Review

Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions

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T lymphocytes bearing the αβ T cell receptor (TCR) but lacking CD4, CD8, and markers of natural killer (NK) cell differentiation are known as 'double-negative' (DN) T cells and have been described in both humans and rodent models. We and others have shown that DN T cells can act as regulatory T cells (Tregs) that are able to prevent allograft rejection, graft-versus-host disease, and autoimmune diabetes. In the last few years, new data have revealed evidence of DN Treg function *in vivo* in rodents and humans. Moreover, significant advances have been made in the mechanisms by which DN Tregs target antigen-specific T cells. One major limitation of the field is the lack of a specific marker that can be used to distinguish truly regulatory DN T cells (DN Tregs) from non-regulatory ones, and this is the central challenge in the coming years. Here, we review recent progress on the role of DN Tregs in transplantation and autoimmunity, and their mechanisms of action. We also provide some perspectives on how DN Tregs compare with Foxp3$^+$ Tregs.

Keywords: double-negative T cells, regulatory T cells, transplantation, autoimmunity, graft-versus-host disease, type 1 diabetes mellitus

Introduction

Double-negative (DN) T cells express the αβ T cell receptor (TCR) but do not express CD4, CD8, or natural killer (NK) cell markers. They exist as a small (1%–5%) population of lymphocytes in the peripheral blood and lymphoid organs of normal rodents and humans. Peripheral T cells with a DN phenotype have been shown to be involved in immune regulation and tolerance as well as in host defense and inflammation. For example, whereas many studies have examined the regulatory function of DN T cells (detailed later), Crispin and colleagues described IL-17-secreting DN T cells derived from a CD8$^+$ precursor in lupus patients (Crispin et al., 2008; Crispin and Tsokos, 2009). In a similar vein, inflammatory DN T cells have been shown to confer protection against a variety of intracellular pathogens in murine models (Viret and Janeway, 2003; Cowley et al., 2005, 2010; Riol-Blanco et al., 2010). Since the role of DN T cells in host defense and inflammation has been reviewed recently (D'Acquisto and Crompton, 2011), this review will focus on recent advances in regulatory DN T cells (DN Tregs).

In 1989, Strober et al. (1989) reported that cloned 'natural suppressor' T cells from murine spleens that did not express CD4 or CD8 could suppress allogeneic mixed lymphocyte reactions, thus providing the first evidence that CD3$^+$, CD4$^-$, and CD8$^-$ DN T cells may possess immune regulatory function. Since NK lineage markers were not examined in that study, one could not exclude the possibility of NK T cells being responsible for the observed suppression. A contemporaneous study showed that graft-versus-host disease (GVHD) in mice transplanted with bone marrow and splenocytes across a minor histocompatibility barrier was reduced when the donors were preimmunized with recipient splenocytes. The authors demonstrated that this reduction in GVHD was attributable to an αβ TCR-expressing population that persisted despite depletion of CD4, CD8, and asialo-GM1 populations (Bruley-Rosset et al., 1990). These early studies provided indirect evidence suggesting the DN T cells may act as Tregs, although their antigen-specificity and mechanisms of action were not determined.

Ours was the first laboratory to characterize αβ-TCR$^+$CD4$^-$CD8$^-$NK1.1$^-$ cells as antigen-specific DN Tregs *in vitro* and *in vivo* (Zhang et al., 2000). We also described alloantigen-specific regulatory properties in the DN T cell

compartment of lymphoproliferative (*lpr*) mice (Zhang et al., 2000; Ford et al., 2002) and non-transgenic mice (Zhang et al., 2000; Young et al., 2002; Chen et al., 2003a). Subsequent reports from various laboratories confirmed (Priatel et al., 2001) and extended these findings in other disease models in mice (Priatel et al., 2001; Ford et al., 2007; Ma et al., 2008; Duncan et al., 2010), in rats (Hill et al., 2011), and in humans (Fischer et al., 2005; Voelkl et al., 2011). Many of the earlier studies on DN Tregs focused on the suppression of $CD8^+$ T cell responses, but it has also been shown that DN Tregs can suppress $CD4^+$ T cells (Ford et al., 2002; Chen et al., 2003a, 2005, 2007), B cells (Ma et al., 2008; Hillhouse et al., 2010), NK cells (He et al., 2007), and dendritic cells (DCs) (Gao et al., 2011). In this review, we summarize some of the recent progress in our understanding of the biology of DN Tregs.

Ontogeny of DN Tregs

The origin of DN Tregs is not well understood. Some studies suggest that peripheral DN T cells can derive from the thymus (Egerton and Scollay, 1990; Mixter et al., 1999) but others support the notion that they develop in the periphery. During T cell development, thymocytes pass through a DN stage and then a $CD4^+CD8^+$ stage prior to committing to a $CD4^+$ or $CD8^+$ lineage (Anderson and Jenkinson, 2001). This observation led to the hypothesis that peripheral DN T cells may represent primitive αβ T cells that bypassed the thymus entirely (Halder et al., 2001; Johansson and Lycke, 2003), or alternatively, that they escaped further development into $CD4^+$ or $CD8^+$ T cells by traversing a rare alternative developmental pathway (Egerton and Scollay, 1990; Takahama et al., 1991). A competing hypothesis is that peripheral DN T cells may arise *de novo* in the periphery from a $CD4^+$ or $CD8^+$ precursor that has downregulated its coreceptor (Schonrich et al., 1991; Merino et al., 1995; Mehal and Crispe, 1998). We have shown that DN Tregs can develop in thymectomized, irradiated C57BL/6 mice which were reconstituted with either TCR transgenic 2C or C57BL/6 bone marrow expressing green fluorescent protein (Ford et al., 2006). Interestingly, the regulatory function of the DN T cells in these mice was enhanced in comparison with DN T cells isolated from sham-thymectomized mice (Ford et al., 2006). However, the frequency and total number of DN T cells in thymectomized mice are significantly reduced compared with sham-thymectomized controls (Ford et al., 2006). These findings suggested that some DN Tregs can develop via a pathway that does not require a thymus. Furthermore, it has been reported that murine DN Tregs can be derived from $CD4^+$ T cells by stimulating the latter with allogeneic bone marrow-derived DCs in the presence of IL-2 or IL-15 (Zhang et al., 2007).

The DN T cells of *lpr* mice are believed to have arisen from a peripheral $CD8^+$ precursor (Landolfi et al., 1993; Giese and Davidson, 1994; Maldonado et al., 1995). Lymphoproliferation in *lpr* mice is greatly alleviated by neonatal thymectomy, including the accumulation of DN T cells (Steinberg et al., 1980; Wofsy et al., 1982). Moreover, human DN Tregs seem to have proliferated extensively and have a low number of T cell receptor excision circles (TRECs) in comparison with $CD4^+$ and $CD8^+$ T cells (Fischer et al., 2005), which is in keeping with a process of thymic development followed by proliferation in the periphery. Therefore, there are four possible scenarios that can be envisioned: (i) DN Tregs may develop as a separate lineage in the thymus; (ii) DN Tregs develop via a non-thymic pathway; (iii) DN Tregs may arise from a peripheral T cell under circumstances that remain to be determined; or (iv) there may be different types of DN T cells that arise from more than one of these pathways. The lack of a specific marker or transcription factor that would permit identification of DN Tregs within the broader population of DN T cells (see next section) has unfortunately greatly hampered efforts in this regard. Nevertheless, in mice at least, we favor a model in which some DN Tregs arise primarily via an extrathymic pathway but are admixed with less regulatory or non-regulatory DN T cells to a greater (*lpr*) or lesser (C57BL/6, 2C) extent.

Phenotype of DN Tregs

It is important to distinguish DN Tregs from other $CD4^-CD8^-$ populations. Importantly, mucosa-associated invariant T (MAIT) cells and invariant NK T (iNKT) cells are frequently $CD4^-CD8^-$ but only express a limited repertoire of αβ TCRs (Treiner and Lantz, 2006), in contrast to human DN Tregs, which express a polyclonal TCR repertoire (Fischer et al., 2005). Both iNKT cells and NKT cells with a variant TCR recognize lipid antigens presented by CD1d molecules (Bendelac et al., 2007). Most of the literature examining αβ DN Tregs in humans and mice has indicated that DN Tregs are restricted by MHC class I or class II molecules. However, a study by van Laethem et al. (2007) revealed that αβ DN T cells could be generated in mice deficient in CD4, CD8, and MHC class I and II molecules, and that these cells could respond to non-MHC ligands, suggesting that the coreceptors function to limit the αβ TCR repertoire to the subset capable of recognizing peptide–MHC complexes. It is possible that the DN T cells studied by van Laethem et al. (2007) are of a fundamentally different nature from DN Tregs as a result of an altered developmental pathway.

