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$CD4^+CD25^+$ regulatory T cells mediate acquired transplant tolerance

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Abstract

The Holy Grail of clinical organ transplantation is the safe induction of allograft tolerance. Transplant tolerance has been successfully induced in animal models. Since T cells play a pivotal role in graft rejection, modulating T cell function has been the primary focus of studies aimed at inducing transplant tolerance. Rodent models of transplant tolerance induction include central deletion and peripheral mechanisms involving activation-induced cell death (AICD), anergy, immune deviation, and production of regulatory T cells. These mechanisms are not mutually exclusive. Although clonal deletion and anergy limit self-reactive T cells in the thymus, these mechanisms alone are not sufficient for controlling self-reactive T cells in the periphery. There is now evidence that the adult animal harbors two functionally distinct populations of $CD4^+$ T cells; one mediates autoimmune disease and the other dominantly inhibits it. The latter cells express CD4, CD25 and CTLA-4. These thymus-derived T cells have recently been shown to mediate the induction and maintenance of transplant tolerance. These $CD4^+CD25^+$ T cells are similar in origin, phenotype, and function to those that maintain natural self-tolerance and T cell homeostasis in the periphery. Against this background, is it possible that alloantigen specific regulatory T cells might be generated and expanded ex vivo before organ transplantation and then infused to induce long-term tolerance, perhaps without the need for chronic immunosuppression?

Keywords: Thymus; Tolerance; Regulatory T cells; Mixed chimerism; Co-stimulatory blockade; Dendritic cells; Allorecognition and allopeptides

1. Introduction

Presentation of self-peptides bound to MHC molecules plays a critical role in the process of negative and positive selection of immature T cells in the developing thymus [1–3]. Similarly, in adults, T cell recognition of self-peptides continuously displayed on antigen presenting cells (APC) regulate the immune response to foreign antigens (Ag) and contribute to maintenance of self-tolerance in the periphery [3–5]. Although clonal deletion and/or anergy may control self reactive T cells that are present in the thymus, these mechanisms alone may not be sufficient for controlling self-reactive T cells especially those that react with self-Ags expressed outside the thymus. Self-reactive T cells that have somehow escaped thymic negative selection or recognize Ags expressed only extrathymically are further subjected to control in the periphery by either T cell anergy [6], T cell ignorance/indifference [7] or deletion upon encounter with self-Ags in the periphery [5]. However, anergic or ignorant T cell populations have the potential to be activated when their target self-Ags are released into the circulation during the course of an infection or when they are activated by cross-reactive epitopes present on infectious agents [8]. Thus, these passive mechanisms for self-tolerance may not be sufficient to completely control potentially pathogenic T cells. Evidence accumulated in the past decade suggests a dominant control mechanism in which a distinct T cell subset actively down-regulates the activation/proliferation of self-reactive T cells that have escaped the passive mechanisms of tolerance [4,5,9,10].

In rodent strains that do not develop autoimmunity, a variety of organ-specific autoimmune diseases can be induced by interfering with normal T cell maturation or by rendering the animals partially T cell deficient [11]. Further, since adoptive transfer of a well defined T cell subset from syngeneic healthy donors can prevent the development of autoimmunity in lymphopenic recipi-

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ents, it is reasonable to suggest that the normal immune system contains immunoregulatory T cells that can prevent the activation of autoreactive T cells [10]. The existence of regulatory T cells has been demonstrated by adoptive transfer of $CD4^+$ T cells from syngeneic normal animals into the NOD mice [12] to prevent the onset of type 1-diabetes. These findings suggest that normal individuals may harbor two functionally distinct populations of $CD4^+$ T cells, one that is capable of mediating autoimmune disease and the other dominantly inhibiting autoimmune disease. The latter T cell population is dominant in the physiologic state and appears to be the key to the maintenance of self-tolerance. A number of recent studies have identified and phenotypically characterized these immunoregulatory T cells in the mouse. Previous reports [4,5,9,10] show that the regulatory T cells arise from the thymus of mature mice, express CD4, CD25 and CTLA-4, and can prevent not only thymectomy (TMX)-induced autoimmune disease [13] but can suppress disease induced by cloned autoantigen-specific effector cells [10]. Thus, these findings indicate that thymic selection may not only delete self-reactive T cells but also give rise to regulatory T cells, presumably specific for self-Ags in the case of natural self-tolerance or regulatory T cells specific for alloantigens in the case of transplant tolerance.

