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Regulation of antigen-expressing dendritic cells by double negative regulatory T cells

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TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$NK1.1$^-$ double negative (DN) Tregs comprise 1–3% of peripheral T lymphocytes in mice and humans. It has been demonstrated that DN Tregs can suppress allo-, xeno- and auto-immune responses in an Ag-specific fashion. However, the mechanisms by which DN Tregs regulate immune responses remain elusive. Whether DN Tregs can regulate DCs has not been investigated previously. In this study, we demonstrate that DN Tregs express a high level of CTLA4 and are able to down-regulate costimulatory molecules CD80 and CD86 expressed on Ag-expressing mature DCs (mDCs). DN Tregs from CTLA4 KO mice were not able to downregulate CD80 and CD86 expression, indicating that CTLA4 is critical for DN Treg-mediated downregulation of costimulatory molecule expression on Ag-expressing mature DCs. Furthermore, DN Tregs could kill both immature and mature allogeneic DCs, as well as Ag-loaded syngeneic DCs, in an Ag-specific manner in vitro and in vivo, mainly through the Fas-FasL pathway. These data demonstrate, for the first time, that DN Tregs are potent regulators of DCs and may have the potential to be developed as a novel immune suppression treatment.

Key words: CTLA4 · DCs · Double negative regulatory T cells · Fas-FasL pathway

Supporting Information available online

Introduction

Tregs play an important role in a variety of diseases [1, 2]. As a subset of Tregs, peripheral TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$NK1.1$^-$ Tregs, termed double negative (DN) Tregs, comprise 1–3% of peripheral T cells in normal mice and humans [3, 4]. DN Tregs can suppress Ag-specific auto-, allo- or xeno-reactive CD8$^+$ [3, 5, 6], CD4$^+$ T cells [6–9] or B cells [10, 11] in several animal models and humans. The number of DN Tregs is significantly reduced in both NOD mice as well as in TCR Tg autoimmune-diabetes-prone mouse models compared with autoimmune-diabetes-resistant strains [5, 10]. A single transfer of DN Tregs could prevent the onset of type I diabetes in mice [5]. Furthermore, the number of DN Tregs is increased in accepted allo- and xeno-grafts [12, 13]. Adoptive transfer of Ag-activated DN Tregs can prolong donor-specific skin, heart and islet allo- or xeno-graft survival [3, 8, 14]. Unlike allogeneic CD4$^+$ or CD8$^+$ T cells, infusion of allogeneic DN Tregs induces skin allograft tolerance without causing graft-versus-host disease (GVHD) [15]. Furthermore, DN Tregs can selectively suppress anti-host T cells

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and attenuate $CD8^+$ T-cell-induced GVHD [15, 16]. Consistent with these findings in mice, human DN Tregs have been shown to suppress autologous T cells in an Ag-specific fashion in vitro [4, 7]. A recent study suggested that the frequency and total number of DN Tregs were significantly decreased in BM transplant patients who developed GVHD and that the CD8/DN Treg ratio directly correlated with severity of GVHD [17]. DN Tregs have also been implicated to play a role in anti-tumor immunity [18]. Collectively, these findings clearly indicate the important role that DN Tregs may play in various diseases.

Although the mechanisms by which $CD4^+CD25^+$ Tregs mediate immune suppression have been studied extensively [19], the mechanisms by which DN Tregs mediate Ag-specific suppression remain elusive. Previous studies have shown that both murine and human DN Tregs can suppress immune responses through direct cytolysis of activated Ag-specific T effector cells [3, 7, 8]. We recently found that the number of DN Tregs was increased in the spleen and in accepted cardiac xenografts after pretransplant donor-specific lymphocyte infusion plus a short course of anti-CD4 mAb treatment in mice [13]. DCs from tolerant cardiac xenografts remained in an immature state and failed to stimulate T-cell proliferation in vitro compared to DCs from rejecting grafts [13]. These findings suggest that in addition to direct cytolysis of activated Ag-specific T cells, DN Tregs may inhibit graft rejection by suppressing DCs. However, whether DN Tregs can directly regulate DCs has not been previously investigated.

In this study, we investigated whether DN Tregs can exert their regulatory effects directly on DCs and examined the mechanisms by which DN Tregs regulate DCs. We demonstrate that DN Tregs can downregulate CD80 and CD86 expression on mature DCs (mDCs) in a CTLA4-dependent manner. In addition, allo-Ag activated DN Tregs can kill both immature DCs (iDCs) and mDCs in an Ag-specific fashion. These findings suggest that DN Tregs are not only able to directly kill activated Ag-specific T effector cells, but can also prevent naïve T cells from becoming activated and proliferating indirectly through inhibition of Ag-expressing DCs. Our data provide a greater understanding of the mechanisms underlying DN Treg-mediated Ag-specific immune suppression and suggest that DN Tregs may be used to treat a variety of immune-mediated disorders.

