Review

The Role and Mechanisms of Double Negative Regulatory T Cells in the Suppression of Immune Responses

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Accumulating evidence has demonstrated that regulatory T (Treg) cells play an important role in the maintenance of immunologic self-tolerance and in down-regulating various immune responses. Thus, there has recently been an increasing interest in studying the biology of Treg cells as well as their potential application in treating immune diseases. Many types of Treg cell subsets have been reported in a variety of disease models. Among these subsets, αβTCR$^+$CD3$^+$CD4$^-$CD8$^-$ double negative (DN) Treg cells are defined by their capability of inhibiting immune responses *via* directly killing effector T cells in an antigen specific fashion. Furthermore, DN Treg cells have been shown to develop regulatory activity after encountering specific antigens, partially mediated by the acquisition of MHC-peptide complexes from antigen presenting cells (APCs). The presentation of acquired alloantigens on DN T cells allows for the specific interaction between DN Treg cells and alloantigen reactive effector T cells. Once the DN Treg and target cells have come into contact, killing is then mediated by Fas/Fas-ligand interactions, and perhaps through other unidentified pathways. Further characterization of the functions, molecular expression and mechanisms of activation of DN Treg cells will help in the development of novel therapies to induce antigen specific tolerance to self and foreign antigens. *Cellular & Molecular Immunology*. 2004;1(5):328-335.

Key Words: regulatory T cell, suppression, transplantation, Fas ligand

Introduction

Suppression of immune responses by regulatory T (Treg) cells is one of the major mechanisms for the induction and maintenance of self-tolerance (1). Treg cells have also been shown to be important in regulating the immune responses in transplant rejection (2-4), tumor immunity (5, 6), infectious diseases (7, 8) and allergy (9, 10). Furthermore, Treg cells can be used to suppress donor T cell-mediated graft-versus-host disease (GVHD) (11, 12). It is now clear that immune regulatory cells consist of many distinct T cell subsets (13, 14). Among them, CD4$^+$ Treg cells have been demonstrated in a wide range of animal models and in humans. CD4$^+$ Treg cells are often characterized by their expression of interleukin-2 (IL-2) receptor α-chain (CD25) (1, 15-18) and have also been shown to express CD45RB^{low}, and the transcription factor FOXP3 (1, 19). Immunosuppressive CD8$^+$CD28$^-$ T cells (20-23), γδTCR$^+$ T cells (24, 25), natural killer (NK) T cells (26-28), CD8$^+$ veto cells (29, 30), and CD4$^-$CD8$^-$ double negative (DN) T cells (31) have also been shown to possess the ability to regulate immune responses.

Mature αβTCR$^+$CD3$^+$CD4$^-$CD8$^-$ NK1.1$^-$ DN Treg cells are a subset of T cells that are present in the periphery in very low numbers representing 1% - 5% and 1% - 2% of peripheral T lymphocytes in mice and humans, respectively. These DN Treg cells express a unique set of cell surface markers and have a unique cytokine profile (31). Recent studies have demonstrated in several mouse models that DN Treg cells are able to suppress CD4$^+$ and CD8$^+$ T cell mediated allogeneic and xenogeneic immune responses as well as response to self antigens (32-35). More recently DN Treg cells have been identified in humans (Mackensen, et al., manuscript submitted, 2004). Here we will review the recent progress in the study of DN Treg cells focusing on their activation, function, mechanism of action, and development and compare the results of these studies to that of other Treg cells.