To date, no specific marker of regulatory DN T cells has been identified. Neither murine (Zhang et al., 2007; Hillhouse et al., 2010; Gao et al., 2011) nor human (Voelkl et al., 2011) DN Tregs express Foxp3. Nevertheless, specific patterns of surface maker expression by DN Tregs have been described. Cloned TCR transgenic DN Tregs express CD25, CD30, and are $CD28^{lo}$ and $CD44^-$ (Zhang et al., 2000). DN Tregs converted from $CD4^+$ T cells, in contrast, are $CD28^+$, $CD25^+$, $CD44^+$, and $CD69^+$ (Zhang et al., 2007). Accumulation of DN T cells in the *lpr* mouse appears to require the transcription factor eomesodermin (Kinjyo et al., 2010), and these cells are $B220^+$ and have a central memory phenotype, being $CD62L^+$ and $CD44^{hi}$ (Giese and Davidson, 1992). How eomesodermin expression in these DN T cells relates to their regulatory properties, if at all, is unknown. Freshly isolated human DN Tregs express CD27, Fas, perforin, and low levels of CD28; in contrast, they largely lack expression of CD25 (Fischer et al., 2005), although CD25 and CD45RO are upregulated after *in vitro* stimulation (Voelkl et al., 2011). They also appear to have undergone extensive proliferation *in vivo*, as indicated by their low content of TRECs (Fischer et al., 2005). These differences in surface marker expression may be a consequence of dissimilar activating stimuli and/or may reflect the

differing developmental histories of the DN T cells, as described above. Identification of marker(s) that can be used to identify and isolate DN Tregs will certainly accelerate our understanding of their biology.

DN Tregs in the induction of donor-specific transplantation tolerance

The ability of DN Tregs to promote tolerance to MHC-mismatched allografts in an antigen-specific manner was first described in 2000 (Zhang et al., 2000). The DN Tregs primarily examined in that study were derived from the 2C transgenic mouse, which has a TCR specific for the L^d MHC class I molecule. Infusion of L^d-expressing splenocytes resulted in the activation and expansion of DN Tregs, which were thus rendered capable of adoptively transferring tolerance to L^d, but not third party, alloantigens. Tolerance was shown to result from the specific killing of L^d-specific $CD8^+$ T cells via the Fas pathway, and the *in vitro* lysis of $CD8^+$ T cells with the same alloantigen specificity as the DN Tregs was shown to depend on acquisition of L^d molecules from donor antigen-presenting cells (APCs). DN Tregs that had acquired L^d were able to bind with and kill activated L^d-specific $CD8^+$ T cells (Zhang et al., 2000; Young et al., 2002; Chen et al., 2003b). Subsequent studies showed that DN Tregs from non-transgenic mice could also suppress syngeneic $CD4^+$ T cells and inhibit the rejection of skin (Ford et al., 2002) and islet (Zhang et al., 2007) allografts as well as cardiac xenografts (Chen et al., 2003a) in a donor-specific manner. These studies revealed an essential role for (i) previous exposure of DN Tregs to cognate alloantigen in order to exert antigen-specific suppression and (ii) DN Treg–effector T cell contact and cytotoxicity mediated by TCR–MHC and Fas ligand–Fas interactions. These key conclusions laid the foundation for further work over the last several years that has expanded our understanding of DN Tregs.

Mice with Fas (*lpr*) and Fas ligand (*gld*) mutations develop massive lymphadenopathy and accumulate a large number of DN T cells (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994). Our data revealed that C57BL/6.*lpr* DN T cells, which express high levels of FasL, were capable of antigen-specifically promoting tolerance to skin allografts by killing syngeneic C57BL/6 Fas^+ $CD4^+$ and $CD8^+$ T cells in a FasL–Fas dependent manner (Ford et al., 2002). In contrast, Hamad et al. (2003) showed that either *lpr* or *gld* DN T cells (the latter do not express functional Fas ligand) could suppress syngeneic T cells in a cell contact but Fas-independent fashion involving suppression of IL-2 and CD25 synthesis in the latter. These studies suggest that the DN compartment of *lpr* mice contains Tregs capable of antigen-specific immune regulation.

Recent studies of DN Tregs in disease models

DN Tregs, GVHD, and bone marrow allograft rejection

GVHD is a major complication of allogeneic bone marrow transplantation (BMT), and occurs when donor T cells present in the marrow graft are activated by recipient alloantigens (Shlomchik, 2007). The result is damage to target organs including the gut, liver, skin, and lungs. While allogeneic $CD4^+$ and $CD8^+$ T cells cause significant GVHD, infusion of allogeneic DN Tregs does not cause GVHD (He et al., 2007; our unpublished data). Furthermore, we have shown that DN Tregs can prevent allogeneic $CD8^+$ and $CD4^+$ T cell-induced GVHD after BMT (Young et al., 2003a; our unpublished data). In the study by Young et al. (2003a), (2C × dm2)F1 DN T cells (H-$2^{b/d}$ lacking L^d) infused into single MHC class I mismatched, lethally irradiated (C57BL/6 × BALB/c)F1 (H-$2^{b/d}$, L^{d+}) recipient mice were able to prevent GVHD caused by $CD8^+$ T cells expressing the same anti-L^d TCR. More recently, we found that *lpr* DN T cells can exert suppression of GVHD mediated by $CD4^+$ T cells in a haplotype mismatched GVHD model (our unpublished data).

Extensive T cell depletion of donor bone marrow to prevent GVHD can increase the risk of relapse of hematopoietic malignancies, because of the loss of a beneficial graft-versus-leukemia (GVL) effect (Kolb, 2008). Interestingly, we have also found, in a single MHC class I mismatched model, that DN T cells can kill allogeneic A20 lymphoma cells *in vivo* in the absence of GVHD (Young et al., 2003b). Hence, in murine models, DN Tregs appear to have the attractive property of being able to inhibit GVHD while mediating GVL. Additional studies are required to determine whether these observations can be extended to more clinically relevant settings characterized by additional MHC mismatches.

Another challenge with allogeneic BMT is the rejection of the bone marrow allograft by recipient NK cells. A recent publication by He et al. (2007) demonstrated that DN Tregs can promote marrow engraftment by inhibiting the NK-mediated rejection of donor bone marrow. This process required perforin expression by the DN Tregs, and the authors found that only DN Tregs capable of expressing this molecule could kill NK cells *in vitro*. Notably, direct NK cell killing *in vivo* by DN Tregs was not demonstrated in this study, but the effect of DN Tregs in promoting bone marrow allograft acceptance was comparable to the effect of NK cell depletion (using anti-asialo-GM1 antibody) on the same process. Adoptive transfer of DN Tregs promoted mixed chimerism in both semiallogeneic and fully MHC mismatched models of BMT, without causing GVHD. Taken together, all of these findings suggest that DN Tregs may be a useful adoptive therapy in clinical BMT.

Autoimmune diabetes

A number of studies have examined the control of autoimmunity by DN Tregs. Chiefly, these have been focused on murine models of autoimmune diabetes. Our group showed, in a TCR-transgenic system, DN Tregs specific for LCMV gp33 protein were able to prevent the development of insulitis and diabetes mellitus in mice expressing gp33 in pancreatic islets (Ford et al., 2007). In this system, diabetes is induced by $CD8^+$ T cells specific for gp33, which become activated and destroy insulin-producing cells upon injection of an agonistic anti-CD40 antibody. Adoptive transfer of pre-activated DN Tregs with the same antigen specifically and effectively prevented the onset of diabetes in animals treated with this antibody (Ford et al., 2007).

Two other groups (Dugas et al., 2010; Duncan et al., 2010; Hillhouse et al., 2010) have explored the ability of DN Tregs to inhibit the development of autoimmune diabetes. Duncan et al. (2010) observed that DN Tregs in non-obese diabetic (NOD) mice could prevent diabetes onset, but this was impaired owing to a progressive loss of DN Tregs with age. Prior to diabetes onset, young NOD mice show a transient expansion of DN T

cells followed by a progressive decline in their numbers. NOD/SCID mice, which lack autoreactive T cells, did not develop diabetes upon adoptive transfer of NOD DN T cells, indicating that this population is not diabetogenic. In contrast, transfer of NOD T cells resulted in diabetes. Cotransfer of NOD DN T cells and NOD T cells did not result in diabetes, indicating that the DN T cells could act as Tregs to inhibit insulitis caused by the NOD T cells. Two key strengths of this study were that (i) the NOD mouse is the model that most closely mimics the pathogenesis of human type 1 diabetes, and (ii) the DN Tregs studied in these animals were not expressing TCR transgenes. Hence, they provide a significant advance in our understanding of DN Treg function in a more clinically relevant context. Interestingly, however, the authors observed that the phenotype of some of the transferred DN T cells changed in the NOD/SCID recipients to become $CD4^+Foxp3^-$ IL-10-secreting cells. The alteration in phenotype might be explained by differences within the DN T cell pool of mice from the NOD background, or perhaps could reflect the influence of stimuli arising from the lymphopenic nature of the NOD/SCID host. It is an interesting phenomenon that, to our knowledge, has not been observed previously, and further investigation is required to determine whether it occurs in other settings.

