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The Role of the Thymus in Tolerance

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Abstract

The thymus serves as the central organ of immunologic self-non-self discrimination. Thymocytes undergo both positive and negative selection resulting in T cells with a broad range of reactivity to foreign antigens but a lack of reactivity to self-antigens. The thymus is also the source of a subset of regulatory T cells that inhibit autoreactivity of T cell clones that may escape negative selection. As a result of these functions, the thymus has been shown to be essential for the induction of tolerance in many rodent and large animal models. Proper donor antigen presentation in the thymus following bone marrow, dendritic cell, or solid organ transplantation has been shown to induce tolerance to allografts. The molecular mechanisms of positive and negative selection and regulatory T cell development must be understood if a tolerance inducing therapeutic intervention is to be designed effectively. In this brief and selective review, we present some of the known information on T cell development and the role of the thymus on experimental models of transplant tolerance. We also cite some clinical attempts to induce tolerance to allografts using either pharmacologic or biologic interventions.

Keywords

Thymus; thymopoiesis; transplantation; tolerance; animal models

Introduction

In the healthy individual, self-non-self discrimination results in a balance between the extremes of immunodeficiency and autoimmunity. This balance represents a singular challenge: the T cell repertoire must respond to a seemingly limitless number of potential foreign antigens while simultaneously not responding to self-antigens expressed in all the various tissues. In order to achieve this, developing T lymphocytes undergo random rearrangement of specific regions of genes coding for their antigen receptors. During T cell development within the thymus, this process results in a small minority of immunologically useful prothymocytes with T cell receptors (TCRs) able to bind antigen–MHC complexes with appropriately low avidity initiating a positive selection pathway culminating in the survival of cells able to react against these antigens. Similarly, random rearrangement of TCR gene segments inevitably yields T cells that bind self-antigen with high avidity, and these potentially autoreactive lymphocytes are eliminated through negative selection

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mechanisms; if they remain the organism is faced with an autoimmune disease state where self-cells are destroyed.

Tolerance, or the state of immunologic non-responsiveness in the presence of a particular antigen, represents the self-discriminatory aspect of immunologic balance. Functional tolerance consists of two coordinated processes: 1) deletion of autoreactive lymphocytes during maturation in the central lymphoid organs before such cells reach maturity, and 2) functional suppression in the periphery of autoreactive lymphocytes that have escaped elimination. These two processes are referred to as central and peripheral tolerance. When this concept is applied to transplantation of tissues or organs, the ultimate aim is to delete donor specific alloreactive lymphocytes to avoid damage to a highly specific tissue or organ, without interfering with the rest of the homeostatic mechanisms. Central tolerance within the thymus classically refers to the scrutiny and negative selection of maturing thymocytes while the positive selection of regulatory T (T_{reg}) lymphocytes, which modulate the peripheral immune response, also occurs within the thymus and thus may be considered a central tolerance mechanism. In order to design a tolerance induction protocol in patients, it is critical to briefly review the available information on the maintenance of self-recognition and reactivity to the non-self. We have organized this brief review with a focus on the known activities in the thymus and on the cell products that may be critical in both induction and maintenance of a specific unresponsive state.

Early thymocyte maturation and positive selection

Upon entering the thymus, immature lymphocytes originating in the bone marrow and committed to the thymocyte lineage undergo T-cell receptor (TCR) β - and α - locus rearrangement (Figure 1). At this stage of development, T cells are characterized as $CD4^{+}/8^{+}$ double-positive (DP), and the cells initiate expression of the $\alpha\beta$ TCR to a modest degree. The fate of each DP cell depends on the ability of its newly rearranged TCR to appropriately interact in a self-peptide–MHC complex; DP thymocytes are programmed to undergo apoptosis unless a ‘rescue signal’ is delivered via TCR: self-peptide–MHC ligation. During the three-day window prior to programmed cell death, sequential rounds of rearrangement at the TCR α locus, referred to as receptor editing, may increase the likelihood of successful self-MHC restriction (1-6). Ultimately, the vast majority (around 95%) of DP cells fail to demonstrate any specificity for an MHC ligand, and these immunologically useless thymocytes undergo “death by neglect” or apoptosis (7,8). A small proportion of DP cells express TCR that are able to bind an MHC ligand with mild avidity, and the resulting signaling cascade diverts those thymocytes from apoptosis (last minute reprieve) and induces DP maturation to the $CD4^{+}8^{-}$ or $CD4^{-}8^{+}$ single-positive (SP) stage(9). This type of positive selection signaling also triggers the expression of chemokines (traffic cop) that direct the T cell for further maturation within the thymic medulla(10-12). The identity and function of these chemokines and their receptors have been reviewed (13).