Activation and expansion of DN Treg cells

Many studies have been performed to determine what stimuli are important for activating Treg cells. It is now

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Abbreviations: Treg, regulatory T; DN, double negative; APC, antigen presenting cell; GVHD, graft-versus-host disease; NK, natural killer.

clear that stimulation through the TCR on Treg cells is critical for their function. The finding that natural occurring $CD4^+CD25^+$ Treg cells constitutively express many activation markers on their surface (36-38), and are able to suppress autoantigen-specific effector T cell proliferation from the first day of *in vitro* TCR stimulation (39) suggests that these Treg cells must be stimulated by self-antigens on a regular basis *in vivo*. On the other hand, in order to mediate suppression of non-self antigen effector T cells, $CD4^+CD25^+$ Treg cells must first be stimulated *via* their TCR. This stimulation can be mediated *in vitro* through specific peptides, but not through third-party antigens (36, 39). For example, anti-OVA TCR transgenic $CD4^+CD25^+$ Treg cells require stimulation by cells pulsed with specific OVA peptides in order to suppress the proliferation of OVA-specific effector T cells (39). Similarly, DN Treg cells also require stimulation through their TCR in order to gain a suppressive phenotype. For instance, MHC class I-L^d-specific DN Treg cells require stimulation with L^{d+} splenocytes in order to proliferate and function *in vitro* and *in vivo* (31, 32, 40). Unlike other Treg cells, DN Treg cells express neither co-receptors (CD4 or CD8) nor costimulatory molecule CD28 (31). Whether DN Treg cells utilize other costimulatory molecules for their activation remains to be determined.

In addition to stimulation through TCR, many studies have shown that cytokines play an important role in activation and expansion of Treg cells. For instance, $CD4^+CD25^+$ T cells require exogenous IL-2 to proliferate both *in vitro* (41) and *in vivo* (42). Several subtypes of $CD4^+$ Treg cells that do not naturally express CD25 can be induced to possess Treg cell function. For instance, Tr1 cells can be induced by chronic stimulation of normal non-regulatory T cells in the presence of IL-10 (43, 44), and Th3 cells can be activated by stimulating $CD4^+$ T cells with myelin-basic protein in the presence of TGF-β (45, 46). Similar to other Treg cells, DN Treg cells can also be activated and expanded *in vitro* by stimulating these cells with allogeneic splenocytes in the presence of exogenous IL-2 and IL-4. It is known that IL-4 protects DN Treg cells from TCR-crosslinking induced apoptosis (47). Whether IL-4 is required for the activation of DN Treg cells is not known. It is also unclear whether other cytokines such as IL-15 (which has been shown to activate $CD4^+$ Treg cells (48) are required for the optimal activation of DN Treg cells. Together, these findings suggest that Treg cells must have access to specific antigens in order to gain and possibly maintain their function. In addition, the cytokine milieu is also important in the control of Treg cell activation.

Characteristics of activated DN Treg cells

Several groups have shown that the transfer of $CD4^+CD25^-$ splenocytes to immunoincompetent animals leads to the spontaneous development of autoimmune disease, whereas the cotransfer of $CD4^+CD25^+$ T cells with $CD4^+CD25^-$ T cells prevents the onset of autoimmune disease (1, 15, 18, 49, 50). As a result of this and several other studies, CD25 has been identified as a marker of naturally occurring $CD4^+$ Treg cells. In addition, several recent studies have shown that $CD4^+CD25^-$ T cells can also down-regulate immune responses to allo and self antigens (51-53). Some of these $CD4^+CD25^-$ Treg cells have been identified by their expression of $CD45RB^{low}$, as well as increased expression of TGF-β or IL-10 (53). In addition, a population of $CD8^+$ Treg cells have been identified that are $CD28^-$, but produce IL-10 following activation and can prevent autoimmune disease development (22, 54). Recently, *Foxp3*, which is expressed by the majority of $CD4^+CD25^+$ cells and a fraction of $CD4^+CD25^-$ T cells, has been considered to be a more reliable marker for identifying Treg cells (1, 19). So far, no cell surface molecule that is uniquely expressed on Treg cells has been identified. Our studies have shown that treatment of DN T cells with a TCR specific peptide, either *in vitro* or *in vivo*, can increase the expression of T cell early activation markers on DN T cells including CD25 and CD69. Similar to activated $CD4^+CD25^+$ Treg, CD62L does not seem to be shed from DN Treg cells following specific TCR ligation. Interestingly, unlike other Treg cells, DN Treg cells do not express the activation markers CD44 or CD28 at any time after activation. *Foxp3* mRNA has been detected in both DN Treg cell clones and their natural mutants that have lost their cytotoxic activity following long-term *in vitro* cultivation (Boris, et al., unpublished data, 2004). Whether the expression of Foxp3 protein is critical for DN Treg cell development and function requires further investigation.