Results

DN Tregs can inhibit the function of Ag-expressing mDCs

It has been demonstrated previously that DN Tregs from both $(2C \times Dm2)_{F1}$ ($2C_{F1}$) Tg mice and normal non-Tg mice are able to suppress the proliferation of naïve syngeneic T cells and kill activated T cells [3]. To determine whether DN Tregs have a regulatory effect on DCs in an Ag-specific manner, we chose the well-characterized $2C_{F1}$ (H-$2^{b/d}$, L^{d-}, anti-L^d Tg TCR^+) mice against $(B6 \times BALB/c)_{F1}$ (CBy, H-$2^{b/d}$, L^{d+}) mice, which allowed us to monitor specific immune response to MHC class I molecule L^d [3]. The majority of DN Tregs and $CD8^+$ T cells in $2C_{F1}$ mice express the 2C-TCR, which specifically recognizes the L^d allo-Ag and can be detected by 1B2 mAb [20]. BM-derived DCs from CBy mice were stimulated with LPS to induce maturation (Supporting Information Fig. 1A). Purified $1B2^+CD4^-CD8^-NK1.1^-$ DN Tregs (Supporting Information Fig. 1B–D) and $1B2^+CD8^+$ T cells from $2C_{F1}$ mice were activated by stimulation with irradiated CBy splenocytes. The L^{d+} mDCs were then cocultured with pre-activated L^d-specific DN Tregs or L^d-specific $CD8^+$ T cells at the DC:DN/CD8 ratio of 1:0.6 for 24 h. Viable $PI^-1B2^-CD11c^+$ DCs, with or without DN Tregs or $CD8^+$ T-cell pre-conditioning, were purified by FACS and used as stimulators. Naïve CFSE-labeled anti-L^d $CD8^+$ T cells (L^{d-}) from $2C_{F1}$ mice were stimulated with either L^{d+} mDCs (Fig. 1A, +mDC) or L^{d+} mDCs that had been pre-conditioned with either $CD8^+$ (Fig. 1A, +CD8-DC) or DN Tregs (Fig. 1A, +DN-DC) at the CD8:DC ratio of 80:1. Naive $CD8^+$ T cells cultured alone were used as a negative control (Fig. 1A, CD8 alone). Proliferation of $CD8^+$ T cells was measured by CFSE dilution. $CD8^+$ T cells proliferated well when stimulated by mDCs or $CD8^+$ T-cell pre-conditioned DCs (Fig. 1A and B). However, when stimulated with DCs that had been conditioned with DN Tregs prior to being used as stimulators, the proliferation of $CD8^+$ T cells was significantly reduced (Fig. 1A and B). These data demonstrate that co-incubation of L^{d+} mDCs with activated L^d-specific DN Tregs can impair the function of allogeneic mDCs to stimulate naive $CD8^+$ T-cell proliferation in vitro.

Next, we addressed the question of whether DN Tregs can also impair the ability of syngeneic mDCs to stimulate $CD8^+$ T-cell proliferation. Peptide SIY (SIYRYYGL) has been shown to bind to the MHC class I molecule K^b and can be recognized by the 2C TCR [21]. When peptide SIY-pulsed syngeneic mDCs from $(B6 \times Dm2)_{F1}$ (H-$2^{b/d}$, L^{d-}) mice were preconditioned by co-culture with SIY peptide-activated DN Tregs, similar results were observed (Fig. 1C and D), indicating that DN Tregs are also able to inhibit the function of syngeneic mDCs. Taken together, these data demonstrate that either incubation of allo Ag-activated DN Tregs with syngeneic mDCs, which express the same Ag, or incubation of preactivated L^d-specific DN Tregs (L^{d-}) with allogeneic mDCs (L^{d+}) can hamper the ability of the mDCs to prime naive $CD8^+$ T-cell proliferation in vitro.

DN Tregs can downregulate mDC CD80 and CD86 expression

Expression of costimulatory molecules on DCs is critical for T-cell priming. To determine the mechanisms by which DN Tregs suppress DC function, we first investigated the effect of DN Tregs on DC costimulatory molecule expression. BM-derived DCs from CBy (L^{d+}) mice were stimulated with LPS to induce maturation. The L^{d+} mDCs expressing high levels of costimulatory molecules (Supporting Information Fig. 1A) were cocultured with an increasing number of pre-activated L^d-specific DN Tregs (L^{d-}). The expression of CD80 and CD86 on PI^-CD11c^+ cells was

