm SN, Ohl L, et al. Increased transplant arteriosclerosis in the absence of CCR7 is associated with reduced expression of Foxp3. *Transplantation.* 2008; 86(4):590–600. [PubMed: 18724230]
54. Andersson J, Tran DQ, Pesu M, et al. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J Exp Med.* 2008; 205(9):1975–1981. [PubMed: 18710931]
55. Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4+CD25–Foxp3– T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol.* 2007; 178(11):6725–6729. [PubMed: 17513718]
56. Zhou L, Lopes JE, Chong MM, et al. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgamma1 function. *Nature.* 2008; 453(7192):236–240. [PubMed: 18368049]
57. Gavin MA, Torgerson TR, Houston E, et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A.* 2006; 103(17):6659–6664. [PubMed: 16617117]
58. Hayashi Y, Tsukumo S, Shiota H, Kishihara K, Yasutomo K. Antigen-specific T cell repertoire modification of CD4+CD25+ regulatory T cells. *J Immunol.* 2004; 172(9):5240–5248. [PubMed: 15100262]
59. Klein L, Emmerich J, d'Cruz L, Aschenbrenner K, Khazaie K. Selection and behavior of CD4+CD25+ T cells in vivo: lessons from T cell receptor transgenic models. *Curr Top Microbiol Immunol.* 2005; 293:73–87. [PubMed: 15981476]
60. Verginis P, McLaughlin KA, Wucherpfennig KW, von Boehmer H, Apostolou I. Induction of antigen-specific regulatory T cells in wild-type mice: visualization and targets of suppression. *Proc Natl Acad Sci U S A.* 2008; 105(9):3479–3484. [PubMed: 18299571]
61. Golshayan D, Jiang S, Tsang J, Garin MI, Mottet C, Lechler RI. In vitro expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. *Blood.* 2006
62. Joffre O, Santolaria T, Calise D, et al. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat Med.* 2008; 14(1):88–92. [PubMed: 18066074]
63. Cobbold SP, Castejon R, Adams E, et al. Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol.* 2004; 172(10):6003–6010. [PubMed: 15128783]
64. Vlad G, Cortesini R, Suciu-Foca N. License to heal: bidirectional interaction of antigen-specific regulatory T cells and tolerogenic APC. *J Immunol.* 2005; 174(10):5907–5914. [PubMed: 15879080]
65. Ochando JC, Homma C, Yang Y, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol.* 2006; 7(6):652–662. [PubMed: 16633346]
66. Zheng SG, Meng L, Wang JH, et al. Transfer of regulatory T cells generated ex vivo modifies graft rejection through induction of tolerogenic CD4+CD25+ cells in the recipient. *Int Immunol.* 2006; 18(2):279–289. [PubMed: 16415106]

67. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol.* 2007; 7(8):610–621. [PubMed: 17627284]
68. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3⁺ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A.* 2008; 105(29):10113–10118. [PubMed: 18635688]
69. Newell KA, Larsen CP. Transplantation tolerance. *Semin Nephrol.* 2007; 27(4):487–497. [PubMed: 17616279]
70. Fehervari Z, Sakaguchi S. CD4⁺ regulatory cells as a potential immunotherapy. *Philos Trans R Soc Lond B Biol Sci.* 2005; 360(1461):1647–1661. [PubMed: 16147529]
71. Roncarolo MG, Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol.* 2007; 7(8):585–598. [PubMed: 17653126]
72. Peters JH, Hilbrands LB, Koenen HJ, Joosten I. Ex vivo generation of human alloantigen-specific regulatory T cells from CD4(pos)CD25(high) T cells for immunotherapy. *PLoS ONE.* 2008; 3(5):e2233. [PubMed: 18493605]