Activated DN Treg cells have been shown to possess a unique array of cytokines that differ from $CD4^+CD25^+$, Th1, Th2 or Th3/Tr1 cells. DN Treg cells produce predominantly INF-γ, TNF-α, and a low amount of TGF-β, but not IL-2, IL-4, IL-10 or IL-13 (31). Unlike $CD4^+$ or $CD8^+$ T cells which are sensitive to activation induced cell death, DN Treg cells are resistant to apoptosis induction both *in vitro* and *in vivo*. Our experiments have shown that when DN Treg and $CD8^+$ T cells are induced to undergo apoptosis by cross-linking their TCR in the presence of IL-4, $CD8^+$ T cells undergo apoptosis but DN Treg cells do not (47, 55). Furthermore, after infusion into alloantigen expressing mice, DN Treg cells are able to persist for a much longer period of time than $CD8^+$ T cells (12), suggesting that DN Treg cells are resistant to activation induced cell death *in vivo*. This feature may allow DN Treg cells to function for a prolonged period of time to regulate immune responses *in vivo*. Nonetheless, when DN Treg cells are incubated *in vitro* with IL-10, their ability to resist apoptosis is abolished (55, 56). This sensitivity to IL-10 may represent a pathway that is used to modulate the function of DN Treg cells *in vivo*. It also suggests that the Th1/Th2 cytokine balance in a recipient animal may be important to the successful administration of DN Treg cells as a cellular therapy.

Functions of DN Treg cells

DN Treg cells in transplantation

Numerous studies have indicated that $CD4^+$ Treg cells are able to regulate a variety of immune responses (1, 57, 58), whereas studies on the function of peripheral DN Treg cells are relatively limited. We have demonstrated in a single MHC class I mismatched models that DN Treg cells

isolated from DLI-treated mice can suppress and kill $CD8^+$ and $CD4^+$ anti-donor T cells in an antigen specific manner *in vitro*, and prolong donor-specific allograft survival when adoptively transferred into naïve syngeneic mice (31). Recently, similar findings have been seen with DN Treg cells in a semi-allogeneic transplantation model (Torrealba, et al., unpublished data, 2004). We have successfully cloned DN Treg cells from the spleens of both tolerant and naïve mice, and shown that infusion of these *in vitro*-activated, recipient-derived DN T cells alone leads to significant prolongation of donor-specific skin (31) and heart allograft (59) survival in mice. Interestingly, both DN T cells and DN Treg cell clones are able to accumulate in tolerant grafts in an antigen specific manner (32). Moreover, we have demonstrated *in vitro* that graft infiltrating DN Treg cells are much more potent in the suppression of anti-donor T cell proliferation than those DN Treg cells isolated from the spleens of the same recipients (Chen, et al., unpublished data, 2004). These findings indicate that activated DN Treg cells can suppress allo responses both systemically in the lymphatic system and locally in grafts, suggesting that either the local allograft environment can potentiate the function of DN Treg cells, or potent DN Treg cells can preferentially migrate into the allograft tissue.

DN Treg cells have also been shown to function in a model of xenograft tolerance. We found that mice that have been given a short course of anti-CD4 depleting mAb, together with DLI, can permanently accept concordant cardiac xeno grafts. DN Treg cells isolated from tolerant xenograft recipients are able to suppress anti-donor T cell proliferation *in vitro* (33). These DN Treg cells can also suppress the *in vivo* proliferation and cytokine production of anti-donor T cells and prolong cardiac xenograft survival when co-infused with anti-donor $CD4^+$ T cells into recipient mice (Chen, et al., unpublished data, 2004). Together, these findings suggest that DN Treg cells could provide a novel therapy for specifically tolerizing transplant recipients to donor type antigens.