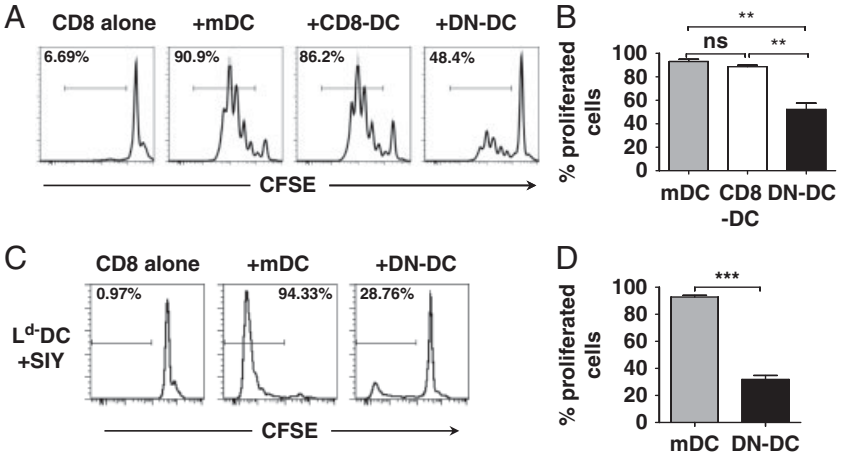

Figure 1. DN Tregs can inhibit the function of Ag-expressing mDCs. (A, B) $1B2^+CD4^-CD8^-NK1.1^-$ DN Tregs or $1B2^+CD8^+$ T cells were purified from $2C_{F1}$ (L^{d-}) mice and activated for 4 days in the presence of IL-2, IL-4 and irradiated CBy (L^{d+}) splenocytes. BM-derived mDCs from CBy mice were cultured in the presence or absence of pre-activated L^d-specific DN Tregs or L^d-specific $CD8^+$ T cells (DC:DN/CD8 = 1:0.6). CFSE-labeled naïve $2C_{F1}$ $CD8^+$ T cells were either cultured alone (left panel), or with purified mDCs (+mDC), or mDCs that had been cocultured with $CD8^+$ T cells (+CD8-DC) or DN Tregs (+DN-DC) at CD8:DC = 80:1. (C, D) Purified DN Tregs from $2C_{F1}$ mice were activated for 4 days in the presence of IL-2, IL-4 and 10μM 2C-TCR specific peptide SIY. L^{d-} mDCs pulsed with SIY were cultured either alone or with 10μM SIY-pulsed DN Tregs. CFSE-labeled naïve $2C_{F1}$ $CD8^+$ T cells were either cultured alone (left panel), or with purified mDCs (+mDC), or mDCs that had been cocultured with DN Tregs (+DN-DC, DC:DN = 1:0.6) at CD8:DC = 80:1. (A–D) 24 h after mDCs were cocultured with DN Tregs or $CD8^+$ T cells, cells were stained with anti-CD11c, anti-1B2 mAb and PI. Viable $PI^-1B2^-CD11c^+$ cells were sorted and used as stimulators. After 3–4 days, cells were stained with anti-CD8, anti-1B2 mAbs, PI and analyzed by flow cytometry. The $CD8^+$ T-cell proliferation is shown by CFSE dilution. The experiments were repeated at least three times each with 2 or 3 mice and similar results were obtained. The bar graphs summarize the data from at least three independent experiments. Error bars represent SEM. ns, not significant, $0.001< {}^{**}p<0.01$, $^{***}p<0.001$, Student's *t*-test.

determined by flow cytometry (Supporting Information Fig. 2 and Fig. 2). We found a dose-dependent downregulation of CD80 (Fig. 2A, top panels and Fig. 2B) and CD86 (Fig. 2A, lower panels and Fig. 2C) expression on L^{d+} viable DCs following 24-h coculture with DN Tregs, compared with mDCs cultured in the absence of DN Tregs. When iDCs were cultured with DN Tregs under similar conditions, no downregulation of CD80 or CD86 expression on iDCs was observed (data not shown). These data indicate that DN Tregs are able to downregulate CD80 and CD86 expression on mDCs, which may contribute to the reduced priming capacity of mDCs preconditioned with DN Tregs observed in Fig. 1.

CTLA4 is critical for DN Treg-mediated downregulation of CD80 and CD86 on mDCs

CTLA4 is constitutively expressed on $CD4^+CD25^+Foxp3^+$ Tregs and is important for their regulatory function [22]. However, the expression and function of CTLA4 in DN Tregs have not been studied previously. We first examined DN Treg CTLA4 expression and found that activated DN Tregs obtained from both normal B6 and TCR transgenic $2C_{F1}$ mice express similar level of surface CTLA4 as that seen on induced $CD4^+CD25^+$ Tregs (Fig. 3A). Next, we investigated whether CTLA4 was critically involved in DN Treg-mediated downregulation of CD80 and CD86 expression on mDCs. $TCR\beta^+CD4^-CD8^-NK1.1^-$ DN Tregs were purified from B6 $CTLA4^{-/-}$ or B6 $CTLA4^{+/+}$ mice, activated in vitro and cultured with allogeneic mDCs. After 24 h of coculture, cells were stained with anti-CD11c, anti-CD80 and anti-CD86 mAbs, and then analyzed by flow cytometry. While DN Tregs from wild-type B6 littermate control mice induced a significant reduction in CD80 and CD86 expression, $CTLA4^{-/-}$ DN Tregs were unable to downregulate expression of these molecules on viable PI^- mDCs (Fig. 3B–D). These results demonstrate that DN Tregs require CTLA4 to downregulate CD80 and CD86 expression on mDCs.

DN Tregs can kill both iDCs and mDCs that express a DN Treg-TCR-specific allo Ag in vitro

It was noticed that not all DCs showed downregulation of their costimulatory molecules following coincubation with DN Tregs (Fig. 2). This suggests that other mechanisms may be involved in the DN Treg-mediated regulation of DCs. It is known that activated $CD8^+$ T cells can kill Ag-expressing DCs in an Ag-specific manner [23, 24]. To determine whether DN Tregs can also kill DCs in a similar fashion, L^d-specific DN Tregs and L^d-specific $CD8^+$ T cells were activated in vitro, purified and used as effectors. Either mDCs or iDCs from L^{d+} CBy (allogeneic) or L^{d-} $(B6 \times Dm2)_{F1}$ (syngeneic) mice were used as targets at varying ratios. After coculture of DN Tregs or $CD8^+$ T cells with DCs for 24 h, the cells were stained with anti-CD11c mAb and PI to identify the dead DCs. As shown in Fig. 4A and B, approximately half of the L^{d+} allogeneic $CD11c^+$ cells became PI^+ after 24 h coculture with DN Tregs, whereas only about 15% L^{d+} allogeneic $CD11c^+$ cells died in the absence of DN Tregs. In contrast, when L^{d-} syngeneic DCs were cocultured with DN