In a reproducible model of transplant tolerance induction, we have shown that indirectly presented alloMHC Class I peptide in the adult thymus induces acquired systemic tolerance [14–16]. While the intrathymic model has provided important information on the mechanisms of acquired thymic tolerance, the limitations of this approach in clinical transplantation have led us to explore the use of the intravenous route. Further, we have shown that adoptive transfer of a single alloMHC peptide-primed immature host dendritic cells (DC) induces tolerance to cardiac and islet allografts [17–19]. In our most recent studies, we have shown that adoptive transfer of in vivo generated syngeneic peripheral T cells indirectly primed to an immunodominant alloMHC Class I peptide induces transplant tolerance to cardiac allografts [18] and islets [19].

The finding that TMX prior to transfer of the immunodominant alloMHC peptide-primed T cells abrogates tolerance induction [18,19] suggests that the recent thymic emigrants with regulatory T cells might be essential for tolerance induction in this system. This hypothesis is reinforced by our earlier observation that removal of the thymus following intrathymic inoculation of donor alloantigen into recipients with functioning cardiac allografts consistently caused graft rejection if TMX was performed earlier than 21 days after heart transplantation; whereas TMX 21 days after organ transplantation (i.e. 28 days post intrathymic inoculation of donor Ag) did not affect permanent cardiac allograft survival [20]. These findings suggest that induction of peripheral tolerance in our model is, in part, dependent on regulatory T cells within the recent thymic emigrants. We have addressed this question and showed that co-transfer of syngeneic thymocytes together with in vivo alloMHC peptide-primed T cells restores tolerance to ALS-transiently immunosuppressed TMX recipients [21]. Importantly, our hypothesis is confirmed by the finding that adoptive transfer of in vivo P5-primed syngeneic T cells led to donor-specific graft acceptance in ALS transiently immunosuppressed TMX recipients with renal subcapsular syngeneic thymic grafts [22]. Restoration of tolerance to TMX recipients by either renal subcapsular syngeneic thymic grafts or co-transfer of thymocytes from naïve syngeneic animals indicates that induction of tolerance in this model is dependent on regulatory T cells arising from the host thymus. To test this hypothesis, we isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the naïve host thymus and compared their ability to restore tolerance to ALS-transiently immunosuppressed TMX recipients of in vivo alloMHC peptide-primed syngeneic T cells.

2. Restoration of donor-specific unresponsiveness to TMX recipients by co-transfer of $CD4^+CD25^+$ thymic regulatory cells

The above observations suggest that the presence of new thymic emigrants with regulatory T cell precursors is required to reinforce the regulatory effect of in vivo P5-primed T cells. To test our hypothesis that the induction phase of tolerance in this model is dependent on regulatory cells derived from the thymus, we isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the naïve ACI animals and showed that the $CD4^+CD25^+$ T cell subset constitutes approximately 5% of the thymic T cells and 9–11% of splenic T cells of naïve animals [22]. We next examined the ability of the $CD4^+CD25^+$ thymic T cells to regulate the response of in vivo immunodominant Wistar Furth MHC Class I ($RT1.A^u$) peptide 5 (P5, residues 93-109)-primed T cells to P5 in context of self-MHC in co-culture studies. Our results showed that syngeneic $CD4^+CD25^+$ T cells completely suppressed the response of in vivo P5-primed ACI splenic T cells to P5. In contrast, $CD4^+CD25^-$ T cells co-cultured with P5-primed syngeneic T cells resulted in a robust proliferative response to P5 presented by self-DC (Fig. 1). Whereas $CD8^+CD25^+$ syngeneic T cells were capable of downregulating the response of in vivo P5-primed T cells to P5 by 50%, the suppression was not of the magnitude observed with $CD4^+CD25^+$ T cells. Our data suggest that the regulatory effect of $CD4^+CD25^+$ T cells from the naïve thymus is not Ag-specific. This finding is consistent with the previous report that the suppressive effect of $CD4^+CD25^+$ T cells is antigen non-specific [23]. A possible explanation for our result may be that these regulatory T cells cross-