DN Treg cells in other diseases

Strober, et al. have reported that $\alpha\beta TCR^+CD4^-CD8^-NK1.1^+$ (DN NK) T cells can suppress immune responses both *in vitro* and *in vivo* (60-63). They demonstrated that DN NK T cells or clones generated from the spleen, thymus and bone marrow of adult mice inhibited mixed lymphocyte reactions *in vitro* and prevented GVHD after co-injection with allogeneic bone morrow cells (63-65). Abraham, et al. have identified $NK1.1^-$ DN T cells in the bone marrow of mice that are protected from GVHD by a short course of high dose IL-2 (66). We have shown that DN Treg cells, which are $NK1.1^-$, can inhibit the development of GVHD (12). We found that immunodeficient mice that were infused with single MHC class I-mismatched splenocytes did not develop GVHD. Interestingly, donor-derived DN Treg cells increase following the infusion and these DN Treg cells are able to suppress the proliferation of anti-host $CD8^+$ T cells *in vitro*. Furthermore, co-injection of *in vitro* propagated DN T cells can prevent $CD8^+$ T cell-mediated GVHD (12). Therefore, these experiments demonstrate that DN Treg cells can suppress syngeneic T cells that are primed against a variety of MHC molecules not only in transplant models, but also in a GVHD model.

Experiments performed in other labs have corroborated our assertion that DN T cells are a subset of potent immune regulatory cells. For example, Priatel, et al. (35) have demonstrated that DN Treg cells are able to down-regulate $CD8^+$ T cell-mediated immune responses in an autoimmune disease model. In their experiments, DN T cells from mice that expressed a transgenic TCR specific for a self expressed MHC class I antigen showed an increased ability to lyse syngeneic $CD8^+$ T cells when compared to DN T cells from antigen-free mice. This study suggests that DN Treg cells may also be involved in down-regulating autoimmune disease. Furthermore, our own work using the Fas mutant lpr mouse model demonstrates that the DN T cells that accumulate in these mice also possess Treg cell activity and can suppress the proliferation of syngeneic $CD4^+$ and $CD8^+$ T cells that express functional Fas receptor (34). Since our studies demonstrate that DN T cells can kill Fas^+ but not Fas^- target cells, we hypothesize that DN T cells may accumulate in the lpr mouse in a failed attempt to control the systemic autoimmune disease that develops in these mice with age.

In another study, DN T cells were found to increase in tissues following burn injuries. Interestingly, these DN T cells produced both Th1 and Th2 type cytokines (IFN-γ, IL-2, IL-4 and IL-10), and were shown to have a regulatory effect in mixed lymphocyte cultures (67). In addition, work done on a model of Listeria monocytogenes infection has shown that DN T cells accumulate in the peritoneal cavity following infection and can contribute to early protection from bacterial infection by secreting cytokines that induce macrophage activation and accumulation (68). This suggests that DN T cells may also play a role in defence against foreign pathogens. Collectively, these studies indicate that DN Treg cells are potent regulators of a variety of immune responses.