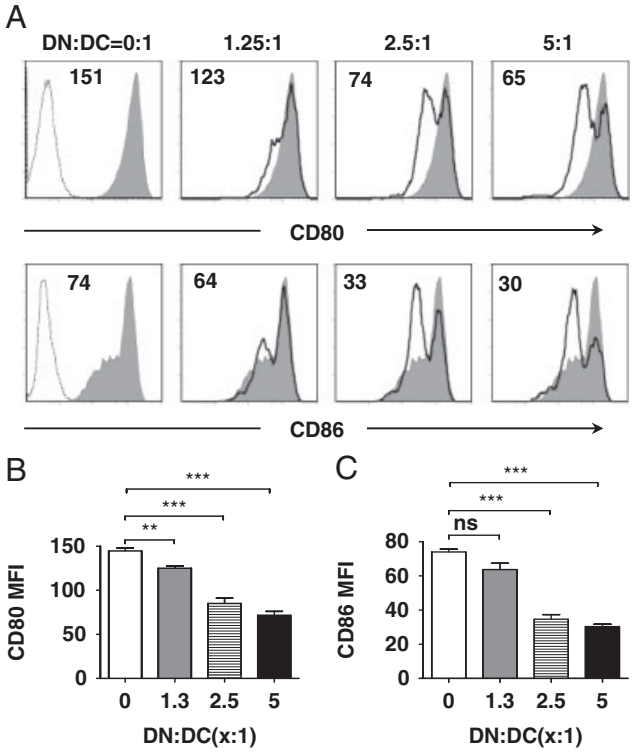

Figure 2. DN Tregs can downregulate mDC CD80 and CD86 expression. DN Tregs from $2C_{F1}$ mice were activated by irradiated L^{d+} CBy splenocytes as described in Fig. 1. BM-derived mDCs from CBy (L^{d+}) mice were cocultured either alone or together with pre-activated DN Tregs at the indicated ratios. After 24 h, cultures were stained with PI, anti-CD11c, anti-CD80 and anti-CD86 mAbs, and then analyzed by flow cytometry. (A) The plots shown are gated on PI^-CD11c^+ cells (grey area: CD80 or CD86 expression on mDCs cultured alone; black line: CD80 or CD86 expression on mDCs after coculture with DN Tregs; dotted line: isotype control). The numbers in the left upper corner of the plots are median fluorescence intensity (MFI) of CD80 and CD86 on viable PI^-CD11c^+ cells. These experiments were repeated at least four times each with 2 or 3 mice and similar results were obtained. The bar graphs show the MFI of CD80 (B) and CD86 (C). The error bars represent SEM. ns, not significant, $0.001 < **p < 0.01$, $***p < 0.001$, Student's *t*-test.

Tregs, no significant increase in the number of $CD11c^+PI^+$ cells was observed. Similar results were obtained when the standard chromium-release assay was used to measure the ability of DN Tregs to kill L^{d+} allogeneic DCs and L^{d-} syngeneic DCs (Fig. 4C). Activated $CD8^+$ T cells effectively killed both allogeneic iDCs and mDCs, but killed the syngeneic DCs to a much lesser extent (Fig. 4D). These data indicate that anti-L^d DN Tregs can kill L^{d+} allogeneic mDCs and iDCs, although to a lesser degree than $CD8^+$ T cells, but do not kill L^{d-} syngeneic DCs in vitro.

To further determine whether the observed cytotoxicity to allogeneic but not syngeneic DCs is due to the lack of Ag expression on syngeneic DCs, we examined whether peptide-activated DN Tregs could kill syngeneic DCs that had been pulsed with TCR-specific peptides. Peptides SIY (SIYRYYGL) and AAA (AAARYYRL) have been shown to bind to the MHC class I molecule K^b; however, only SIY is recognized by the 2C TCR [21]. DN Tregs were activated using the 2C-TCR-specific peptide SIY and used as effector cells. Syngeneic iDCs and mDCs were pulsed with either SIY or AAA peptides and used as targets at varying ratios in ^{51}Cr-release killing assays (Fig. 4E). We found that the SIY peptide-activated DN Tregs specifically lysed both immature and mature syngeneic DCs that had been pulsed with SIY peptides in a dose-dependent fashion, whereas much lower cytotoxicity against AAA peptide-pulsed iDCs and mDCs was observed (Fig. 4E). These data indicate that DN Tregs can kill both mature and immature syngeneic Ag-loaded DCs in an Ag-specific manner. Taken together, our data suggest that DN Tregs are able to kill both allogeneic and syngeneic DCs that express a TCR-specific Ag in a dose-dependent and Ag-specific manner.