Negative selection: Costimulation and the role of Fas

Negative selection, also referred to as clonal deletion, is the process by which high-avidity TCR:self-peptide–MHC ligation induces cell death. Although distinction between low-avidity TCR binding (leading to positive selection) and high-avidity binding (leading to negative selection) is of critical importance, the mechanisms of this discrimination are not well understood. Negative selection was initially thought to coincide with positive selection within the thymic cortex. However, much of the early evidence for clonal deletion within the cortex is currently viewed as an artifact of experimental models(14-19). Although recent and more physiologically accurate models of clonal deletion demonstrate apoptosis following high-avidity TCR binding(8,20,21), the bulk of negative selection for the remainder of self-

reactive T-cells appears to occur within the medulla(8,22-24). Two mechanistic details arising from these models deserve specific mention: costimulation and the role of Fas. Exposure of DP thymocytes to anti-TCR mAb fails to induce significant apoptosis except in the presence of antigen-presenting cells (APCs)(17,25-27). The interaction between the thymocyte CD28 receptor and B7 APC ligand was subsequently identified as a crucial costimulatory factor(28,29), and DP thymocytes were shown to undergo apoptosis in the combined presence of anti-TCR and anti-CD28 mAb *in vitro*(26,30). It is therefore not surprising that this interaction is of great interest in recent drug development. For the more mature SP thymocytes, mAb against the surface molecules CD5 and CD43 also independently provide sufficient costimulation to cause apoptosis in the presence of anti-TCR mAb, suggesting a degree of costimulatory redundancy(30,31), which is a problem for the clinician when using only one agent for costimulatory blockade. When compared to the cortex, B7 expression is significantly higher in the medulla(32), thus suggesting the latter as the more favorable environment for negative selection(33) (Figure 1). The costimulatory molecules CD40, CD80, and CD86, which are expressed in the medulla by both medullary thymic epithelial cells (mTECs) and dendritic cells (DCs), have also been shown to have a role in clonal deletion(34,35).

An important caveat to the costimulation requirement was discovered as semi-mature SP thymocytes were exposed to high-concentration of anti-TCR mAb: apoptosis was induced by the high-level TCR signaling in the absence of costimulation(30). In Fas⁻ *lpr/lpr* cells [reviewed in (36)], this effect is abrogated(17,26). Since Fas, a member of the TNF-receptor superfamily, contains a death domain and has been shown to play an important role in the physiologic regulation of cell death, this is somewhat surprising. It thus appears that apoptosis following moderate-avidity TCR binding requires costimulation and has been described as Fas-independent. With strong TCR ligation, apoptosis is Fas-dependent and proceeds in the absence of costimulation(17,36). In that case the interaction of TCR receptor with its ligand leads to the formation of a death-inducing signaling (Fas-associated death domain protein – FADD) and the various caspases which lead to a caspase cascade and downstream apoptosis. Due to the lack of costimulatory APCs within the thymic cortex, it is likely that negative selection within the cortex is limited to high-intensity TCR signaling leading to priming of the cells for subsequent death on contact with APCs at the corticomedullary junction(37). Further mechanisms of the apoptotic sequence in clonal deletion are the subject of recent reviews (38,39).

Negative selection and tissue-specific antigens

The successful elimination of potentially self-reactive thymocytes through clonal deletion requires a complete representation of self-antigens within the thymus. The comprehensive nature of antigen representation within the thymus was first noted with the discovery that genes for certain tissue specific antigens (TSAs), such as pancreatic proteins, are expressed ectopically (or ‘promiscuously’) in the thymic medulla(40-42) and the significance of this phenomenon was subsequently clarified and appreciated(42-45). Control of TSA expression appears to be partially controlled by the genes such as AIRE, whose deficiency in mice and even in man results in an autoimmune disorder (46-51). Current analyses suggest that mTECs express an excess of 500 to 1200 genes when compared with cTEC (cortical epithelial cells) controls(49); expression of representative genes has been observed for TSAs from every tissue in the body controlling the reactivity to self (52). Despite the surprisingly large extent of pGE within thymic mTECs, a number of proteins have been identified which are not expressed within the thymus, or are expressed at such low levels that they are not detected(44,52). Such proteins are often targets for autoimmunity(53). For example, the pancreatic protein GAD65 is expressed within the thymus at levels significantly lower than