Indeed, a further fascinating aspect of the study by Duncan et al. (2010) was the role played by IL-10 in the function of the transferred cells, since neutralization of this cytokine abrogated their ability to prevent diabetes onset. This observation may indicate that the NOD environment triggers a different cytokine expression profile in DN Tregs from that seen in the aforementioned transplantation settings, which have been primarily performed on the C57BL/6 background. Alternatively, NOD DN Tregs may represent a cell type distinct from 2C or C57BL/6 DN Tregs.

The Lesage group (Hillhouse et al., 2010) shed additional light on this issue when they observed that 3A9 TCR transgenic NOD DN Tregs secrete much higher levels of IL-10 than 3A9 B10.Br DN Tregs. However, in their study IL-10 promoted apoptosis of 3A9 NOD DN Tregs. DN Tregs from both backgrounds could exert similar cytotoxic function against B cells expressing the hen egg lysozyme (HEL) cognate antigen in the context of the MHC class II molecule $I\text{-}A^k$. Interestingly, cytotoxicity directed toward $CD4^+$ or $CD8^+$ T cells was not observed, leading the authors to speculate that the MHC-class II restriction imposed by the 3A9 TCR might redirect DN Treg cytotoxic function toward APCs. The authors also speculated that the secretion of IL-10 by NOD DN Tregs likely leads to an impairment in their survival *in vivo*, which ultimately leads to a reduction in their numbers and the onset of diabetes. In keeping with this explanation, we found that 2C DN Tregs are generally resistant to activation-induced cell death (Khan et al., 1999) but IL-10 increases the susceptibility of DN Tregs to this process (Marra et al., 2004). Nevertheless, it seems difficult to reconcile this possibility with the observations of Duncan et al. (2010), since the secretion of IL-10 by NOD DN Tregs was also central to their ability to suppress diabetes development. The explanation for this apparent contradiction may lie in the fact that Duncan et al. (2010) employed adoptive transfer to NOD/SCID mice, which, being lymphopenic, may have provided survival stimuli that might retard the pro-apoptotic effects of IL-10 in this setting. Alternatively, there may be a critical ambient IL-10 concentration below which diabetes suppression occurs, but above which DN Treg apoptosis ensues.

Lesage and co-workers (Dugas et al., 2010) also found that CD47 deficiency promoted diabetes onset in 3A9 TCR transgenic BALB.K mice engineered to express HEL under the control of the insulin promoter (i.e. restricted to the β cells of the islets of Langerhans). When CD47 was expressed, only about 20% of mice developed diabetes by 15 weeks of age; however, when CD47 was absent, diabetes onset was much earlier (6 weeks of age) and occurred in over 90% of mice. Intriguingly, these CD47-deficient mice showed no differences in $CD4^+$, $CD8^+$, or $Foxp3^+$ Treg cell numbers, but were markedly deficient in DN Tregs. Moreover, diabetes-resistant mouse strains had relatively higher numbers of DN Tregs in the secondary lymphoid organs, and adoptive transfer of DN Tregs from CD47-sufficient mice to CD47-deficient mice prevented diabetes onset. These findings suggest a role for CD47 in maintaining peripheral DN Treg numbers, and imply that this pathway may be crucial in preventing the onset of autoimmune diseases.

Taken together, these studies suggest that DN Tregs play an important role in preventing type 1 diabetes. Other cell populations, including $Foxp3^+$ Tregs (Green et al., 2003) and NKT cells (Hong et al., 2001) have previously been implicated in the same phenomenon, and so it remains to be seen which of these population(s) are most relevant to the control of autoimmunity and whether these Tregs interact with each other *in vivo*.

Progress in human DN Tregs

DN Tregs have been identified in humans (Fischer et al., 2005) and have been shown to have many of the same phenotypic properties as their murine counterparts, including secretion of interferon gamma (IFNγ) and the acquisition of alloantigen from donor APCs followed by recognition and killing of activated $CD8^+$ T cells specific for the same alloantigen–peptide complex (Fischer et al., 2005). In a recent study, Voelkl et al. (2011) showed that human DN Tregs activated by allogeneic APCs or beads coated with anti-CD3 and anti-CD28 antibodies were capable of suppressing the proliferation of $CD4^+$ and $CD8^+$ T cells. Interestingly, this process was independent of FasL and perforin/granzyme, and the suppression was reversible in that, upon sorting of responder T cells from the culture and restimulation with fresh APCs, vigorous proliferation ensued. Furthermore, suppression could be exerted in the presence of anti-CD3/anti-CD28-coated beads, indicating that human DN Tregs can exert suppression via an APC-independent pathway. As discussed below, this observation stands in contrast to the predominant *in vivo* suppressive mechanism described for $CD4^+Foxp3^+$ Tregs (Bluestone and Tang, 2005).

Circumstantial evidence for the role of human DN Tregs in transplantation tolerance comes from patients undergoing allogeneic BMT. Intriguingly, McIver et al. (2008) observed, in a cohort of 40 patients who had undergone allogeneic BMT, that there was an inverse correlation between the frequency of circulating DN T cells and the severity of clinical GVHD. Patients with

few DN T cells in the peripheral blood tended to have more severe illness, while those with >1% DN Tregs in the peripheral blood did not develop GVHD. Low numbers of DN T cells were associated with increased number of clonal T cell expansions in the peripheral blood, consistent with the notion that DN T cells might be acting as Tregs to inhibit the expansion of alloreactive T cells during GVHD. Although this was a study with relatively small number of patients and the findings represent an association, rather than evidence of a causative effect, it is the first report showing a relationship between DN T cells and a positive clinical outcome in human beings. It should therefore prompt further mechanistic studies on human DN Tregs.

New insights into the mechanisms of DN Treg function

The past several years have seen a remarkable increase in our knowledge of how DN Tregs exert their regulatory effects. These are described in this section and summarized in Figure 1. Notably, studies examining DN Treg function in widely differing model systems have revealed distinct phenotypic and mechanistic properties of these cells. Table 1 provides an overview of the similarities and differences between DN Tregs in these varied settings.

Acquisition of alloantigen by trogocytosis

Since the last time the role of DN Tregs in transplantation was reviewed in 2006 (Thomson et al., 2006), further studies have elucidated the mechanisms by which murine DN Tregs suppress immune responses. One major question that arose from earlier work was whether acquisition of alloantigen by DN Tregs is a relevant phenomenon *in vivo*. Trogocytosis, or the acquisition of membrane fragments from one cell by another, is a well-described phenomenon but its relevance to cellular function remains uncertain (Joly and Hudrisier, 2003; Hudrisier et al., 2009). We (Zhang et al., 2000) and others (Fischer et al., 2005) had shown that both murine and human DN Tregs can acquire allogeneic MHC-peptide through their TCR and use acquired MHC-peptide to trap and kill syngeneic $CD8^+$ T cells with the same antigen specificity *in vitro*. To test whether this might occur by trogocytosis *in vivo,* mice with the 2C TCR transgene were infused with splenocytes expressing a TCR that specifically recognizes MHC class I molecule L^d. At various time points after injection, acquisition of L^d by DN Tregs and $CD8^+$ T cells of the recipient were determined. We found that although both populations were capable of acquiring L^d molecules, $CD8^+$ T cells largely lost surface expression of L^d within 48 h, while DN Tregs maintained surface L^d expression