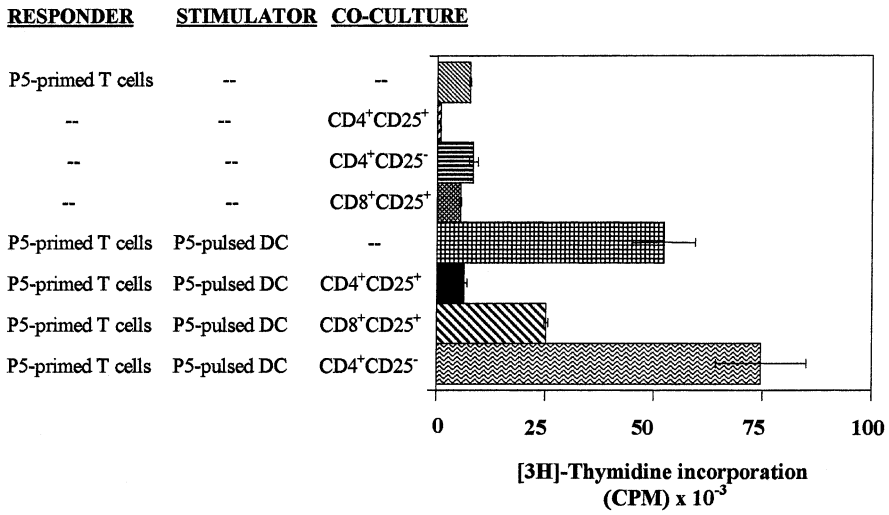

Fig. 1. Inhibition of the proliferative response of in vivo P5-primed ACI splenic T cells co-cultured with P5-pulsed ACI immature myeloid DC by syngeneic thymic $CD4^+CD25^+$ T cells. Splenic T cells were obtained from ACI animals immunized with i.v. injection of 1×10^6 WF alloMHC Class I ($RT1.A^u$) peptide 5-pulsed ACI DC for 7 days. The P5-primed ACI splenic T cells were co-cultured with P5-pulsed syngeneic DC in the presence of $CD4^+CD25^+$ or $CD4^+CD25^-$ syngeneic thymic T cells. Proliferation was determined after 4 days of culture by the addition of ^{3}[H] thymidine for the final 16 h. A representative result of three separate experiments is shown.

react with the immunodominant alloMHC peptide on self-MHC via the indirect pathway.

Furthermore, we found that $CD4^+CD25^+$ T cell subset obtained from the naïve thymus regulates in vitro co-culture alloimmune response and in vivo allograft rejection. To determine the role of the native thymus in the induction of tolerance in this model, we performed TMX 4 weeks before pretreatment with in vivo alloMHC peptide-primed syngeneic T cells. Thymectomy abrogated the induction of tolerance in animals given i.v. injection of alloMHC primed T cells and ALS at 7 days prior to cardiac transplantation (Fig. 2). Whereas adoptive transfer of enriched $CD4^+CD25^+$ (2×10^5) ACI thymic T cells combined with in vivo P5-primed syn-

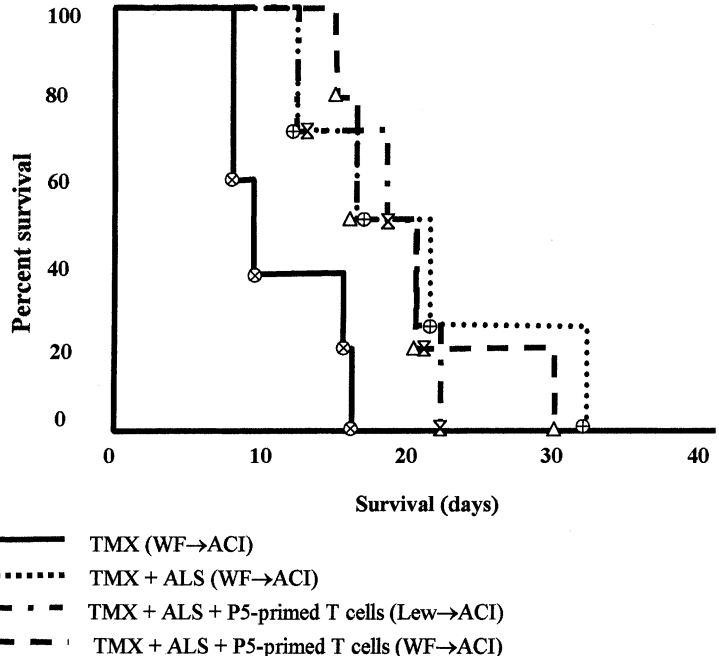

Fig. 2. Cardiac allograft survival in thymectomized (TMX) ACI recipients of alloMHC WF Class I peptide 5 (P5)-primed syngeneic T cells. There are five to six animals in each experimental group.