DN Tregs are able to kill Ag-expressing DCs in vivo

To further determine whether DN Tregs can kill Ag-expressing DCs in vivo, we adoptively transferred 5×10^6 purified viable BM-derived $CD11c^+$ cells from CBy ($D^{b/d}$, $K^{b/d}$, L^{d+}) mice into sex-matched $Rag2^{-/-}Pfp^{-/-}$ recipients ($D^{b/b}$, $K^{b/b}$, L^{d-}), which exhibit a severe depletion of NK-cell function through the disruption of the perforin (Pfp) gene [25], and lack mature T and B lymphocytes through disruption of the Rag2 gene [26]. Two hours later, the $Rag2^{-/-}Pfp^{-/-}$ recipients were intravenously injected with either PBS or 5×10^6/mouse purified pre-activated L^d-specific $2C_{F1}$ DN Tregs ($D^{b/d}$, $K^{b/d}$, L^{d-}). Survival of the DCs was determined 4 days later by staining recipient splenocytes with PI and mAbs against H-$2K^d$ (to detect DCs), and 1B2 (to detect $2C_{F1}$ DN Tregs), and analyzing the cells by flow cytometry. As shown in Fig. 5A and B, there was a significant decrease in the proportion of H-$2K^{d+}$ cells (gated on PI^-1B2^- cells) in the spleens of the mice co-injected with L^{d+} DCs and DN Tregs, compared with those injected with L^{d+} DCs and PBS. However, when the same number of purified L^{d-} DCs from $(B6 \times Dm2)_{F1}$ mice was injected into $Rag2^{-/-}Pfp^{-/-}$ recipients, no significant difference in the percentages (Fig. 5B) and total numbers (Fig. 5C) of L^{d-} DCs in the spleens was observed, regardless of whether PBS or DN Tregs were subsequently injected. This experiment was repeated using DCs from CD45.1 congenic mice and similar results were obtained (data not shown). These data are consistent with what was observed in vitro and indicate that DN Tregs can selectively kill Ag-expressing DCs in vivo.

The Fas-FasL pathway is involved in DN Treg-mediated killing of Ag-expressing DCs

Next, we investigated the mechanisms that DN Tregs use to kill Ag-expressing DCs. Both perforin and FasL have been shown to be involved in DN Treg-mediated cytolysis [3, 11]. The kinetics of DN Treg-mediated killing of DCs is shown in Fig. 6A. We found a significant killing of both iDCs and mDCs that took place at 24 h after co-incubation of DN Tregs with DCs, compared with the cultures of DCs alone. In addition, both iDCs and mDCs express

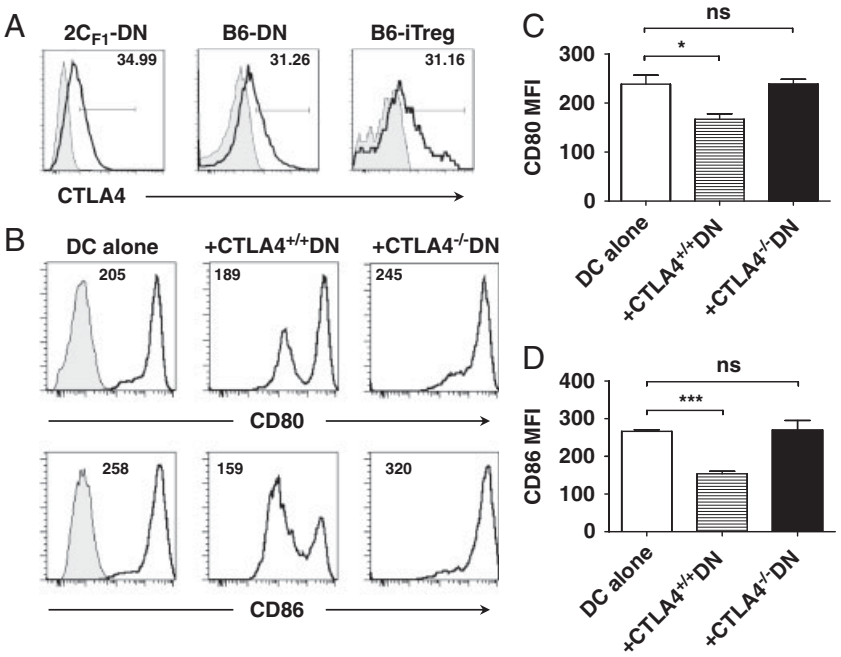

Figure 3. CTLA4 is critical for DN Treg-mediated downregulation of CD80 and CD86 on mDCs. (A) CTLA4 expression on Tregs. DN Tregs were cultured as described in the *Materials and methods* section, then collected and stained with anti-CD4, anti-CD8, anti-NK1.1, anti-CTLA4, anti-1B2 or anti-TCRβ mAbs. $CD4^+CD25^+$ induced Tregs (iTregs) were prepared as described by Fantini et al. [33]. The expression of CTLA4 on $CD4^-CD8^-NK1.1^-1B2^+$ cells (for $2C_{F1}$-DN Tregs), $CD4^-CD8^-NK1.1^-TCR\beta^+$ (for B6-DN Tregs) cells or $CD4^+CD25^+$ iTregs was analyzed by flow cytometry (solid line: CTLA4 expression; shaded areas: fluorescence minus one control). (B–D) LPS activated mDCs were cultured either alone or with pre-activated $CTLA4^{+/+}$ or $CTLA4^{-/-}$ DN Tregs (DN:DC = 2:1). After 24 h, cultures were stained with PI, anti-CD11c, anti-CD80, anti-CD86 mAbs and analyzed by flow cytometry. The data shown are gated on viable PI^-CD11c^+ cells (shaded area: isotype control; black line: CD80 or CD86 expression). The bar graphs show the MFI of CD80 (C) or CD86 (D) of three independent experiments. Error bars indicate SEM. ns, not significant, $0.01< {}^*p<0.05$, $0.001< {}^{**}p<0.01$, $^{***}p<0.001$, Student's *t*-test.