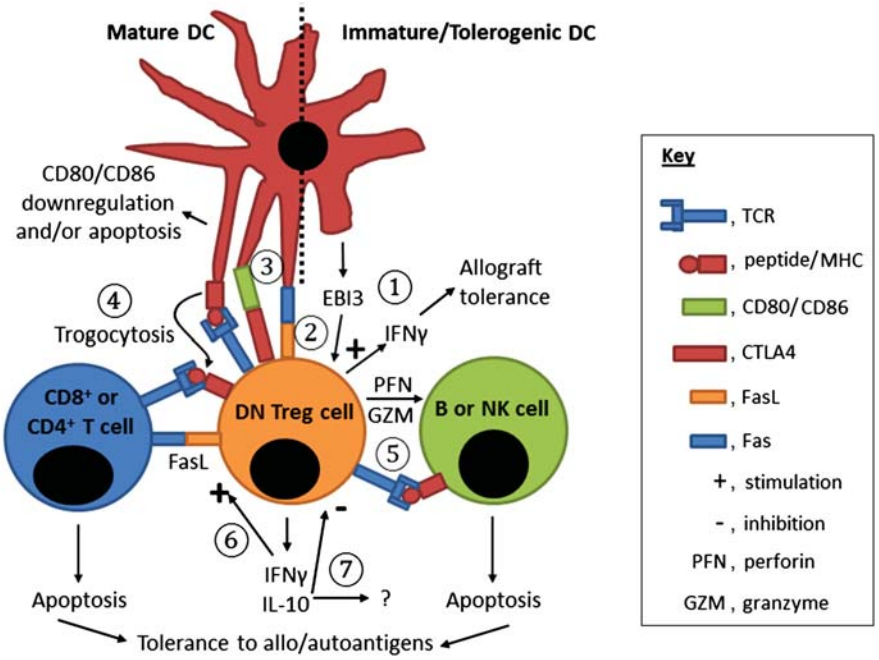

Figure 1 DN Tregs employ multiple mechanisms to exert antigen-specific control over immune responses. This figure summarizes the major mechanisms discussed in the text. Note that the different mechanisms have been demonstrated to be operative in different models and contexts. (**1**) Immature/tolerogenic DCs secreting EBI3 can elicit IFNγ from DN Tregs, resulting in allograft tolerance by an undetermined mechanism (Hill et al., 2011). (**2**) Mature or immature DCs presenting alloantigen to DN Tregs are susceptible to apoptosis via, at least partially, the Fas pathway (Gao et al., 2011). (**3**) DN Treg-expressed CTLA4 also interacts with CD80/CD86 on mature DCs causing downregulation of these molecules on the DC surface, making the DCs less capable of priming allogeneic T cell responses (Gao et al., 2011). (**4**) DN Tregs can acquire alloantigen from DCs via trogocytosis and present them to T cells bearing the same TCR specificity as the DN Tregs. The T cell is then susceptible to Fas-mediated apoptosis (Ford McIntyre et al., 2008). This mechanism has only been demonstrated for $CD8^+$ T cells, but $CD4^+$ T cells are also susceptible to killing by DN Tregs. (**5**) Similarly, B cells presenting specific antigen in the context of MHC class II are susceptible to killing by DN Tregs (Hillhouse et al., 2010), and this appears to be via a perforin/granzyme-mediated process (Ford McIntyre et al., 2010). NK cells can also be killed by DN Treg perforin/granzyme (He et al., 2007). (**6**) The ability of DN T cells from *lpr* mice to kill syngeneic Fas^+ T cells is under the control of autocrine IFNγ, secreted upon TCR stimulation (our unpublished data). (**7**) IL-10 secreted by DN Tregs can inhibit autoimmune diabetes development by an as-yet unknown mechanism (indicated by the question mark) while also limiting DN Treg expansion (Dugas et al., 2010).

Table 1 Phenotype, target cells, and mechanisms of regulation by DN Tregs in humans and mice.

Type of DN Tregs	Phenotype	Suppressive mechanism(s)	Target cell(s)	References
TCR transgenic mice				
2C (SIY/ L^d-specific)	$CD25^+CD28^-CD44^-$	Fas ligand, trogocytosis of MHC class I	Syngeneic alloreactive $CD8^+$ T cells responding to L^d	Zhang et al. (2000); Young et al. (2002)
	$CTLA4^+$	Downregulation of CD80/ CD86 expression on DCs	Allogeneic DCs expressing L^d	Gao et al. (2011)
3A9 (HEL/ I-A^k-specific)	$CD44^{lo}CD25^{lo}CD5^+CD47^+$ IL-10^+Granzyme$B^+CD107a^+$	Perforin/granzyme (?)	Syngeneic B cells presenting HEL peptide in I-A^k	Dugas et al. (2010); Hillhouse et al. (2010)
P14 (gp33/ D^b-specific)	$CD25^{lo}CD69^{lo}$	Unknown	Syngeneic $CD8^+$ T cells responding to gp33 presented by D^b in islets	Ford et al. (2007)
Inbred mice				
C57BL/6	$B220^+CD25^+$	Perforin/granzyme	Xenoreactive B cells, T cells	Zhang et al. (2006)
		Perforin/granzyme	NK cells	He et al. (2007)
	$CTLA4^+$	Downregulation of CD80/ CD86 expression on DCs	Allogeneic DCs	Gao et al. (2011)
C57BL/6, converted	$CD4^-$(downregulated)$CD28^+$ $CD44^+$ $CD25^+$ $CD69^+$	Perforin/granzyme	Alloreactive $CD4^+$ T cells	Zhang et al. (2007)
Humans				
Human	$CD25^{lo}CD28^{lo}CTLA4^-$	Perforin/granzyme (?)	Alloreactive $CD8^+$ T cells	Fischer et al. (2005)
	$CD38^{lo}$	Cell-cell contact-dependent inhibition of proliferation	Alloreactive $CD4^+$ and $CD8^+$ T cells	Voelkl et al. (2011)
Lymphoproliferative mice				
lpr	$CD44^{hi}CD62L^{+a}CD28^{+d}$Perforin^{+e}	Fas ligand[f,g]	Alloreactive Fas$^+$ $CD4^+$ and $CD8^+$ T cells[b,c]	[a]Giese and Davidson, (1992); [b]Ford et al. (2002); [c]our unpublished data; [d]Giese et al. (1993); [e]Hammond et al. (1993); [f]Chu et al. (1995); [g]Watanabe et al. (1995)
lpr/gld		Inhibition of CD25 and IL-2 synthesis in responder T cells	Alloreactive $CD4^+$ and $CD8^+$ T cells	Hamad et al. (2003)

for up to 7 days; furthermore, we found that the process of trogocytosis was antigen-specific in that DN Tregs only efficiently acquired L^d from splenocytes pulsed with the high affinity 2C TCR-binding peptide QL9, and not a non-specific peptide; finally, sorted $L^{d\ hi}$ but not $L^{d\ lo}$ DN Tregs were capable of killing activated L^d-specific, but not H-2^k-specific, $CD8^+$ T cells indicating that trogocytosis of alloantigen is required for the recognition and killing of alloreactive $CD8^+$ T cells (Ford McIntyre et al., 2008). These results demonstrate that DN Tregs with a defined alloantigen specificity promote tolerance by acquiring alloantigen from donor APCs and then binding with and killing $CD8^+$ T cells with the same alloantigen specificity. Further research is needed to determine whether trogocytosis of class I is both necessary and sufficient for cytotoxicity toward $CD8^+$ T cells, or whether, for instance, it simply confers antigen specificity on this process. Additionally, whether DN Tregs are able to acquire MHC class II molecules from APCs, and if so, the consequences of this process remain unknown. In the study by Hillhouse et al. (2010), 3A9 TCR-transgenic DN Tregs specific for an MHC class II molecule were able to kill B cells in an antigen-specific fashion but could not kill either $CD4^+$ or $CD8^+$ T cells. Hence, this study suggests that DN Tregs either cannot acquire MHC class II molecules, or that such acquisition does not confer upon them an ability to trap and kill $CD4^+$ T cells. Clearly, further work is needed in this regard.

INFγ as an immunoregulatory cytokine secreted by DN Tregs

It has been known for many years that DN Tregs in mice and humans secrete IFNγ (Zhang et al., 2000; Fischer et al., 2005), but the role of this cytokine in the regulatory function of these cells has not been explored until recently. Indeed, there is growing recognition that this prototypical inflammatory cytokine can have tolerance-promoting effects in transplantation and autoimmune disease (Brok et al., 1993; Yang et al., 1998; Gran et al., 2004; Wood et al., 2007). $Foxp3^+$ Tregs require IFNγ to control allogeneic immune responses (Sawitzki et al., 2005; Wang et al., 2006; Wei et al., 2010) and, in parallel with these observations, IFNγ is emerging as a key player in the function of DN Tregs.

Using a rat cardiac allograft model, Hill et al. (2011) recently demonstrated that adoptive transfer of allogeneic bone marrow-derived DCs in combination with the novel immunosuppressive agent LF15-0195 dosed daily for 10 days resulted in significant prolongation of allograft survival in comparison with LF15-0195 treatment alone. The spleens of rats receiving both treatments contained 3-fold higher concentrations of IFNγ compared with those of rats receiving either treatment alone. The source of IFNγ was determined to be DN Tregs which were found in close contact with the DCs in recipient spleens. DN Treg secretion of IFNγ was augmented by EBI3 secretion by the DCs, and interference with this pathway using siRNA or neutralizing antibody resulted in decreased DN Treg IFNγ secretion and augmented allograft rejection. These findings indicate that splenic DN Tregs, acting via IFNγ, play an important role in solid organ allograft tolerance. Further studies will be required (Zhang and Thomson, 2011) to determine precisely how they use IFNγ to achieve this effect.