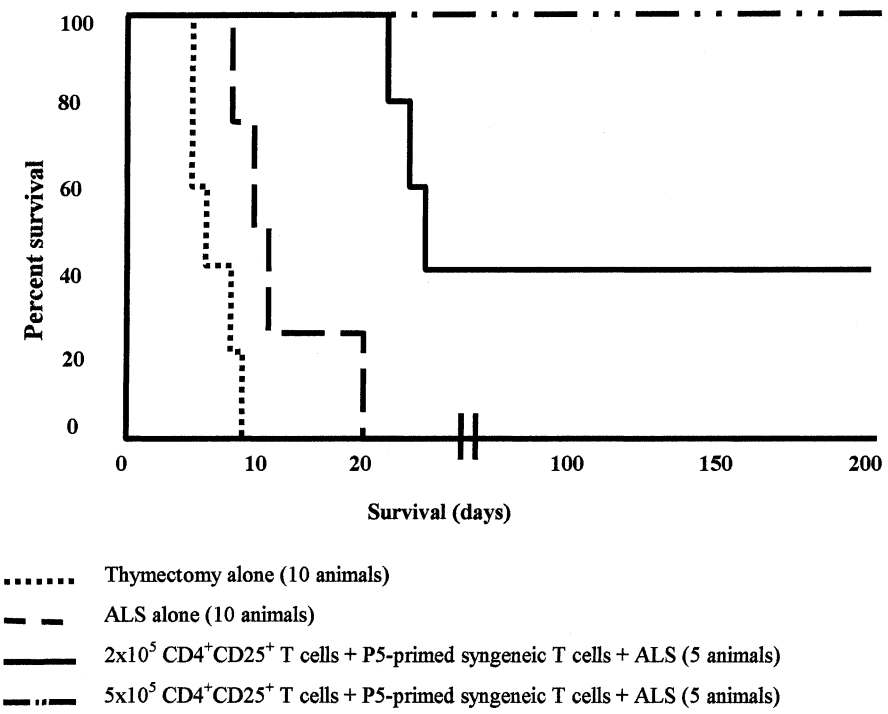

Fig. 3. Dose dependent effect of co-transfer of $CD4^+CD25^+$ syngeneic thymic T cells on cardiac allograft survival in TMX recipients pretreated with in vivo P5-primed syngeneic T cells.

geneic T cells (2×10^7) and 0.5 ml ALS on day 7 led to acute graft rejection (MST of 17.0 days compared to 24.0 ± 2.5 days in ALS alone treated animals), co-transfer of $CD4^+CD25^+$ host thymic T cells (2×10^5) with P5-primed syngeneic peripheral T cells restored permanent graft survival (>200 days) in 2 of 5 recipients. Increasing the dose of $CD4^+CD25^+$ host thymic T cells in the co-transfer inoculum to 5×10^5 resulted in 100% permanent graft survival (Fig. 3). Similar treatment led to acute rejection of third-party (Lewis) cardiac allografts (MST ± S.D. of 19.6 ± 2.8 days vs. 17 ± 2.1 days in ALS treated controls). Importantly, our results showed that 5×10^5 of $CD4^+CD25^-$ or $CD8^+CD25^+$ thymic T cells did not restore unresponsiveness to TMX recipients. Of interest is our observation that adoptive transfer of P5-pulsed immature myeloid host DC combined with $CD4^+CD25^+$ T cells restored donor-specific unresponsiveness to cardiac allografts in TMX recipients.