73. DiGiusto DL, Cooper LJ. Preparing clinical grade Ag-specific T cells for adoptive immunotherapy trials. *Cytotherapy.* 2007; 9(7):613–629. [PubMed: 17943498]
74. Hoffmann P, Eder R, Boeld TJ, et al. Only the CD45RA⁺ subpopulation of CD4⁺CD25^{high} T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion. *Blood.* 2006; 108(13):4260–4267. [PubMed: 16917003]
75. Kleinewietfeld M, Starke M, Mitri DD, et al. CD49d provides access to 'untouched' human Foxp3⁺ Treg free of contaminating effector cells. *Blood.* 2008
76. Liu W, Putnam AL, Xu-Yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med.* 2006; 203(7):1701–1711. [PubMed: 16818678]
77. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4⁺FOXP3⁺ T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood.* 2007; 110(8):2983–2990. [PubMed: 17644734]
78. June CH, Blazar BR. Clinical application of expanded CD4⁺25⁺ cells. *Semin Immunol.* 2006; 18(2):78–88. [PubMed: 16458015]
79. Jiang S, Camara N, Lombardi G, Lechler RI. Induction of allopeptide-specific human CD4⁺CD25⁺ regulatory T cells ex vivo. *Blood.* 2003; 102(6):2180–2186. [PubMed: 12775574]
80. Nomura M, Plain KM, Verma N, et al. The cellular basis of cardiac allograft rejection. IX. Ratio of naive CD4⁺CD25⁺ T cells/CD4⁺CD25[–] T cells determines rejection or tolerance. *Transpl Immunol.* 2006; 15(4):311–318. [PubMed: 16635754]
81. Demirkiran A, Hendrikx TK, Baan CC, van der Laan LJ. Impact of immunosuppressive drugs on CD4⁺CD25⁺FOXP3⁺ regulatory T cells: does in vitro evidence translate to the clinical setting? *Transplantation.* 2008; 85(6):783–789. [PubMed: 18360256]
82. Lin W, Haribhai D, Relland LM, et al. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol.* 2007; 8(4):359–368. [PubMed: 17273171]
83. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol.* 2007; 8(3):277–284. [PubMed: 17220892]
84. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3⁺ T regulatory cells. *J Immunol.* 2007; 178(7):4022–4026. [PubMed: 17371955]
85. Zheng SG, Wang J, Horwitz DA. Cutting edge: Foxp3⁺CD4⁺CD25⁺ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. *J Immunol.* 2008; 180(11):7112–7116. [PubMed: 18490709]
86. Luo X, Tarbell KV, Yang H, et al. Dendritic cells with TGF-beta1 differentiate naive CD4⁺CD25[–] T cells into islet-protective Foxp3⁺ regulatory T cells. *Proc Natl Acad Sci U S A.* 2007; 104(8):2821–2826. [PubMed: 17307871]
87. Yamazaki S, Bonito AJ, Spisek R, Dhodapkar M, Inaba K, Steinman RM. Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3⁺ CD4⁺ regulatory T cells from peripheral Foxp3 precursors. *Blood.* 2007; 110(13):4293–4302. [PubMed: 17699744]

88. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci U S A*. 2008; 105(27):9331–9336. [PubMed: 18599457]
89. Yamazaki S, Dudziak D, Heidkamp GF, et al. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J Immunol*. 2008; 181(10):6923–6933. [PubMed: 18981112]
90. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med*. 2007; 204(8):1775–1785. [PubMed: 17620362]
91. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med*. 2007; 204(8):1757–1764. [PubMed: 17620361]
92. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. 2006; 24(2):179–189. [PubMed: 16473830]
93. Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. 2007; 317(5835):256–260. [PubMed: 17569825]
94. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *J Exp Med*. 2007; 204(7):1543–1551. [PubMed: 17591856]
95. Polansky JK, Kretschmer K, Freyer J, et al. DNA methylation controls Foxp3 gene expression. *Eur J Immunol*. 2008; 38(6):1654–1663. [PubMed: 18493985]