the related protein GAD67(54,55); corresponding antibody levels against GAD65 are significantly higher than GAD67 in type 1 diabetes mellitus patients(56).

Antigen presentation and negative selection

Effective negative selection requires the costimulatory support provided by APCs. (DCs constitutively express B7) (25,57-59). Accordingly, dendritic cells, which function in antigen cross-presentation within the medulla (25,60,61), have been thought to play a major role in antigen presentation for negative selection. Indeed, studies demonstrated that DCs are indeed required for full tolerance induction (62-67). The results also show that in certain cases, however, mTECs may autonomously induce tolerance, particularly for CD8⁺ cells (40,60-62,68,69). Strong agonist signaling from high-avidity TCR:self-peptide-MHC binding possibly plays a role in this process, which would be characterized as Fas-dependent and thus proceeds in the absence of costimulation (39,60). The overall significance of mTEC-induced negative selection, however, is probably low.

Several studies have established dendritic cell acquisition of TSA from mTECs (61,70-72). A number of mechanisms for this process have been proposed. First, as DCs efficiently phagocytize apoptotic cells, the frequent turnover of mature mTECs yields an abundance of TSA-rich cell fragments for DC processing and cross-presentation (73-76). Second, DCs may acquire protein material from living mTECs either via exosomes (77) or through an imprecisely characterized process of nibbling, whereby membrane-enclosed blebs of intracellular material are removed from living mTECs (70-72,78,79). Finally, the observed transfer of intracellular material via gap junctions has been described as a method of antigen transfer (80).

Dominant mechanisms of tolerance

Despite the extensive mechanisms promoting negative selection of self-reactive thymocytes, autoreactive cells have been shown to regularly escape into the periphery (81-86). An additional critical function of the thymus is the selection of thymocytes responsible for antigen-specific tolerance regulation. The naturally occurring CD4⁺CD25⁺ regulatory T (T_{reg}) lymphocyte subset is responsible for abolishing the response of autoreactive effector T cells for which negative selection mechanisms have failed (87), primarily by interrupting transcription of the *IL-2* gene on effector thymocytes (88,89). T_{reg} cells express TCR with medium to high avidity for self-antigens (90-92), yet are diverted away from the negative selection pathway by an uncertain mechanism (93). Such non-deletional tolerance is also referred to as dominant, transferable, or infectious tolerance because T_{reg} cells are able to induce tolerance to a corresponding foreign antigen after administration (passive transfer dependent on quantitative considerations) to naïve recipients. T_{reg} cells may also compensate for deficits in TSA expression within the thymus by attuning autoimmune responses in an antigen-nonspecific manner referred to as bystander suppression(94-97). These cells through cell-to-cell contact inhibit activation of naïve CD4⁺ T cells (98) and do not proliferate in response to antigens(99). This suppressor function can be eliminated by exposing Tregs to anti-CD3 and anti-CD28 mAbs(100) while IL2 is necessary for their activation (101) – both these findings may be important in development and selection of new immunosuppressive strategies.

A role for mTECs in T_{reg} development has been proposed (90,91,102) since mTEC function may result in a reduced T_{reg} repertoire (103-105). Additionally, the FoxP3 family of transcription factors [reviewed in (13,38,40,52)], found primarily in CD4⁺CD25⁺ T_{reg} cells (at least in mice) and is found primarily in the thymic medulla. It appears now that FOXP3 acts a “master control gene” for the development and function of T_{regs} (96). The concept that T_{regs} that have the CD4⁺/CD25⁺/FoxP3⁺ phenotype are critical for maintenance of