3. Role of $CD4^+CD25^+$ regulatory T cells in the induction of transplant tolerance

The finding that induction of acquired transplant tolerance in our model is dependent on the presence of $CD4^+CD25^+$ regulatory T cells in the recent thymic emigrants is consistent with the observation that adoptive transfer of $CD4^+CD45RB^{low}$ T cells obtained from naïve animals regulates pancreas rejection mediated by $CD4^+CD45RB^{high}$ [24]. Similarly, $CD4^+CD25^+$, but not $CD4^+CD25^-$ T cells from naïve syngeneic mice have been shown to prevent naïve splenocytes from

Table 1
Role of regulatory T cells in the induction of transplant tolerance

Conditioning	Graft	Adoptive transfer	Outcome	Ref. #
Tmx, ALS P5-host DC	Rat heart	Naïve $CD4^+CD25^+$ thymocytes Naïve $CD4^+CD25^-$ thymocytes	Acceptance Rejection	[21,22]
–	Murine Neonatal Pancreas	Naïve $CD4^+CD45RB^{low}$ Naïve $CD4^+CD45RB^{high}$ $CD45RB^{low}$ and $CD45RB^{high}$	Acceptance Rejection Acceptance	[24]
Tmx, Campath-1H (TCD)	Murine skin	Naïve $CD4^+CD25^+$ Naïve $CD4^+CD25^-$	Acceptance Rejection	[25]

Tmx, thymectomy; TCD, T cell depleted; P5, WF MHC Class I ($RT1.A^u$) peptide 5 (93–109).

Table 2
Role of regulatory T cells in the maintenance of transplant tolerance in syngeneic secondary hosts

Tolerance induction	Graft	Adoptive transfer	Outcome	Ref. #
Tmx, Campath-1H (TCD)	Murine skin	Tolerant $CD4^+CD25^+$ Tolerant $CD4^+CD25^-$	Acceptance Rejection	[25]
Vit D_3 and MMF	Murine islets	Tolerant $CD4^+CD25^+$ Tolerant $CD4^+CD25^-$ Naïve $CD4^+CD25^+$	Acceptance Rejection Rejection	[27]
Tmx (TCD) AntiCD4 DST	Murine skin	Tolerant $CD4^+CD45RB^{high}$ Tolerant $CD4^+CD45RB^{low}$	Rejection Acceptance	[28]
Tmx (TCD) AntiCD4 DST	Murine skin	Tolerant $CD4^+CD25^+$ and $CD4^+CD25^-$ Naïve $CD4^+CD25^+$ and $CD4^+CD25^-$	Acceptance Rejection	[29]

MMF, mycophenolate mofetil; DST, donor-specific transfusion; Vit D_3, 1α, 25-dihydroxyvitamin D_3.

rejecting skin allografts [25]. Our observation together with these two reports (Table 1) parallels the findings in the autoimmune animal model where $CD4^+CD25^+$ T cells obtained from naïve animals regulate the effects of $CD4^+CD45RB^{high}$ and prevent autoimmune disease [26]. In contrast to the reports in Table 1, other studies have shown that $CD4^+CD25^+$ T cells obtained from the spleen and lymph nodes of naïve animals fail to regulate murine islet [27] and skin graft [28,29] rejection. The latter finding raises the possibility that the precursor frequency of $CD4^+CD25^+$ regulatory T cells obtained from the peripheral lymphoid compartments of naïve animals might be too low to regulate allograft rejection in a similar fashion to $CD4^+CD25^+$ T cells obtained from naïve syngeneic thymus that prevent autoimmunity. Additionally, the conflicting data regarding the potential use of $CD4^+CD25^+$ T cells to regulate graft rejection might be related to the differences in the dose of $CD4^+CD25^+$ T cell inoculum used in the various experimental protocols. The Ag specificity of the $CD4^+CD25^+$ T cells that mediate allograft acceptance has not yet been defined. $CD4^+CD25^+$ T cells have been shown to suppress $CD4^+CD25^-$ T cell proliferation to different Ags in MLR, as long as the $CD4^+CD25^+$ cells are themselves preactivated [23]. It has also been suggested that if the $CD4^+CD25^+$ T cells contain receptors directed toward self-Ag, then there is the possibility that self-reactive regulators might mediate graft acceptance through linked suppression [25]. Alternatively, these investigators have hypothesized that the receptor repertoire of $CD4^+CD25^+$ T cells might show cross-reactivity to donor-type peptides from the graft which are indirectly presented by self-MHC [25].