96. Sauer S, Bruno L, Hertweck A, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, mTOR. *Proc Natl Acad Sci U S A*. 2008; 105(22):7797–7802. [PubMed: 18509048]
97. Janson PC, Winerdal ME, Marits P, Thorn M, Ohlsson R, Winqvist O. FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS ONE*. 2008; 3(2):e1612. [PubMed: 18286169]
98. Watanabe M, Mencil RL, Cramer DV, Starnes VA, Barr ML. Transforming growth factor-beta/interleukin-2-induced regulatory CD4+ T cells prolong cardiac allograft survival in rats. *J Heart Lung Transplant*. 2005; 24(12):2153–2159. [PubMed: 16364865]
99. Kapp JA, Honjo K, Kapp LM, Xu X, Cozier A, Bucy RP. TCR transgenic CD8+ T cells activated in the presence of TGFbeta express FoxP3 and mediate linked suppression of primary immune responses and cardiac allograft rejection. *Int Immunol*. 2006; 18(11):1549–1562. [PubMed: 16966495]
100. Singh RP, La Cava A, Wong M, Ebling F, Hahn BH. CD8+ T cell-mediated suppression of autoimmunity in a murine lupus model of peptide-induced immune tolerance depends on Foxp3 expression. *J Immunol*. 2007; 178(12):7649–7657. [PubMed: 17548601]
101. Fan TM, Kranz DM, Flavell RA, Roy EJ. Costimulatory strength influences the differential effects of transforming growth factor beta1 for the generation of CD8+ regulatory T cells. *Mol Immunol*. 2008; 45(10):2937–2950. [PubMed: 18321576]
102. Lan YY, Wang Z, Raimondi G, et al. "Alternatively activated" dendritic cells preferentially secrete IL-10, expand Foxp3+CD4+ T cells, and induce long-term organ allograft survival in combination with CTLA4-Ig. *J Immunol*. 2006; 177(9):5868–5877. [PubMed: 17056511]
103. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol*. 2007; 178(11):7018–7031. [PubMed: 17513751]
104. Min WP, Zhou D, Ichim TE, et al. Inhibitory feedback loop between tolerogenic dendritic cells and regulatory T cells in transplant tolerance. *J Immunol*. 2003; 170(3):1304–1312. [PubMed: 12538690]
105. Zhang X, Li M, Lian D, et al. Generation of therapeutic dendritic cells and regulatory T cells for preventing allogeneic cardiac graft rejection. *Clin Immunol*. 2008; 127(3):313–321. [PubMed: 18358783]
106. Chatenoud L, Bluestone JA. CD3-specific antibodies: a portal to the treatment of autoimmunity. *Nat Rev Immunol*. 2007; 7(8):622–632. [PubMed: 17641665]

107. Hirsch R, Eckhaus M, Auchincloss H Jr, Sachs DH, Bluestone JA. Effects of in vivo administration of anti-T3 monoclonal antibody on T cell function in mice. I. Immunosuppression of transplantation responses. *J Immunol.* 1988; 140(11):3766–3772. [PubMed: 3286764]
108. Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat Med.* 2003; 9(9):1202–1208. [PubMed: 12937416]
109. You S, Leforban B, Garcia C, Bach JF, Bluestone JA, Chatenoud L. Adaptive TGF-beta-dependent regulatory T cells control autoimmune diabetes and are a privileged target of anti-CD3 antibody treatment. *Proc Natl Acad Sci U S A.* 2007; 104(15):6335–6340. [PubMed: 17389382]
110. Nicolls MR, Aversa GG, Pearce NW, et al. Induction of long-term specific tolerance to allografts in rats by therapy with an anti-CD3-like monoclonal antibody. *Transplantation.* 1993; 55(3):459–468. [PubMed: 8456460]
111. Perruche S, Zhang P, Liu Y, Saas P, Bluestone JA, Chen W. CD3-specific antibody-induced immune tolerance involves transforming growth factor-beta from phagocytes digesting apoptotic T cells. *Nat Med.* 2008; 14(5):528–535. [PubMed: 18438416]