tolerance to both self and non-self antigens in rodents has been described by many investigators (87) (106). Although the finding of CD4⁺/CD25⁺/FOXP3⁺ T cells supports the idea of tolerance induction and maintenance through formation of suppressor or regulatory cells, it still remains likely that other mechanisms of tolerance induction such as anergy or deletion are operating simultaneously or independent of the suppressor/regulatory pathways (107). Additionally, the finding in humans that some markers of T_{regs}, such as FoxP3, CTLA4, and CD25 are shared by other T cells which represent a stage in the differentiation of activated T cells complicates the analysis of the importance of T_{regs} in humans when evaluated by markers rather than by direct function (108) (109). It has also been suggested in studies of T_{regs} that CD25 represents a major marker of FoxP3 T cells with suppressive function (87) (106,110) (111). This has raised the question at the clinical level of whether the use of anti-CD25 monoclonal antibodies, such as Daclizumab or Basiliximab, which are used as induction agents in organ transplantation, may interfere with the function or deplete T_{regs} in clinical situations. A recent study by our group in heart transplant recipients (112) has shown that the use of the anti-CD25 Daclizumab did not impair the development of T_{regs} in these patients and that there was a positive correlation between the serial presence of markers of T_{regs} and suppressor T cells (Ts) such as FoxP3, CTLA-4 and IL-10 in peripheral blood and the lack of rejection episodes.

As with effector T cell selection, evidence for the role of DCs in T_{reg} development has been recently described (113-116). A number of distinct populations of DCs exist within the thymus (117-119), and it is postulated that these subsets assume distinct tolerogenic roles, i.e. directing potentially self-reactive T cells to the T_{reg} lineage or to clonal deletion (120-122). Induced T_{regs} which are antigen-specific CD8⁺T suppressor cells (Ts) and CD4⁺CD25⁺ T_{regs} can be generated by stimulation with immature myeloid DCs (imDC) or by mature plasmacytoid DCs (mpDC) (123). Human T cell clones produced *in vivo* or *in vitro* in the presence of IL-10 and imDCs inhibit antigen-induced activation of naïve T cells which can lead to experimental allograft acceptance in rodents (124) and in humans (125). Such CD4⁺CD25⁺ T_{regs} suppress normal T cell populations, making them anergic, and these cells in turn further suppress syngeneic CD4⁺ T cells via inhibitory cytokines (126). Suciu-Foca and her colleagues recently described another population of antigen-specific CD8⁺CD28⁻ T suppressor cells that express FoxP3 and act directly on professional antigen presenting cells (APCs), including imDCs to direct them towards tolerogenic state (127). On the basis of their studies, this group suggests that T cell suppression is induced by initial “tolerization” of APCs by CD8⁺ T cells which then induce anergic T helper cells that then acquire regulatory capacity as they increase FoxP3 expression and upregulate inhibitory receptors ILT3 and ILT4. Since DCs have a key role controlling the immune response and are also involved through costimulatory molecules and other factors in the maintenance of peripheral tolerance, it is not surprising that DC-based therapies for induction of immune regulation in autoimmunity and in tolerance are now of paramount interest. DCs express various receptors including at least one member of the inhibitory ILT family (immunoglobulin-like transcripts that contain a tyrosine-based inhibitory motif) and which are important in immunoregulation. One such receptor, ILT3, has been now shown to negatively regulate APC activation and its upregulation is required to induce CD4⁺Foxp3⁺ T_{regs}.

In vitro observations on intrathymic DCs proposes that the CD11c⁺CD11b⁻ DC subset is activated by thymic stromal lymphopoietin (TSLP) from epithelial cells in Hassall's corpuscles (128) and that this subset subsequently compels T cells to the T_{reg} lineage (113,129). This may also be accomplished by the expression of specific cytokines by dendritic cells and Hassall's corpuscles leading maturing SP cells toward T_{reg} development (130-134). T_{reg} cell development also requires costimulatory molecules CD28, CD80, and CD86, as well as CD40 and the CD40 ligand expressed by the medulla (135-137). This

could therefore hinder the development of T_{regs} in protocols that rely on costimulatory blockade for tolerance induction. Finally, it should be noted that T differentiation is not limited to the thymic environment; mature, naïve CD4⁺ reg T cells can be induced in the periphery to develop into CD4⁺CD25⁺ T_{reg} cells (138-140).