high levels of Fas on their surface before coculture with DN Tregs (Fig. 6B), suggesting that the Fas-FasL pathway may play an important role in DN Treg-mediated cytolysis of DCs. To test this hypothesis, FasL on $2C_{F1}$ DN Tregs was blocked by preincubation with Fas-Fc fusion protein before coculture with DCs. This resulted in a dose-dependent reduction in cytotoxicity toward Ag-expressing (L^{d+}) iDCs and mDCs (Fig. 6C). To further assess the contribution of the Fas-FasL pathway in DN Treg-mediated killing of Ag-expressing DCs, DN Tregs from Gld ($FasL^{-/-}$) mice were purified and their ability to kill DCs was compared with DN Tregs obtained from wild-type B6 ($FasL^{+/+}$) mice. As shown in Fig. 6D–F, the Gld DN Tregs mounted a significantly lower degree of cytotoxicity toward L^{d+} iDCs and mDCs compared with B6 $FasL^{+/+}$ DN Tregs. Taken together, these data demonstrate that the Fas-FasL pathway is involved in the DN Treg-mediated killing of DCs.

Discussion

Previous studies have demonstrated that DN Tregs exhibit a potent immune suppressive effect in an Ag-specific manner in multiple disease models both in vitro and in vivo [5, 8–10, 13, 15, 17, 27, 28]. Furthermore, mice that were rendered tolerant to cardiac xenografts had increased numbers of DN Tregs in accepted cardiac xenografts [13]. Interestingly, DCs isolated from tolerant cardiac xenografts remained in an immature state and failed to stimulate T-cell proliferation in vitro, compared to DCs obtained from rejecting grafts [13]. These findings provide indirect evidence suggesting that DN Tregs may inhibit graft rejection by suppressing DCs. In this study, we demonstrated that coculture of DN Tregs with DCs, which expressed DN Treg-specific Ags (either TCR-specific peptide-loaded syngeneic DCs or DCs that expressed the allo-Ag recognized by the DN Tregs), significantly impaired the ability of DCs to prime naïve $CD8^+$ T-cell proliferation. In contrast, viable DCs that were pre-incubated with activated $CD8^+$ T cells retained their ability to stimulate naive $CD8^+$ T-cell proliferation (Fig. 1A and B). These results provide the first direct evidence indicating that DN Tregs can inhibit immune responses by directly suppressing Ag-expressing DCs.

Furthermore, we have dissected the mechanisms by which DN Tregs suppress DCs in an Ag-specific manner. We demonstrated that DN Tregs could downregulate the expression of co-stimulatory molecules CD80 and CD86 on LPS-induced allogeneic mDCs (Fig. 2). This process required CTLA4, as CTLA4-deficient DN Tregs were completely unable to downregulate CD80 and CD86 on mDCs (Fig. 3B–D). $Foxp3^+CD25^+$ nTregs have been shown to be able to restrain the immature state of DCs through CTLA4, but are not capable of suppressing LPS-induced mDCs [22].