112. Ochi H, Abraham M, Ishikawa H, et al. Oral CD3-specific antibody suppresses autoimmune encephalomyelitis by inducing CD4+ CD25- LAP+ T cells. *Nat Med.* 2006; 12(6):627–635. [PubMed: 16715091]
113. Taylor PA, Noelle RJ, Blazar BR. CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med.* 2001; 193(11):1311–1318. [PubMed: 11390438]
114. Hara M, Kingsley CI, Niimi M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol.* 2001; 166(6):3789–3796. [PubMed: 11238621]
115. Jarvinen LZ, Blazar BR, Adeyi OA, Strom TB, Noelle RJ. CD154 on the surface of CD4+CD25+ regulatory T cells contributes to skin transplant tolerance. *Transplantation.* 2003; 76(9):1375–1379. [PubMed: 14627918]
116. Quezada SA, Bennett K, Blazar BR, Rudensky AY, Sakaguchi S, Noelle RJ. Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J Immunol.* 2005; 175(2):771–779. [PubMed: 16002673]
117. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol.* 2000; 164(5):2405–2411. [PubMed: 10679076]
118. Wang Z, Larregina AT, Shufesky WJ, et al. Use of the inhibitory effect of apoptotic cells on dendritic cells for graft survival via T-cell deletion and regulatory T cells. *Am J Transplant.* 2006; 6(6):1297–1311. [PubMed: 16686754]
119. Peche H, Renaudin K, Beriou G, Merieau E, Amigorena S, Cuturi MC. Induction of tolerance by exosomes and short-term immunosuppression in a fully MHC-mismatched rat cardiac allograft model. *Am J Transplant.* 2006; 6(7):1541–1550. [PubMed: 16827854]
120. Miyake Y, Asano K, Kaise H, Uemura M, Nakayama M, Tanaka M. Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. *J Clin Invest.* 2007; 117(8):2268–2278. [PubMed: 17657313]
121. Morelli AE, Larregina AT, Shufesky WJ, et al. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood.* 2003; 101(2):611–620. [PubMed: 12393562]
122. Kennedy MK, Tan LJ, Dal Canto MC, et al. Inhibition of murine relapsing experimental autoimmune encephalomyelitis by immune tolerance to proteolipid protein and its encephalitogenic peptides. *J Immunol.* 1990; 144(3):909–915. [PubMed: 1688591]
123. Tan LJ, Kennedy MK, Miller SD. Regulation of the effector stages of experimental autoimmune encephalomyelitis via neuroantigen-specific tolerance induction. II. Fine specificity of effector T cell inhibition. *J Immunol.* 1992; 148(9):2748–2755. [PubMed: 1374098]
124. Vanderlugt CL, Neville KL, Nikcevich KM, Eagar TN, Bluestone JA, Miller SD. Pathologic role and temporal appearance of newly emerging autoepitopes in relapsing experimental autoimmune encephalomyelitis. *J Immunol.* 2000; 164(2):670–678. [PubMed: 10623809]

125. Fife BT, Guleria I, Gubbels Bupp M, et al. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. *J Exp Med*. 2006; 203(12):2737–2747. [PubMed: 17116737]
126. Jenkins MK, Ashwell JD, Schwartz RH. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. *J Immunol*. 1988; 140(10):3324–3330. [PubMed: 2834436]
127. Luo X, Pothoven KL, McCarthy D, et al. ECDI-fixed allogeneic splenocytes induce donor-specific tolerance for long-term survival of islet transplants via two distinct mechanisms. *Proc Natl Acad Sci U S A*. 2008; 105(38):14527–14532. [PubMed: 18796615]
128. Bailey-Bucktrout SL, Caulkins SC, Goings G, Fischer JA, Dzionek A, Miller SD. Cutting edge: central nervous system plasmacytoid dendritic cells regulate the severity of relapsing experimental autoimmune encephalomyelitis. *J Immunol*. 2008; 180(10):6457–6661. [PubMed: 18453561]