Thymus in experimental models of tolerance

The report of successful tolerance induction by cotransplantation of donor bone marrow has brought us to the threshold of tolerance inducing protocols being used in the clinic (141). Given that the thymus is the origin of the self-non-self distinction, it is not surprising that it is integral in the experimental induction of tolerance to organ transplants. Even in experimental models using a mixed chimerism approach for tolerance induction, the presence of the thymus is required to maintain tolerance after the induction of chimerism(107). It also appears to be required for at least 3 weeks in the experimental models in rats using immature DC's (imDC) primed in vitro with immunodominant allopeptide where the DC's appear to re-circulate through the recipient thymus where they become "educated" (142). The subsequent development of CD4+CD25+FoxP3 anti-donor regulatory T cells is dependent on the presence of the thymus for at least 2-3 weeks after injection of the primed imDC and induction of tolerance to hearts, islets, or bone marrow is prevented by early thymectomy (143). Other studies in miniature swine have sought to determine reliable markers of successful tolerance induction following hematopoietic stem cell transplantation prior to transplantation of a solid organ. While peripheral T cell chimerism was not found to be entirely reliable(144), the presence of thymic chimerism and donor bone marrow engraftment correlated precisely with tolerance to subsequent solid organ transplants(145), suggesting that the thymus plays an active role in inducing and maintaining tolerance in hematopoietic cell transplant models.

Models of transplantation tolerance that do not rely on chimerism to induce tolerance have also demonstrated a dependency on the thymus for the induction of tolerance. In MGH miniature swine, class I mismatched renal transplants are uniformly accepted after a 12-day course of Cyclosporine(146). Mechanistic studies have shown that regulatory mechanisms are involved in mediating this tolerance (147-151). However, when the recipients were thymectomized 21 days prior to renal transplant, or when recipients were aged with involuted thymii, tolerance was not induced(152,153). This finding has also been shown in other rodent models of tolerance(154,155), suggesting that thymic-dependant tolerance strategies may be more successful in the pediatric population. We have also shown that inoculating the thymus directly with allopeptides or peptide pulsed self dendritic cells can induce tolerance to the dominant peptides and solid organ transplants(156-159). Together, these studies suggest that migration of donor antigen to an active thymus can induce transplantation tolerance in rodent and large animal models. Once central tolerance is induced experimentally, almost always in the presence of the thymus, the appearance and persistence of the regulatory mechanisms appears sufficient to maintain the state of donor-specific tolerance even when the thymus is ablated (usually 2- 3 weeks (160,161) after tolerance induction to allow for initial "education" of T regs and prevention of subsequent generation of new alloreactive T cells. It must be mentioned that some models have demonstrated the ability to induce tolerance in thymectomized animals, likely by peripheral generation of Tregs, and this tolerance appears to be robust, again likely due to a lack of generation of new alloreactive T cells(162).

Another strategy for the induction of tolerance that has shown promise in animal models is the transplantation of allogeneic or xenogeneic thymus tissue. The transplantation of xenogenic thymus tissue to mice has been extensively studied by Sykes et al(163-169). These studies have shown that transplantation of porcine thymus tissue to mice leads to the

development of donor-specific tolerance *in vitro*(168) and resulted in permanent donor-matched porcine skin graft survival, while allogeneic murine skin was rejected(169). Studies by Yamada et al showed that if donor thymus was transplanted as a vascularized graft, tolerance could be induced across class I and full MHC mismatched barriers to renal and cardiac grafts(170-175). The development of the ability to transplant the thymus as a vascularized graft also allowed for studies to determine if a rejuvenated adult thymus could regain the ability to induce tolerance in the miniature swine model. When thymi from aged swine were transplanted to thymectomized juvenile recipients, they regained the architecture of a juvenile thymus and also the ability to facilitate the induction of tolerance to allogeneic renal grafts(176). This suggests that age related thymic involution can be reversed by a juvenile milieu. Multiple attempts to chemically rejuvenate the thymus have been attempted including administration of IL-7(177,178) and chemical castration via LH-RH antagonists(179). Using various fractions of the thymic hormone, thymosin(180), to initiate or to maintain the rejuvenation process has not been investigated and may offer a productive approach. The role of the thymus in T cell maturation may partially depend on its hormonal function and therefore its radioresistant reticuloepithelial scaffold(181). The latter plays an important function in the development of T cell ontogeny, not only in utero and infancy, but throughout the lifetime of the individual albeit at a reduced tempo(182). Since postnatal thymus tissue transplantation has been used successfully in juvenile patients with DiGeorge's syndrome(183-185) with *in vitro* evidence for tolerance to the donor thymus(186), it is reasonable to assume that clinical application of thymus transplantation for tolerance induction in the adult population would be possible if a reliable means of rejuvenating the adult donor thymus is found.