4. Role of $CD4^+CD25^+$ regulatory T cells in the maintenance of transplant tolerance

A number of studies in rodents demonstrate that immunomodulation of the recipients with donor Ags, non-depleting anti-CD4 and anti-CD8 mAb, and costimulatory blockade can induce a robust form of peripheral tolerance [30–35]. To understand the mechanisms of tolerance in these animals, adoptive transfer studies were performed and demonstrated that T cells obtained from long-term tolerant animals can prevent graft rejection in secondary syngeneic recipients [36–39]. These findings suggest that donor-specific unresponsiveness is mediated by a specific T cell subpopulation [39,40]. To define the function and phenotype of the regulatory T cell subset(s) present in long-term graft recipients which is involved in the maintenance of transplant tolerance, a number of studies have compared the ability of adoptively transferred $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets from tolerized animals to regulate graft rejection. These recent studies show that maintenance of murine skin [25,28,29] and islet [27] allografts is associated with an increased frequency of $CD4^+CD25^+$ regulatory T cells in the peripheral lymphoid compartments of the recipients that can adoptively transfer transplant tolerance to secondary syngeneic hosts (Table 2). These results suggest that $CD4^+CD25^+$ regulatory T cells that are specific for the induction and maintenance of transplant tolerance are similar in origin, phenotype, and function to those involved in the maintenance of self-tolerance and the prevention of autoimmunity. Additionally, they raise the possibility that Ag-specific regulatory T cells could be generated and expanded ex vivo before transplantation to induce long-term operational tolerance without the need for prolonged immunosuppression.

5. Underlying mechanisms of regulatory T cells

Although the underlying mechanisms by which regulatory T cells suppress self-reactive T cells in autoimmune disease and alloreactive T cells in transplantation have not been fully defined, recent reports suggest that the dominant control of other cells by regulatory cells may be dependent on their ability to suppress IL-2 production [9] and/or on their expression of CTLA-4 [4,41]. Most recent studies in the rodent autoimmune disease model suggest various mechanistic ways by

which regulatory T cells mediate suppression of immune response to maintain self-tolerance. First, the underlying mechanism involves CTLA-4-dependent cell-to-cell contact whereby regulatory $CD4^+CD25^+$ T cells activated through CTLA-4 suppress other T cells [41]. $CD4^+CD25^+$ T cells, unlike other T cells, constitutively express CTLA-4 which is essential for regulation of cell cycle progression mediated by production of TGFβ [42]. When $CD4^+CD25^+$ T cells are stimulated via TCR in vitro, they potentially suppress antigen-specific and polyclonal activation of other T cells. Blockade of CTLA-4 abrogates this suppression [41]. Second, regulatory T cells control other T cells through a 'three-cell model' of suppressor function. In this model, regulatory T cells directly inhibit the functional state of APC, which in turn fail to engage other T cells in a bi-directional process that will normally induce full T cell activation [43]. This effect of regulatory T cells on APC emphasizes the important role of APC in determining the outcome of T cell activation. Finally, the immunoregulatory function of $CD4^+CD25^+$ T cells has been shown to be dependent on cell-to-cell contact via TCR. In this model, Thornton et al. [9,23] have shown that $CD4^+CD25^+$ cells are resistant to stimulation via TCR and suppress polyclonal T cell proliferation of $CD4^+CD25^-$ T cells in co-culture studies by inhibiting the production of IL-2. This suppression is cytokine independent, cell-to-cell contact dependent, and requires activation of the suppressors via their TCR. Such immunosuppression can be overcome by the addition of IL-2 or anti-CD28 mAb because the regulatory T cells function by blocking the delivery of a co-stimulatory signal.

6. Conclusion

In conclusion, animal models have demonstrated that the induction of transplant tolerance is an achievable goal. It appears that intrinsic immunoregulatory mechanisms that activate T cell tolerance are self-sustaining without the need for further therapy. Understanding the mechanisms of such regulation is vital to the development of clinical transplant tolerance strategies. With present insight into the mechanisms of operational tolerance in the rodent model, it appears that maintenance of tolerance is, in part, dependent on generation of $CD4^+CD25^+$ regulatory cells.

Acknowledgments

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