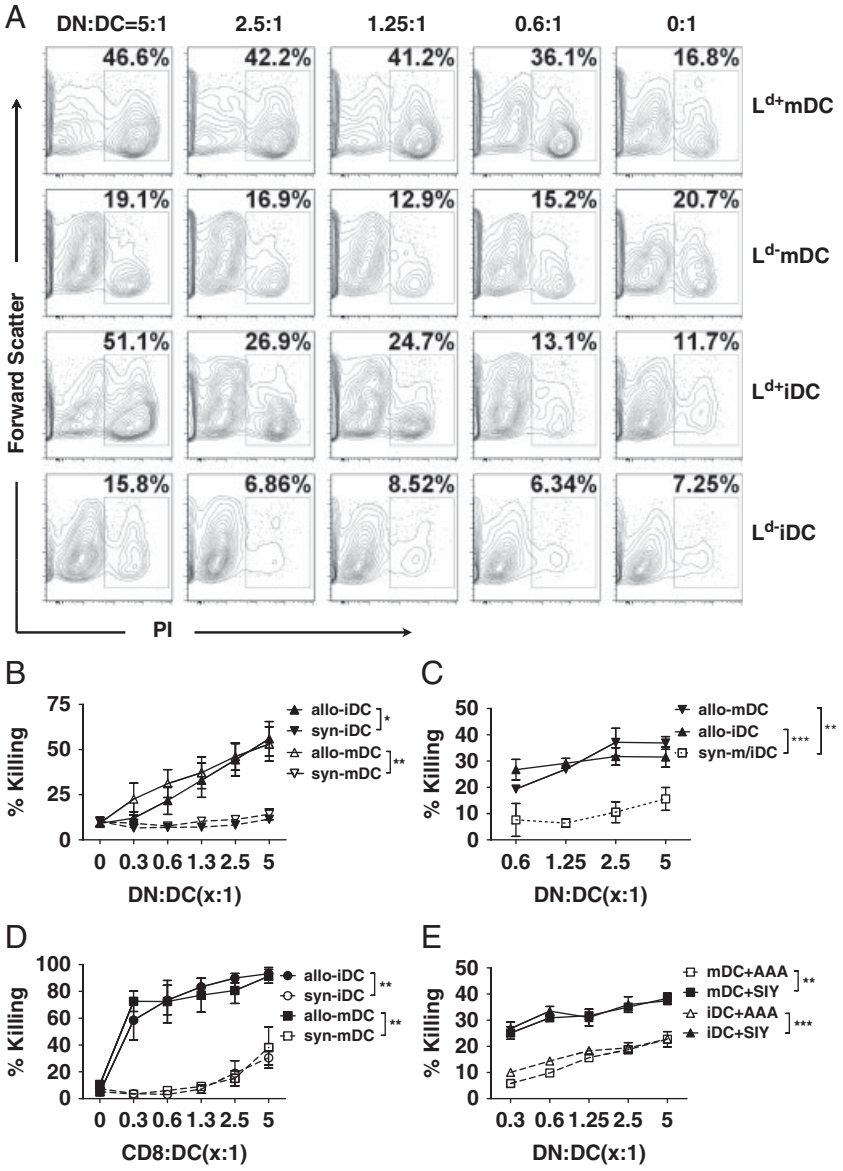

Figure 4. DN Tregs can kill both iDCs and mDCs that express a DN Treg-TCR-specific alloantigen in vitro. L^d-specific DN Tregs (A–C) or L^d-specific $CD8^+$ T cells (D) from $2C_{F1}$ mice were activated as described in Fig. 1 and used as effectors. BM-derived mDCs or iDCs from L^{d+} (solid lines) or L^{d-} (dashed lines) mice were cocultured either alone or together with DN Tregs (A, B) or $CD8^+$ T cells (D) at different ratios. After 24 h of coculture, cells were stained with PI and anti-CD11c mAbs. Percentages of PI^+ cells in total $CD11c^+$ cells are shown (A). Data shown in (B) are from six independent experiments and data shown in (D) are from three independent experiments. (C) L^{d+} mDCs (inverted triangle), L^{d+} iDCs (triangle) and L^{d-} DCs (open square) were labeled with ^{51}Cr and cocultured at 5×10^4 cells/well with increasing numbers of pre-activated L^d-specific $2C_{F1}$ DN Tregs for 18–20 h. Spontaneous release was less than 25% of the maximum release. (E) DN Tregs from $2C_{F1}$ mice were activated for 4 days in the presence of IL-2, IL-4 and 10μM SIY peptides, and then used as putative cytotoxic cells. mDCs (squares) or iDCs (triangles) were pulsed with either 10μM SIY (Ag-specific, solid lines) or AAA (control, dashed lines) peptides, labeled with ^{51}Cr and used as targets in ^{51}Cr-release assays. Percentage specific killing was assessed after 18–20 h of coculture. These experiments were performed three times and similar results were obtained. Error bars indicate SEM. $0.01< {}^*p<0.05$, $0.001< {}^{**}p<0.01$, $^{***}p<0.001$, Student's *t*-test.

Conditional knockout of CTLA4 in $Foxp3^+$ cells rendered $Foxp3^+$ nTregs unable to suppress proliferation of naïve T cells to allo-Ag stimulation in vitro and induced lethal autoimmune disease [22]. However, unlike CTLA4 KO mice, which die from multi-organ autoimmune disease at 2–3 wk, mice that are deficient in CTLA4 only in $Foxp3^+$ Tregs remain healthy for at least 7 wk [22]. Thus, in addition to $Foxp3^+$ Tregs, other Treg subsets that do not express Foxp3 but require CTLA4 to suppress immune responses may contribute to the significant phenotypic difference between CTLA4 KO and the Foxp3 conditional KO mice. Consistent with this notion, we found that activated DN Tregs do not express Foxp3 (Supporting Information Fig. 1C), but expressed a high level of CTLA4 (Fig. 3A). When DN Tregs from CTLA4-deficient mice were coincubated with mDCs, the downregulation of both CD80 and CD86 on mDCs was completely abolished (Fig. 3B–D). Taken together, these data indicate that downregulation of CD80