An alternative use of thymus transplantation could eventually lead to the induction of xenogeneic tolerance now that it has been demonstrated that transplantation of vascularized porcine thymus tissue to baboons was associated with *in vitro* evidence for donor-specific T cell unresponsiveness(187). When α 1,3-galactosyltransferase knockout swine were used as the thymus and kidney donors, the recipients had survivals greater than 80 days with normal renal function, compared to recipients of kidneys alone who rejected their grafts in 30 days(188).

Clinical Approaches to Induction of “Operational” Tolerance to Organ Transplantation

It is at present still difficult to discuss tolerance induction in a clinical setting without the use of immunosuppressive agents. Many clinical studies have been conducted as part of this search for the “holy grail” and so far almost all have been unsuccessful. Clinicians have used various terms to define a “partial tolerance” where maintenance immunosuppression is minimized but not completely withdrawn. One such term has been “Operational Tolerance”, while a more recent one has been “Prope Tolerance” coined by Sir Roy Calne (189).

One clinical approach that attempted to induce tolerance to renal allografts consisted of using co-stimulation blockade with anti CTLA4-Ig antibody (Belatacept – Bristol Myers Squibb) which offered sufficient experimental data in non-human primates to proceed with clinical trials (190). Initial Phase II studies reported by Vincenti et al (191) and a more recent report (192) of a Phase III multi-institutional trial involving more than 1000 patients randomized to receive an immunosuppressive regimen based on either Belatacept or cyclosporine showed that the incidence of patient and graft survival was similar and that the Belatacept group had significantly improved GFR (191). There has been no evidence that the Belatacept recipients were immunologically less responsive to their grafts than the control patients.

The use of donor bone marrow infusion to induce tolerance was initially explored by Monaco and colleagues. Experimental models in rodents demonstrated that donor marrow infusion had a salutary effect on skin graft and islet transplant survival and tolerance induction. (193-195) Mechanistic studies performed in these models showed that allograft survival correlated with the presence of donor class II mRNA in the recipient's thymus (196). Studies in canines by the Monaco group demonstrated that donor bone marrow infusion induced prolonged graft survival in a large animal model(197). When this strategy was applied in a primate model using an anti-CD3 immunotoxin and donor bone marrow, prolonged graft survival was again observed(198). Small studies using donor bone marrow infusions were encouraging that this may be a useful adjunct to prolong graft survival and perhaps induce tolerance (199,200). Attempts at clinical application of these important experimental and preliminary clinical findings have been made by the Pittsburgh group under the guidance of Dr. Thomas Starzl starting with the idea of inducing macrochimerism by injecting unmodified donor bone marrow along with the donor allograft. When these studies were repeated and expanded by Ciancio et al, it was found that acute rejection of cadaveric kidneys and the outcomes for recipients of living donors were no different in controls even when macrochimerism was detected in the recipients' bone marrow two years after transplantation (201). More importantly, no correlation has been found between the degree of microchimerism and renal allograft survival in several studies (202). Although the Pittsburgh Group initially postulated that any favorable effect of simultaneous donor bone marrow transplantation with the donor organ was due to development and persistence of microchimerism, the authors feel that it would be more likely that altered antigen presentation in the recipient's thymus by the APC's in the transplanted marrow may have a transient favorable effect on subsequent increase in the production of donor-specific regulatory T cells. Since the quantitative aspects of this manipulation appeared to be minimal relative to the existing T cell pool, the results of allograft prolongation remain controversial.