DN T cells from *lpr* mice, which have previously been shown to regulate syngeneic T cells (Ford et al., 2002; Hamad et al., 2003),

are nevertheless traditionally considered to be pathogenic in these mice and in humans with autoimmune lymphoproliferative syndrome (ALPS). We recently found, in a semiallogeneic BMT model, that *lpr* DN T cells can suppress GVHD mediated by syngeneic T cells without causing significant illness. Moreover, this process was dependent upon autocrine IFNγ: *lpr* DN T cells that failed to secrete or respond to this cytokine were unable to upregulate surface FasL expression and could not suppress GVHD (our unpublished data). Interestingly, we also observed that IFNγ-deficient *lpr* DN T cells were more impaired in suppression of $CD4^+$ T cell proliferation *in vitro* than IFNγ receptor-deficient *lpr* DN T cells, suggesting that there may be additional regulatory effects of DN T cell-secreted IFNγ on other cells.

Interaction of DN Tregs with APCs

We have also recently shown that DN Tregs can cause downregulation of the costimulatory molecules CD80 and CD86 on DCs (Gao et al., 2011). Using 2C TCR transgenic DN Tregs, we observed that L^d-expressing lipopolysaccharide (LPS)-matured DCs that had been exposed to DN Tregs were unable to prime the proliferation of $CD8^+$ T cells. This was due to a decrease in CD80 and CD86 expression on the DC surface, and this process required cytotoxic T lymphocyte antigen 4 (CTLA4) expression by DN Tregs, since $CTLA4^{-/-}$ DN Tregs were unable to downregulate the expression of these molecules on the DC surface. Furthermore, we found that alloantigen-expressing LPS-mature and immature DCs were susceptible to killing by DN Tregs, as were syngeneic DCs that had been pulsed with the 2C-specific peptide SIY (which can also be presented by the syngeneic MHC class I molecule K^b). Additional studies by us (Ford McIntyre et al., 2010) and others (Ma et al., 2008; Hillhouse et al., 2010) have demonstrated that DN Tregs were able to kill both allogeneic and syngeneic B cells via a perforin-dependent pathway. These findings indicate that DN Tregs can suppress immune responses by exerting an effect on APCs, and hence significantly add to the repertoire of mechanisms by which DN Tregs can modulate allograft rejection.

Another aspect of the relationship between DN Tregs and APCs is the finding that DN Tregs acquire regulatory potency through prior activation by exposure to cognate antigen. For example, in the case of DN Tregs expressing the 2C TCR, donor lymphocyte infusion (DLI) of L^{d+} splenocytes can facilitate acceptance of L^{d+} skin and cardiac allografts (Zhang et al., 2000; Young et al., 2002). We have also extended this observation to C57BL/6 DN Tregs accepting Lewis rat cardiac xenografts (Chen et al., 2003a). In that study, DLI with rat splenocytes combined with a short course of *in vivo* depletion of $CD4^+$ T cells led to permanent cardiac xenograft survival, whereas either treatment alone was insufficient. Subsequent studies showed that DLI significantly increased both total number of recipient DN Tregs and their suppressive function (Chen et al., 2005, 2007). Furthermore, DLI-induced DN Tregs could suppress proliferation of xenoreactive $CD4^+$ and $CD8^+$ T cells and inhibit DC function (Chen et al., 2007). In addition, xenoantigen-activated DN Tregs could kill xenoreactive B and T cells (Zhang et al., 2006). We have also observed that a DLI of allogeneic splenocytes resulted in DN Treg expansion in *lpr* mice. These *lpr* DN Tregs, when adoptively transferred to B6 mice, could enhance the survival of skin grafts from the same donor strain as the DLI (but not from a third party) (Ford et al., 2002). In these systems, exposure of the DN Tregs to allo- and xenoantigens resulted in the expansion of DN Tregs in the recipient, which were rendered capable of suppress other T cells responding to the same foreign antigens. Exposure to activating antigens either *in vitro* or *in vivo* also appears to be required for 3A9 (Hillhouse et al., 2010) and P14 (Ford et al., 2007) TCR transgenic DN Tregs to exert regulatory functions toward their target cells.

The importance of prior activation has also been shown for human DN Tregs by the group of Mackensen and colleagues (Fischer et al., 2005). They have shown that freshly isolated DN Tregs can acquire antigens presented by MHC class I molecules via trogocytosis from APCs, and that this process confers on them an ability to suppress autologous $CD8^+$ T cells responding to the same antigen; DN Tregs not primed in this fashion were unable to exert suppression. Similarly, alloantigen-primed DN Tregs, but not resting DN Tregs, could suppress the proliferation of autologous $CD4^+$ and $CD8^+$ T cells responding to the same alloantigens (Voelkl et al., 2011). Hence, it appears that DN Tregs must be primed by specific antigen in order to exert antigen-specific regulatory phenomena. This property makes them an attractive target for manipulation in both transplantation and autoimmune disease.

Relationship of DN Tregs to $CD4^+CD25^+Foxp3^+$ Tregs

$CD4^+$ T cells expressing $Foxp3^+$ are the best-characterized immunoregulatory population in mice and humans, having been shown to inhibit T cell-mediated autoimmune, alloimmune, antitumour, and other responses (Shevach, 2009; Sakaguchi et al., 2010). Neither rodent (Zhang et al., 2007; Hillhouse et al., 2010) nor human DN Tregs (Voelkl et al., 2011) express Foxp3. In addition to $Foxp3^+$ Tregs and DN Tregs, other cells with regulatory properties have been demonstrated, including Tr1 cells, $CD8^+$ Tregs, and others (Shevach, 2006). An important question is what physiologic roles these different populations have *in vivo* during disease pathogenesis.

Arguably, $Foxp3^+$ Tregs have the best-documented physiologic function, as their absence or dysfunction as a result of Foxp3 deficiency (*scurfy* mice, humans with immune dysregulation, polyendocrinopathy X-linked, or IPEX syndrome) or CTLA4 deficiency results in lethal lymphoproliferation and autoimmunity (Fontenot et al., 2003; Wing et al., 2008). The absence of similar mutations that might specifically impair or delete other identified Treg subsets currently precludes making any firm conclusions about their *in vivo* physiologic roles.

Another important issue is whether there may be functional interactions between Treg subsets *in vivo*. Zhang et al. (2011) recently showed that, compared with a 9-day course of rapamycin alone, adoptive transfer of DN Tregs plus rapamycin in a fully MHC mismatched model of cardiac transplantation extended allograft survival time from 39 to 101 days. Interestingly, this improvement in survival was associated with the accumulation of $CD4^+CD25^+Foxp3^+$ Tregs in the recipient spleen, and depletion of these cells with an anti-CD25 antibody resulted in a partial abrogation of the survival benefit provided by DN Tregs. A second adoptive transfer of DN Tregs also further increased the number of $Foxp3^+$ Tregs. This preliminary study hence suggests that, at

least in this model, Treg subsets may collaborate *in vivo* to constrain immune responses. The mechanism by which Foxp3$^+$ Tregs accumulated in this model was not clear, and specifically whether the phenomenon was mediated by direct or indirect interactions between DN Tregs and Foxp3$^+$ Tregs, was not determined. Clearly, further studies are required to explore the nature of this relationship and its importance.

A study by Zheng's group showed that CD4$^+$ T cells incubated with allogeneic DCs could, after several rounds of cell division, downregulate CD4 and become DN Tregs (Zhang et al., 2007). DN Tregs sorted from these cultures were able to suppress syngeneic T cell responses to the same alloantigen. Interestingly, both conventional CD4$^+$ T cells and CD4$^+$Foxp3$^+$ Tregs could be converted to DN Tregs in this fashion, but Foxp3$^+$ cells became Foxp3$^-$ during the conversion process. These findings are especially interesting because they suggest that DN Tregs might be derived *ex vivo* from a patient's own CD4$^+$ T cells. While provocative, they require further exploration.