Mechanisms involved in DN Treg cell-mediated suppression

DN Treg cell-mediated suppression is antigen specific and requires cell-cell contact

Numerous studies have demonstrated that $CD4^+CD25^+$ Treg cells require cell-cell contact in order to mediate suppression (36, 39, 69, 70). Since the expression of co-stimulatory molecules on APC such as CD80, CD86, CD54 and CD40 is unchanged following incubation with $CD4^+CD25^+$ Treg cells (38) and that suppression was not abrogated when APC were fixed with paraformaldehyde prior to use (39, 70), these data suggest that the mechanism of suppression is unlikely mediated through modulation of APC. Following contact with other T cells, $CD4^+CD25^+$ Treg cells have been shown to inhibit IL-2 production by T cells at the gene transcription level (36, 39, 69). Most studies have shown that $CD4^+CD25^+$ Treg cell-mediated suppression is antigen non-specific (38, 39). For example, $CD4^+CD25^+$ Treg cells from TCR-transgenic mice that recognize a specific ovalbumin peptide are able to suppress responder $CD4^+CD25^-$ T cells that recognize both the same and different peptides (39). More recently, some studies have shown that $CD4^+CD25^+$ Treg cells can mediate

antigen-specific suppression *in vivo* (2, 71). We have demonstrated that DN Treg cells can suppress immune responses in an antigen specific manner both *in vitro* and *in vivo*. DN Treg cells from anti-L^d transgenic mice are able to inhibit T cells that are activated by L^d antigens, but not those that are activated by third-party alloantigens (31, 40). We have demonstrated that DN Treg cells do not suppress $CD8^+$ T cell responses by competing with $CD8^+$ T cells for growth factors or surface area on APC (31, 40). Similar to $CD4^+CD25^+$ Treg cells, DN Treg cell-mediated immune suppression also requires cell-cell contact since DN Treg cells that are separated from responder cells using a transwell system cannot suppress the proliferation of $CD8^+$ responder cells (31). It remains to be determined how cell-cell contact leads to Treg cell-mediated suppression of syngeneic T cells.

DN Treg cells mediate antigen-specific suppression by acquisition of MHC-peptides from APC

The ability to acquire proteins from neighbouring cells has been demonstrated in a variety of cell types including T cells (72-77), B cells (78) and dendritic cells (79). We have found that L^d-negative DN Treg cells are able to acquire allo L^d peptides from L^{d+} APC *in vitro* and present the acquired L^d peptides on their surface. The acquired L^d molecules on DN Treg cells may then be directly recognized by the anti-L^d TCR on the responder T cells. Blocking the acquired antigen on DN Treg cells or blocking the expression of TCR on responder T cells using mAbs eliminates DN Treg cell-mediated suppression (31). These studies suggest that the acquisition of antigen by DN Treg cells is important to their function. We have also demonstrated that DN Treg cells can acquire alloantigen *in vivo* by tracking the increased expression of L^d on naturally L^d negative DN T cells that have been injected into L^{d+} hosts (Young, et al., unpublished data, 2004). In addition, A. Mackensen's group has recently demonstrated that human HLA-A2 negative DN Treg cells can acquire allo HLA-A2-Melan-A peptides from $HLA\text{-}A2^+$ dendritic cells that have been pulsed *in vitro* with Melan-A peptides. Moreover, these human DN Treg cells can use the acquired Melan-A peptides to specifically trap and kill Melan-A-specific, but not third-party gp120 peptide-specific, $CD8^+$ T cells (Mackensen, et al., manuscript submitted, 2004). These findings indicate that murine and human DN Treg cells may use a similar mechanism to mediate antigen-specific suppression of immune responses.

There are several interesting features of the antigen acquisition mediated by DN Treg cells. First, both mouse and human DN Treg cells are able to retain expression of the acquired molecules on their surface for several days, in contrast to $CD8^+$ T cells which also acquired allo-MHC-peptides but reduced their expression to basal levels within a 12 hour time period (31, 77). This extended period of expression may give DN Treg cells an increased window of opportunity in which they can suppress other T cells by bringing the T cells that are able to recognize the acquired allo MHC-peptides into cell contact. Second, experiments have shown that the acquisition of allo MHC-peptides by DN Treg cells is antigen specific, and that pre-incubation of DN Treg cells with anti-TCR blocking mAb abrogates acquisition (31). The ability of DN Treg cells to specifically acquire allo-MHC molecules is particularly important since it dictates which cells can come into contact with DN Treg cells and be suppressed. Hence, the antigen-specific acquisition of MHC molecules by DN Treg cells helps to ensure that the regulation of T cells by DN Treg cells is antigen specific.