Intentional allograft tolerance with full withdrawal of maintenance immunosuppression has been demonstrated only by the Boston group, initially in two patients with multiple myeloma who required bone marrow transplantation and a renal allograft (203). Immunosuppression was discontinued after 73 and 77 days. Multilineage chimerism of 5-80% of CD3 cells was maintained for approximately 12 weeks and then declined to undetectable levels after 105-123 days while kidney function remained normal from day 3 to more than 2 and 4 years without any immunosuppression. Subsequently the same group transplanted 5 patients with combined bone marrow and kidney transplants from HLA single-haplotype mismatched living donors after non-myeoablative conditioning. While one patient had irreversible humoral rejection, all immunosuppression could be discontinued in the 4 other patients 9 to 14 months after transplantation. Patients have continued to maintain stable renal function for 2 to 5.3 years. The T cells in these recipients were donor unresponsive *in vitro* while there were high levels of FoxP3 messenger RNA in graft biopsies (141). Although the Boston group did not achieve long term mixed chimerism (less than 3 weeks), they demonstrated durable renal allograft survival in 4 out of 5 patients. This finding is not surprising since induction of unresponsiveness is not necessarily equivalent or may even not be related to maintenance of chimerism. It may, instead, depend heavily, if not exclusively, on appearance and maintenance of robust regulatory mechanisms; these may need to be periodically reinforced ("boosted").

The Stanford group has also attempted to use mixed chimerism to induce tolerance to renal allografts in 4 patients (204). Recipients conditioned with total-lymphoid irradiation (10 doses of 80 cGy) and given anti-thymocyte globulin and maintained on cyclosporine and prednisone received cytokine mobilized CD34+ hematopoietic progenitor cells from living donors. Three of these four recipients developed transient macrochimerism for up to 3

months without evidence of graft-versus-host-disease(205). Two patients were weaned from immunosuppression but had to be restarted due to mild rejection episodes. A later report of 1 patient who was treated with a similar regimen with the addition of mycophenolate mofetil for 1 month after the administration of the cytokine mobilized CD34+ progenitor cells received an HLA matched kidney. Durable mixed chimreism was achieved and the patient had been off immunosuppression for 28 months without evidence of rejection (206). The mechanism responsible for tolerance in this patient remains to be elucidated.

The standard immunosuppression that is being used at present consists primarily of calcineurin inhibitors, cyclosporin or tacrolimus, both found to inhibit activated T cell proliferation, including Tregs, and in most experimental models tested found to inhibit experimental tolerance induction. Although tolerance has not been inhibited by calcineurin administration in the porcine model by Sachs et. al nor in humans subjected to tolerance induction by the Boston and Stanford groups, there is some evidence that cyclosporine inhibits AIRE expression and decreases the number of mTEC cells in mouse thymus(207). This might theoretically decrease the ability of thymus to perform appropriate negative selection that could lead to central tolerance. Other studies have shown a detrimental effect of cyclosporine on FoxP3 gene transcription(208) and Treg function *in vivo*(209). Alternatively, the use of sirolimus has been found to promote the shift to Tregs in both experimental models(210,211) and in man(210). Although the agent has not been very successful in promoting any type of clinical tolerance induction, it has that potential on the basis of several experimental studies in animals and in non-human primates(211).

We have tried to provide the reader with an overview of central tolerance, focusing initially on the mechanisms involved and then to very briefly summarize the existing clinical efforts in translating that knowledge to a clinical setting. To develop new strategies of selective immunosuppression it should be clear that much better understanding of the mechanisms of T cell inactivation remains to be translated to clinical usefulness for central tolerance induction in allograft recipients. A broader understanding of the induction and characterization of antigen-specific Tregs and Tsm, as well as mDCs and imDCs, involved in tolerance induction should lead to clinical protocols that will eventually “fool mother nature”, allowing recipients to accept allogeneic, or even xenogeneic, transplants.

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Figure 1.

Positive and negative selection in the thymus. Thymocytes enter the cortex and undergo T cell receptor (TCR) gene rearrangement and display both CD4 and CD8. These cells interact with cortical thymic epithelial cells (CTEC) and undergo apoptosis unless they receive a survival signal generated via TCR/self-peptide—MHC interaction. Positively selected thymocytes progress to single-positive CD4 or CD8 cells and enter the medulla. High-avidity TCR/self-peptide—MHC ligation in the presence of medullary thymic epithelial cell (MTEC) or dendritic cell (DC) co-stimulatory molecules in the medulla lead to negative selection of self-reactive thymocytes. (Modified with permission from Palmer E. Negative selection--clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol* 2003; 3 (5): 383.)