In some respects, DN Tregs have an intriguing resemblance to CD4$^+$Foxp3$^+$ Tregs. Both of them (i) can play an important role in controlling the development of autoimmune type I diabetes (Green et al., 2003; Ford et al., 2007; Dugas et al., 2010; Duncan et al., 2010), inhibit GVHD (Hoffmann et al., 2002; Young et al., 2003a; our unpublished data), and prevent allograft rejection (Zhang et al., 2000; Lee et al., 2005; Safinia et al., 2010); (ii) do not produce IL-2, but require IL-2 and TCR stimulation for their activation and function (Zhang et al., 2000; Sakaguchi et al., 2008); (iii) can suppress syngeneic T cell proliferation *in vitro* and *in vivo* (Zhang et al., 2000; Young and Zhang, 2002; Ford McIntyre et al., 2008; Sakaguchi et al., 2008; Shevach, 2009); (iv) suppress in a cell contact-dependent fashion (Zhang et al., 2000; Sakaguchi et al., 2008); (v) express high levels of CTLA4 (Wing et al., 2008; Gao et al., 2011), at least in the case of murine DN Tregs; (vi) can regulate costimulatory molecule expression on DCs in a CTLA4-dependent manner (Wing et al., 2008; Gao et al., 2011); (vii) can kill Ag-loaded DCs through the Fas/FasL pathway (Gorbachev and Fairchild, 2010; Gao et al., 2011); and (ix) at least in the context of regulating alloimmune responses, can employ IFNγ in an autocrine fashion (Wei et al., 2010; our unpublished data). On the other hand, there are also differences between CD4$^+$Foxp3$^+$ Tregs and DN Tregs. Unlike CD4$^+$CD25$^+$ Tregs, neither rodent nor human DN Tregs express Foxp3 (Zhang et al., 2007; Hillhouse et al., 2010; Gao et al., 2011; Voelkl et al., 2011). The suppression mediated by CD4$^+$Foxp3$^+$ Tregs can be either Ag-specific or Ag-non-specific. However, so far all studies on mouse, rat and human DN Tregs have shown that they suppress immune responses in an antigen-specific fashion both *in vitro* and *in vivo*. CD4$^+$CD25$^+$ Tregs have been shown to be able to restrain the immature state of iDCs, but are incapable of suppressing LPS-induced DC maturation (Yamazaki et al., 2003; Fehervari and Sakaguchi, 2004; Onishi et al., 2008). We showed that DN Tregs are potent suppressors of LPS-induced mDCs, through down-regulation of their costimulatory molecule expression as well as direct cytolysis (Gao et al., 2011). DN Tregs are able to kill antigen-expressing, immature and mature DCs, B cells, as well as CD4$^+$ and CD8$^+$ T cells that are activated by the same antigen as the one used to activate DN Tregs. These features make DN Tregs highly potent and antigen-specific suppressor cells. Specifically, the use of trogocytosis to regulate T cells with the same antigen specificity is a unique feature of DN Tregs that makes them an attractive potential cellular therapy for transplantation and autoimmunity. Likewise, an understanding of how DN Tregs use IFNγ to regulate immune responses has also enhanced our knowledge of how these cells function *in vivo*.

Major challenges in DN Treg studies

Significant challenges lie ahead. One of the major obstacles has been the lack of a lineage-specific marker capable of identifying DN T cells with regulatory function, making it difficult to study this population of Tregs. We and others (D'Acquisto and Crompton, 2011) have pointed out that non-NK cells expressing the phenotype αβ-TCR$^+$CD4$^-$CD8$^-$ are likely to contain more than one functional subset. It is possible that only some of these cells are Tregs while others are proinflammatory, in much the same way that CD4$^+$CD25$^+$ cells contain both activated effector T cells and Foxp3$^+$ Tregs. The discovery of a specific marker or transcription factor that identifies DN Tregs within the broader DN T cell population would significantly advance this field.

The origin and developmental regulation of DN Tregs are largely unknown. It is unclear whether DN Tregs arise from a peripheral mature T lymphocyte precursor or they represent a completely thymus-independent lineage. In any case, we and others (Bleesing et al., 2002) speculate that the massive accumulation of DN T cells in the *lpr* mouse probably represents a different process and that true DN Tregs develop in a distinct manner. Another possibility is that, as has been observed for Foxp3$^+$ Tregs (Zhou et al., 2009; d'Hennezel et al., 2011), significant phenotypic plasticity may exist within the DN Treg subset and not all DN T cells may be regulatory in all contexts. These hypotheses await further experimentation.

It is currently recognized that in order for Foxp3$^+$ Tregs to be used as an adoptive cellular therapy for patients undergoing transplantation or with autoimmune diseases, more robust expansion methods will be required, and retention of a regulatory phenotype *in vivo* will need to be assured (Wieckiewicz et al., 2010). For DN Tregs, the challenges are similar, and even greater. In addition to defining DN Treg-specific markers as described above, more clinically relevant animal models need to be developed, in which the function of DN Tregs and their phenotypes are carefully monitored *in vivo* and *ex vivo*. In parallel, expansion methods capable of generating large numbers of autologous DN Tregs will be required for further preclinical development. Despite these challenges, we believe that the potential of DN Tregs deserves further exploration because of their unique ability to confer antigen-specific tolerance, which suggests a potentially greater degree of safety than might be possible with adoptive Foxp3$^+$ Treg therapy.

Conclusions

DN Tregs are unique antigen-specific regulatory cells that can exert control over allograft rejection, GVHD, and autoimmune diseases. Although originally characterized in TCR-transgenic mice, emerging evidence suggests that non-transgenic DN Tregs and

human DN Tregs have similar regulatory properties. Furthermore, DN Tregs can employ different mechanisms to regulate immune responses directed against allografts and autoantigens in both humans and rodents (Figure 1). Although previous studies of DN Tregs were focused on their role in suppressing T cells, recent studies indicate that DN Tregs are also potent regulators of B cells, DCs, and NK cells. Specifically, the use of trogocytosis to regulate T cells with the same antigen specificity is a unique feature of DN Tregs that makes them an attractive potential cellular therapy for transplantation and autoimmunity. Likewise, an understanding of how DN Tregs use IFNγ to regulate immune responses has also enhanced our knowledge of how these cells function *in vivo*. Hopefully, this property will inspire ongoing investigation in the coming years.

Funding

Research in our laboratory has been supported by Canadian Institutes of Health Research Grant (#14431) and Canadian Cancer Society to L.Z. S.C.J. received salary support from a Terry Fox Foundation/National Cancer Institute of Canada Clinical Research Fellowship (#18014) and the Clinician-Scientist Training Program, Department of Medicine, University of Toronto. L.Z. is the Maria H. Bacardi Chair in Transplantation at the University Health Network.

References

Anderson, G., and Jenkinson, E.J. (2001). Lymphostromal interactions in thymic development and function. Nat. Rev. Immunol. *1*, 31–40.

Bendelac, A., Savage, P.B., and Teyton, L. (2007). The biology of NKT cells. Annu. Rev. Immunol. *25*, 297–336.

Bleesing, J.J., Brown, M.R., Novicio, C., et al. (2002). A composite picture of TcR alpha/beta$^+$CD4$^-$CD8$^-$T cells (alpha/beta-DNTCs) in humans with autoimmune lymphoproliferative syndrome. Clin. Immunol. *104*, 21–30.

Bluestone, J.A., and Tang, Q. (2005). How do CD4$^+$CD25$^+$ regulatory T cells control autoimmunity? Curr. Opin. Immunol. *17*, 638–642.

Brok, H.P., Heidt, P.J., van der Meide, P.H., et al. (1993). Interferon-gamma prevents graft-versus-host disease after allogeneic bone marrow transplantation in mice. J. Immunol. *151*, 6451–6459.

Bruley-Rosset, M., Miconnet, I., Canon, C., et al. (1990). Mlsa generated suppressor cells. I. Suppression is mediated by double-negative (CD3$^+$CD5$^+$CD4$^-$CD8$^-$) alpha/beta T cell receptor-bearing cells. J. Immunol. *145*, 4046–4052.

Chen, W., Ford, M.S., Young, K.J., et al. (2003a). Role of double-negative regulatory T cells in long-term cardiac xenograft survival. J. Immunol. *170*, 1846–1853.

Chen, W., Ford, M.S., Young, K.J., et al. (2003b). Infusion of in vitro-generated DN T regulatory cells induces permanent cardiac allograft survival in mice. Transplant. Proc. *35*, 2479–2480.

Chen, W., Zhou, D., Torrealba, J.R., et al. (2005). Donor lymphocyte infusion induces long-term donor-specific cardiac xenograft survival through activation of recipient double-negative regulatory T cells. J. Immunol. *175*, 3409–3416.

Chen, W., Diao, J., Stepkowski, S.M., et al. (2007). Both infiltrating regulatory T cells and insufficient antigen presentation are involved in long-term cardiac xenograft survival. J. Immunol. *179*, 1542–1548.

Chu, J.L., Ramos, P., Rosendorff, A., et al. (1995). Massive upregulation of the Fas ligand in lpr and gld mice: implications for Fas regulation and the graft-versus-host disease-like wasting syndrome. J. Exp. Med. *181*, 393–398.

Cowley, S.C., Hamilton, E., Frelinger, J.A., et al. (2005). CD4$^-$CD8$^-$ T cells control intracellular bacterial infections both in vitro and in vivo. J. Exp. Med. *202*, 309–319.

Cowley, S.C., Meierovics, A.I., Frelinger, J.A., et al. (2010). Lung CD4$^-$CD8$^-$ double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with Francisella tularensis live vaccine strain. J. Immunol. *184*, 5791–5801.

Crispin, J.C., and Tsokos, G.C. (2009). Human TCR-alpha beta$^+$CD4$^-$CD8$^-$ T cells can derive from CD8$^+$ T cells and display an inflammatory effector phenotype. J. Immunol. *183*, 4675–4681.