DN Treg cells can kill target cells through Fas/FasL interaction

Ligation of Fas receptors by FasL has been shown to be a major pathway of apoptosis induction in T cells (80, 81) and is one of the mechanisms whereby peripheral T cell tolerance is maintained. Various types of T cells have been shown to be able to mediate suppression of immune responses by killing $CD4^+$ or $CD8^+$ T cells through Fas/FasL interactions (82, 83). However, Fas/FasL interactions mediated by $CD4^+$ and $CD8^+$ T cells have also been shown to result in non-specific bystander killing of $CD4^+$ and $CD8^+$ T cells (82, 83). We have demonstrated that DN Treg cells can directly kill target T cells through Fas/FasL interactions since blocking FasL on DN Treg cells using mAb or fusion proteins significantly inhibits DN T cell mediated killing (31). Furthermore, DN T cells showed an impaired ability to suppress the proliferation of, or directly kill, $CD8^+$ T cells that did not express functional Fas receptors when compared to wild-type Fas expressing $CD8^+$ T cells. Moreover, DN T cells from gld mice that express mutant FasL molecules showed a reduced ability to kill CD8 T cell targets when compared to wild-type FasL expressing DN T cells (34). These data demonstrate that FasL expression on the DN T cell, and Fas expression on the target T cell, are important for DN T cell-mediated regulation.

However, several pieces of data suggest that DN T cells may also use other mechanisms to mediate suppression of immune responses. First, we have observed that the ability of DN Treg cells to suppress responder T cell proliferation is often much more potent than their ability to cytotoxically lyse T cell targets. Second, although blocking FasL with Fas-Fc fusion protein significantly reduced DN T cell-mediated killing, blocking of killing is usually not complete. Finally, DN T cells obtained from autoimmune disease-prone lpr mice could inhibit $CD4^+$ and $CD8^+$ T cell proliferation, although to a much lesser extend, even when cell-cell contact was inhibited using a transwell system (Ford, et al., unpublished data, 2004). These data suggest that DN Treg cells may also suppress through pathways other than FasL/Fas.

Interestingly, the neutralization of cytokines such as IL-4, IL-10 or TGF-β in some rodent models of autoimmunity has been shown to abrogate T cell mediated suppression, suggesting that some Treg cells may mediate suppression by releasing these cytokines (84-86). For instance, the ability of Tr1 cells to suppress the proliferation of naïve $CD4^+$ T cells, antibody production by B cells and antigen presentation by monocytes and dendritic cells (DCs) *in vitro* can be reversed with neutralizing anti-IL-10 and/or anti-TGF-β antibodies (44, 84, 87, 88). Our transwell experiments have shown that wild-type DN Treg cell-mediated suppression requires cell-cell contact. However, DN T cells from autoimmune-prone *lpr* mice are able to partially suppress T cell

responses in the absence of cell-cell contact. This suggests that perhaps if DN T cells become chronically activated they may produce some soluble factors that can mediate suppression of T cell responses. Both human and mouse DN Treg cells express high levels of IFN-γ, and our preliminary data suggest that blocking IFN-γ expression on DN T cells can inhibit their ability to kill target cells *in vitro*. The potential role that IFN-γ and other cytokines expressed by DN Treg cells may play in their ability to mediate immune regulation requires further investigation.