Crispin, J.C., Oukka, M., Bayliss, G., et al. (2008). Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. J. Immunol. *181*, 8761–8766.

D'Acquisto, F., and Crompton, T. (2011). CD3$^+$CD4$^-$CD8$^-$ (double negative) T cells: saviours or villains of the immune response? Biochem. Pharmacol. *82*, 333–340.

d'Hennezel, E., Yurchenko, E., Sgouroudis, E., et al. (2011). Single-cell analysis of the human T regulatory population uncovers functional heterogeneity and instability within FOXP3$^+$ cells. J. Immunol. *186*, 6788–6797.

Dugas, V., Beauchamp, C., Chabot-Roy, G., et al. (2010). Implication of the CD47 pathway in autoimmune diabetes. J. Autoimmun. *35*, 23–32.

Duncan, B., Nazarov-Stoica, C., Surls, J., et al. (2010). Double negative (CD3+4−8−) TCR alphabeta splenic cells from young NOD mice provide long-lasting protection against type 1 diabetes. PLoS One *5*, e11427.

Egerton, M., and Scollay, R. (1990). Intrathymic selection of murine TCR alpha beta$^+$CD4$^-$CD8$^-$ thymocytes. Int. Immunol. *2*, 157–163.

Fehervari, Z., and Sakaguchi, S. (2004). Control of Foxp3$^+$CD25$^+$CD4$^+$ regulatory cell activation and function by dendritic cells. Int. Immunol. *16*, 1769–1780.

Fischer, K., Voelkl, S., Heymann, J., et al. (2005). Isolation and characterization of human antigen-specific TCRalphabeta$^+$CD4$^-$CD8$^-$ double-negative regulatory T cells. Blood *105*, 2828–2835.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4$^+$CD25$^+$ regulatory T cells. Nat. Immunol. *4*, 330–336.

Ford, M.S., Young, K.J., Zhang, Z., et al. (2002). The immune regulatory function of lymphoproliferative double negative T cells in vitro and in vivo. J. Exp. Med. *196*, 261–267.

Ford, M.S., Zhang, Z.X., Chen, W., et al. (2006). Double-negative T regulatory cells can develop outside the thymus and do not mature from CD8$^+$ T cell precursors. J. Immunol. *177*, 2803–2809.

Ford, M.S., Chen, W., Wong, S., et al. (2007). Peptide-activated double-negative T cells can prevent autoimmune type-1 diabetes development. Eur. J. Immunol. *37*, 2234–2241.

Ford McIntyre, M.S., Young, K.J., Gao, J., et al. (2008). Cutting edge: in vivo trogocytosis as a mechanism of double negative regulatory T cell-mediated antigen-specific suppression. J. Immunol. *181*, 2271–2275.

Ford McIntyre, M.S., Gao, J.F., Li, X., et al. (2010). Consequences of double negative regulatory T cell and antigen presenting cell interaction on immune response suppression. Int. Immunopharmacol. *11*, 597–603.

Gao, J.F., McIntyre, M.S., Juvet, S.C., et al. (2011). Regulation of antigen-expressing dendritic cells by double negative regulatory T cells. Eur. J. Immunol. *41*, 2699–2708.

Giese, T., and Davidson, W.F. (1992). Evidence for early onset, polyclonal activation of T cell subsets in mice homozygous for lpr. J. Immunol. *149*, 3097–3106.

Giese, T., and Davidson, W.F. (1994). Chronic treatment of C3H-lpr/lpr and C3H-gld/gld mice with anti-CD8 monoclonal antibody prevents the accumulation of double negative T cells but not autoantibody production. J. Immunol. *152*, 2000–2010.

Giese, T., Allison, J.P., and Davidson, W.F. (1993). Functionally anergic lpr and gld B220$^+$ T cell receptor (TCR)-alpha/beta$^+$ double-negative T cells express CD28 and respond to costimulation with phorbol myristate acetate and antibodies to CD28 and the TCR. J. Immunol. *151*, 597–609.

Gorbachev, A.V., and Fairchild, R.L. (2010). CD4$^+$CD25$^+$ regulatory T cells utilize FasL as a mechanism to restrict DC priming functions in cutaneous immune responses. Eur. J. Immunol. *40*, 2006–2015.

Gran, B., Chu, N., Zhang, G.X., et al. (2004). Early administration of IL-12 suppresses EAE through induction of interferon-gamma. J. Neuroimmunol. *156*, 123–131.

Green, E.A., Gorelik, L., McGregor, C.M., et al. (2003). CD4$^+$CD25$^+$ T regulatory cells control anti-islet CD8$^+$ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. Proc. Natl Acad. Sci. USA *100*, 10878–10883.

Halder, R.C., Kawamura, T., Bannai, M., et al. (2001). Intensive generation of NK1.1-extrathymic T cells in the liver by injection of bone marrow cells isolated from mice with a mutation of polymorphic major histocompatibility complex antigens. Immunology *102*, 450–459.

Hamad, A.R., Mohamood, A.S., Trujillo, C.J., et al. (2003). B220$^+$ double-negative T cells suppress polyclonal T cell activation by a Fas-independent mechanism that involves inhibition of IL-2 production. J. Immunol. *171*, 2421–2426.

Hammond, D.M., Nagarkatti, P.S., Gote, L.R., et al. (1993). Double-negative T cells from MRL-lpr/lpr mice mediate cytolytic activity when triggered through adhesion molecules and constitutively express perforin gene. J. Exp. Med. *178*, 2225–2230.

He, K.M., Ma, Y., Wang, S., et al. (2007). Donor double-negative Treg promote allogeneic mixed chimerism and tolerance. Eur. J. Immunol. *37*, 3455–3466.

Hill, M., Thebault, P., Segovia, M., et al. (2011). Cell therapy with autologous tolerogenic dendritic cells induces allograft tolerance through interferon-gamma and epstein-barr virus-induced gene 3. Am. J. Transplant. *11*, 2036–2045.

Hillhouse, E.E., Beauchamp, C., Chabot-Roy, G., et al. (2010). Interleukin-10 limits the expansion of immunoregulatory CD4$^-$CD8$^-$ T cells in autoimmune-prone non-obese diabetic mice. Immunol. Cell Biol. *88*, 771–780.

Hoffmann, P., Ermann, J., Edinger, M., et al. (2002). Donor-type CD4$^+$CD25$^+$ regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. J. Exp. Med. *196*, 389–399.

Hong, S., Wilson, M.T., Serizawa, I., et al. (2001). The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat. Med. *7*, 1052–1056.

Hudrisier, D., Clemenceau, B., Balor, S., et al. (2009). Ligand binding but undetected functional response of FcR after their capture by T cells via trogocytosis. J. Immunol. *183*, 6102–6113.

Johansson, M., and Lycke, N. (2003). A unique population of extrathymically derived alpha beta TCR$^+$CD4$^-$CD8$^-$ T cells with regulatory functions dominates the mouse female genital tract. J. Immunol. *170*, 1659–1666.

Joly, E., and Hudrisier, D. (2003). What is trogocytosis and what is its purpose? Nat. Immunol. *4*, 815.

Khan, Q., Penninger, J.M., Yang, L., et al. (1999). Regulation of apoptosis in mature alphabeta$^+$CD4$^-$CD8$^-$ antigen-specific suppressor T cell clones. J. Immunol. *162*, 5860–5867.

Kinjyo, I., Gordon, S.M., Intlekofer, A.M., et al. (2010). Cutting edge: Lymphoproliferation caused by Fas deficiency is dependent on the transcription factor eomesodermin. J. Immunol. *185*, 7151–7155.

Kolb, H.J. (2008). Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood *112*, 4371–4383.

Landolfi, M.M., van Houten, N., Russell, J.Q., et al. (1993). CD2$^-$CD4$^-$CD8$^-$ lymph node T lymphocytes in MRL lpr/lpr mice are derived from a CD2$^+$CD4$^+$CD8$^+$ thymic precursor. J. Immunol. *151*, 1086–1096.

Lee, B.P., Mansfield, E., Hsieh, S.C., et al. (2005). Expression profiling of murine double-negative regulatory T cells suggest mechanisms for prolonged cardiac allograft survival. J. Immunol. *174*, 4535–4544.

Ma, Y., He, K.M., Garcia, B., et al. (2008). Adoptive transfer of double negative T regulatory cells induces B-cell death in vivo and alters rejection pattern of rat-to-mouse heart transplantation. Xenotransplantation *15*, 56–63.

Maldonado, M.A., Eisenberg, R.A., Roper, E., et al. (1995). Greatly reduced lymphoproliferation in lpr mice lacking major histocompatibility complex class I. J. Exp. Med. *181*, 641–648.

Marra, L.E., Zhang, Z.X., Joe, B., et al. (2004). IL-10 induces regulatory T cell apoptosis by up-regulation of the membrane form of TNF-alpha. J. Immunol. *172*, 1028–1035.