Origin and development of DN Treg cells

Although the function of DN T cells has been extensively characterized, the origin of peripheral DN T cells is still unclear. The heterogeneity of markers expressed by different DN T cell subtypes suggests that several maturation pathways may exist. Wang, et al. showed that thymic double positive cells can down-regulate CD4 and CD8 and become DN T cells when stimulated with high affinity antigens in reaggregate cultures. These DN T cells were also able to suppress the proliferative response of naïve T cells (89). However, these DN T cells produced large amount of IL-10, a feature that has not been demonstrated in our extensively studied DN Treg clones. Furthermore, we have shown that incubation of DN Treg clones with IL-10 abrogates their regulatory function (55). In other experiments, $CD8^+$ T cells treated with IL-4 and ionomycin have also been shown to differentiate into DN cells that produce IL-4 and other Th2 cytokines (90). Furthermore, the CD8 gene was found to be demethylated in peripheral DN T cells (91, 92), suggesting that DN T cells may arise from down regulation of CD8 molecule expression during their maturation and expansion. However, we have recently demonstrated that DN T cells exist in $CD8^{-/-}$ mice, and these cells can be activated and suppress syngeneic $CD8^+$ T cells. Moreover, the DN Treg cells that have been identified in our models do not have any CD8 messenger ribonucleic acid (mRNA) transcripts. In addition, we have been unable to produce DN Treg cells from $CD8^+$ T cell populations either *in vitro* by culture in the presence of IL-2 and/or IL-4, or *in vivo* by injecting highly purified $CD8^+$ T cells into antigen expressing mice (Zhang, et al., unpublished data, 2004). These data suggest that DN Treg cells are not derived from $CD8^+$ T cell precursors and demonstrate that CD8 coreceptor expression is not required for the development of DN Treg cells *in vivo*. Currently, we are attempting to further characterize the molecules expressed by DN Treg cells with the goal of finding DN Treg cell specific antibodies. This will possibly allow us to monitor DN Treg cell development *in vivo*.

Several experiments have attempted to determine where DN T cell development occurs *in vivo*. Tissue analysis has shown that DN T cells are present in the spleen, lymph node, bone marrow, thymus, liver and appendix (93). However, ours and others data have only shown that spleen, lymph node and bone marrow derived DN T cells possess suppressor activity (31, 94). Further characterization of molecular expression and function of DN T cells from other tissues is required in order to determine whether these DN T cells are related to DN Treg cells found in the spleen and lymph nodes. Although the majority of $CD4^+$ and $CD8^+$ T cells found in the peripheral lymphatic system are known to require thymic selection in order to functionally mature, studies have suggested that DN αβ-TCR^+ cells might mature extra-thymically from organs such as the bone marrow, the appendix or the liver (93-96). First, DN T cells have been shown to be present in athymic nude mice, and their number have been demonstrated to increase with age (97). Furthermore, our preliminary data suggest that thymectomy does not prevent the development of DN T cells in the peripheral lymphatic system following reconstitution with syngeneic bone marrow. Taken together, these data suggest that at least some portion of the DN Treg cell population can develop extrathymically.

Concluding remarks

There are several potential clinical uses for DN Treg cells that warrant further study. We have shown that DN Treg cells are able to infiltrate skin and cardiac allograft, and that the presence of DN Treg cells allografts is correlated with graft acceptance (32). These findings indicate that the number of DN Treg cells in allografts may be a useful prognostic indicator for graft rejection. We and others have demonstrated that functional DN Treg cells can be generated *in vitro*, and that *in vitro* generated DN Treg cells can be used directly to prevent graft rejection and GVHD in mice (31, 59). This technology could pave the way for using DN Treg cells in transplantation, since DN Treg cells with various specificities could be pre-generated using donor antigens, and then suitable clones could be chosen and infused into recipients at the time of transplantation.

Overall, we demonstrate that DN Treg cells can be activated both *in vitro* and *in vivo* upon antigen stimulation. Activated DN Treg cells can play an important role in preventing donor-specific graft rejection and development of GVHD through inhibition of antigen-specific $CD4^+$ and $CD8^+$ T cells. A better understanding of the molecular mechanisms leading to DN Treg cell activation and function offers a possibility that DN Treg cells may be used in the clinical setting as a novel therapy for a vast array of immune mediated diseases.

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