McIver, Z., Serio, B., Dunbar, A., et al. (2008). Double-negative regulatory T cells induce allotolerance when expanded after allogeneic haematopoietic stem cell transplantation. Br. J. Haematol. *141*, 170–178.

Mehal, W.Z., and Crispe, I.N. (1998). TCR ligation on CD8$^+$ T cells creates double-negative cells in vivo. J Immunol. *161*, 1686–1693.

Merino, R., Fossati, L., Iwamoto, M., et al. (1995). Effect of long-term anti-CD4 or anti-CD8 treatment on the development of lpr CD4$^-$ CD8$^-$ double negative T cells and of the autoimmune syndrome in MRL-lpr/lpr mice. J. Autoimmun. *8*, 33–45.

Mixter, P.F., Russell, J.Q., Morrissette, G.J., et al. (1999). A model for the origin of TCR-alphabeta$^+$ CD4$^-$CD8$^-$ B220$^+$ cells based on high affinity TCR signals. J. Immunol. *162*, 5747–5756.

Onishi, Y., Fehervari, Z., Yamaguchi, T., et al. (2008). Foxp3$^+$ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc. Natl Acad. Sci. USA *105*, 10113–10118.

Priatel, J.J., Utting, O., and Teh, H.S. (2001). TCR/self-antigen interactions drive double-negative T cell peripheral expansion and differentiation into suppressor cells. J. Immunol. *167*, 6188–6194.

Riol-Blanco, L., Lazarevic, V., Awasthi, A., et al. (2010). IL-23 receptor regulates unconventional IL-17-producing T cells that control bacterial infections. J. Immunol. *184*, 1710–1720.

Safinia, N., Sagoo, P., Lechler, R., et al. (2010). Adoptive regulatory T cell therapy: challenges in clinical transplantation. Curr. Opin. Organ. Transplant. *15*, 427–434.

Sakaguchi, S., Yamaguchi, T., Nomura, T., et al. (2008). Regulatory T cells and immune tolerance. Cell *133*, 775–787.

Sakaguchi, S., Miyara, M., Costantino, C.M., et al. (2010). FOXP3$^+$ regulatory T cells in the human immune system. Nat. Rev. Immunol. *10*, 490–500.

Sawitzki, B., Kingsley, C.I., Oliveira, V., et al. (2005). IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. J. Exp. Med. *201*, 1925–1935.

Schonrich, G., Kalinke, U., Momburg, F., et al. (1991). Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. Cell *65*, 293–304.

Shevach, E.M. (2006). From vanilla to 28 flavors: multiple varieties of T regulatory cells. Immunity *25*, 195–201.

Shevach, E.M. (2009). Mechanisms of foxp3$^+$ T regulatory cell-mediated suppression. Immunity *30*, 636–645.

Shlomchik, W.D. (2007). Graft-versus-host disease. Nat. Rev. Immunol. *7*, 340–352.

Steinberg, A.D., Roths, J.B., Murphy, E.D., et al. (1980). Effects of thymectomy or androgen administration upon the autoimmune disease of MRL/Mp-lpr/lpr mice. J. Immunol. *125*, 871–873.

Strober, S., Dejbachsh-Jones, S., van Vlasselaer, P., et al. (1989). Cloned natural suppressor cell lines express the CD3$^+$CD4$^-$CD8$^-$ surface phenotype and the alpha, beta heterodimer of the T cell antigen receptor. J. Immunol. *143*, 1118–1122.

Takahama, Y., Kosugi, A., and Singer, A. (1991). Phenotype, ontogeny, and repertoire of CD4$^-$CD8$^-$ T cell receptor alphabeta$^+$ thymocytes. Variable influence of self-antigens on T cell receptor V beta usage. J. Immunol. *146*, 1134–1141.

Takahashi, T., Tanaka, M., Brannan, C.I., et al. (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell *76*, 969–976.

Thomson, C.W., Lee, B.P., and Zhang, L. (2006). Double-negative regulatory T cells: non-conventional regulators. Immunol. Res. *35*, 163–178.

Treiner, E., and Lantz, O. (2006). CD1d- and MR1-restricted invariant T cells: of mice and men. Curr. Opin. Immunol. *18*, 519–526.

van Laethem, F., Sarafova, S.D., Park, J.H., et al. (2007). Deletion of CD4 and CD8 coreceptors permits generation of alphabetaT cells that recognize antigens independently of the MHC. Immunity *27*, 735–750.

Viret, C., and Janeway, C.A., Jr. (2003). Self-specific MHC class II-restricted CD4$^-$CD8$^-$ T cells that escape deletion and lack regulatory activity. J. Immunol. *170*, 201–209.

Voelkl, S., Gary, R., and Mackensen, A. (2011). Characterization of the immunoregulatory function of human TCR-alphabeta$^+$CD4$^-$CD8$^-$ double-negative T cells. Eur. J. Immunol. *41*, 739–748.

Wang, Z., Hong, J., Sun, W., et al. (2006). Role of IFN-gamma in induction of Foxp3 and conversion of CD4$^+$CD25$^-$ T cells to CD4$^+$ Tregs. J. Clin. Invest. *116*, 2434–2441.

Watanabe, D., Suda, T., Hashimoto, H., et al. (1995). Constitutive activation of the Fas ligand gene in mouse lymphoproliferative disorders. EMBO J. *14*, 12–18.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., et al. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature *356*, 314–317.

Wei, B., Baker, S., Wieckiewicz, J., et al. (2010). IFN-gamma triggered STAT1-PKB/AKT signalling pathway influences the function of alloantigen

reactive regulatory T cells. Am. J. Transplant. *10*, 69–80.

Wieckiewicz, J., Goto, R., and Wood, K.J. (2010). T regulatory cells and the control of alloimmunity: from characterisation to clinical application. Curr. Opin. Immunol. *22*, 662–668.

Wing, K., Onishi, Y., Prieto-Martin, P., et al. (2008). CTLA-4 control over $Foxp3^+$ regulatory T cell function. Science *322*, 271–275.

Wofsy, D., Ledbetter, J.A., Roubinian, J.R., et al. (1982). Thymic influences on autoimmunity in MRL-lpr mice. Scand J. Immunol. *16*, 51–58.

Wood, K.J., Feng, G., Wei, B., et al. (2007). Interferon gamma: friend or foe? Transplantation *84*, S4–S5.

Yamazaki, S., Iyoda, T., Tarbell, K., et al. (2003). Direct expansion of functional $CD25^+CD4^+$ regulatory T cells by antigen-processing dendritic cells. J. Exp. Med. *198*, 235–247.

Yang, Y.G., Dey, B.R., Sergio, J.J., et al. (1998). Donor-derived interferon gamma is required for inhibition of acute graft-versus-host disease by interleukin 12. J. Clin. Invest. *102*, 2126–2135.

Young, K.J., and Zhang, L. (2002). The nature and mechanisms of DN regulatory T-cell mediated suppression. Hum. Immunol. *63*, 926–934.

Young, K.J., Yang, L., Phillips, M.J., et al. (2002). Donor-lymphocyte infusion induces transplantation tolerance by activating systemic and graft-infiltrating double-negative regulatory T cells. Blood *100*, 3408–3414.

Young, K.J., DuTemple, B., Phillips, M.J., et al. (2003a). Inhibition of graft-versus-host disease by double-negative regulatory T cells. J. Immunol. *171*, 134–141.

Young, K.J., Kay, L.S., Phillips, M.J., et al. (2003b). Antitumor activity mediated by double-negative T cells. Cancer Res. *63*, 8014–8021.

Zhang, L., and Thomson, A.W. (2011). New partners for tolerogenic dendritic cells. Am. J. Transplant. *11*, 2003–2004.

Zhang, Z.X., Ma, Y., Wang, H., et al. (2006). Double-negative T cells, activated by xenoantigen, lyse autologous B and T cells using a perforin/granzyme-dependent, Fas-Fas ligand-independent pathway. J. Immunol. *177*, 6920–6929.

Zhang, D., Yang, W., Degauque, N., et al. (2007). New differentiation pathway for double-negative regulatory T cells that regulates the magnitude of immune responses. Blood *109*, 4071–4079.

Zhang, Z.X., Yang, L., Young, K.J., et al. (2000). Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. Nat. Med. *6*, 782–789.

Zhou, X., Bailey-Bucktrout, S., Jeker, L.T., et al. (2009). Plasticity of $CD4^+FoxP3^+$ T cells. Curr. Opin. Immunol. *21*, 281–285.

Zhang, Z.X., Lian, D., Huang, X., et al. (2011). Adoptive transfer of DNT cells induces long-term cardiac allograft survival and augments recipient $CD4^+Foxp3^+$ Treg cell accumulation. Transpl. Immunol. *24*, 119–126.