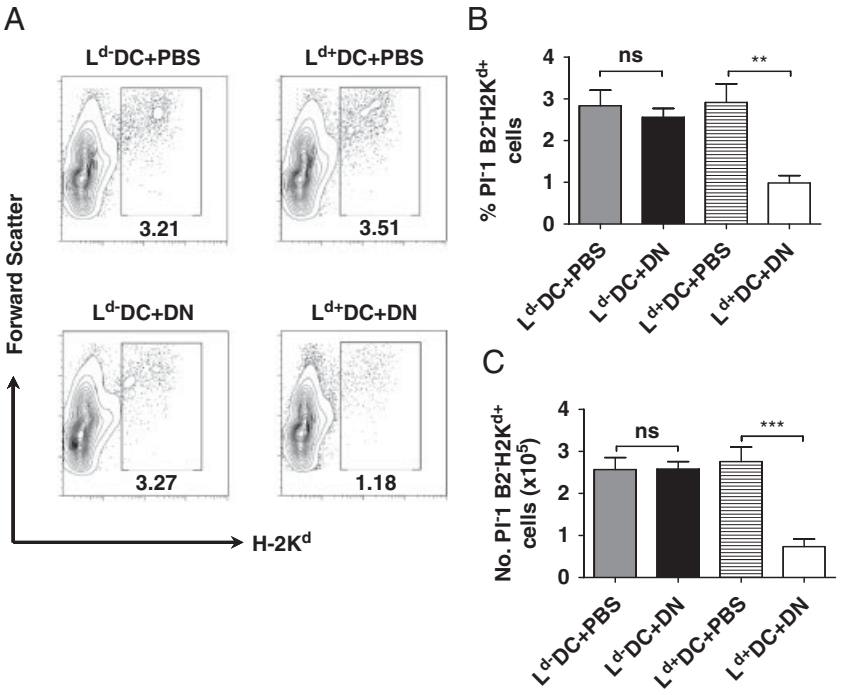

Figure 5. DN Tregs are able to kill Ag-expressing DCs in vivo. $Rag2^{-/-}Pfp^{-/-}$ mice were intravenously injected with 5×10^6/mouse BM-derived DCs, from either CBy (H-$2K^{d+}$, L^{d+}) or $(B6 \times Dm2)_{F1}$ (H-$2K^{d+}$, L^{d-}) mice. Two hours later, each mouse was injected with either 5×10^6 pre-activated L^d-specific DN Tregs from $2C_{F1}$ (L^{d-}) mice or PBS as control. Four days later, the recipient spleen cells were isolated and stained with PI, anti-H-$2K^d$ (to detect DCs) and anti-1B2 (to detect DN Tregs) mAbs. The percentage of H-$2K^{d+}$ cells was analyzed by gating on PI^-1B2^- cells. (A) Representative contour plots show percentage of $PI^-1B2^-H\text{-}2K^{d+}$ cells. Data shown are mean percentages (B) and total numbers (C) of $PI^-1B2^-H\text{-}2K^{d+}$ in the spleen of the recipient mice following injection of L^{d-}DC and PBS (grey bars), L^{d-}DC and DN Tregs (black bars), L^{d+}DC and PBS (hatched bars) or L^{d+}DC and DN Tregs (white bars). Error bars indicate SEM. ns: not statistically significant; $0.001 < **p < 0.01$, $***p < 0.001$, Student's *t*-test. These experiments were performed at least three times and similar results were obtained.

and CD86 expression on Ag-expressing DCs though CTLA4 is one of the mechanisms by which DN Tregs suppress Ag-specific immune responses.

Although DN Tregs could mediate downregulation of costimulatory molecules on mDCs, not all mDCs showed downregulation, even at a 5:1 ratio (Fig. 2). Interestingly, we found that L^d-specific DN Tregs could kill L^{d+} but not L^{d-} iDCs and mDCs in vitro in a dose-dependent manner, albeit at a much lower level than L^d-specific $CD8^+$ T cells (Fig. 4B–D). Furthermore, adoptively transferred L^d-specific DN Tregs reduced the frequency of L^{d+}, but not L^{d-}, DCs in $Rag2^{-/-}Pfp^{-/-}$ mice that were pre-infused with L^{d+} or L^{d-} DCs, respectively (Fig. 5). In addition, DN Tregs that were able to recognize SIY peptides killed SIY peptide-loaded syngeneic DCs to a much greater extent than AAA-loaded DCs (Fig. 4E). Collectively, our data indicate that DN Tregs can not only inhibit DC function by direct cytolysis of iDCs and mDCs in an Ag-specific fashion but also downregulate the costimulatory molecule expression on mDCs.

Previous studies have shown that DN Tregs can kill target cells through different mechanisms depending on the type of target cell. DN Tregs have been demonstrated to be able to kill Ag-expressing syngeneic $CD8^+$ and $CD4^+$ T cells in an Ag-specific manner through Fas-FasL interaction [3, 29]. Furthermore, DN Tregs are able to acquire MHC peptides from APCs through trogocytosis both in vitro and in vivo, and expression of acquired allo-MHC-peptides on the surface of DN Tregs is critical for their cytolysis of Ag-specific $CD8^+$ effector cells [30]. Recent studies have also shown that DN Tregs can kill autologous B cells via the perforin/granzyme pathway [11]. In this study, we demonstrated that DN Tregs are able to kill allo-Ag-expressing DCs in a process that could be inhibited significantly, although incompletely, by Fas–Fc fusion protein (Fig. 6C). Moreover, DN Tregs obtained from FasL-deficient Gld mice had a significantly reduced, but not completely abrogated, ability to kill allogeneic DCs (Fig. 6D–F). These data suggest that DN Tregs kill DCs mainly through Fas–FasL interactions, but other mechanisms may also be involved. Taken together, the data generated by our laboratory and others show that, similar to $CD4^+CD25^+$Tregs, DN Tregs can regulate Ag-specific immune responses by multiple mechanisms. Whether both downregulation of co-stimulatory molecule expression and cytotoxicity to DCs are mediated by the same DN Tregs or by different DN Treg subsets requires further investigation.

In conclusion, our study provides the first direct evidence demonstrating that DN Tregs can control Ag-expressing DCs through both downregulation of costimulatory molecule expression on mDCs and direct cytotoxicity to iDCs and mDCs. Expression of CTLA4 and FasL is important for DN Treg-mediated downregulation of CD80/CD86 and cytolysis of DCs, respectively. These findings further enhance our

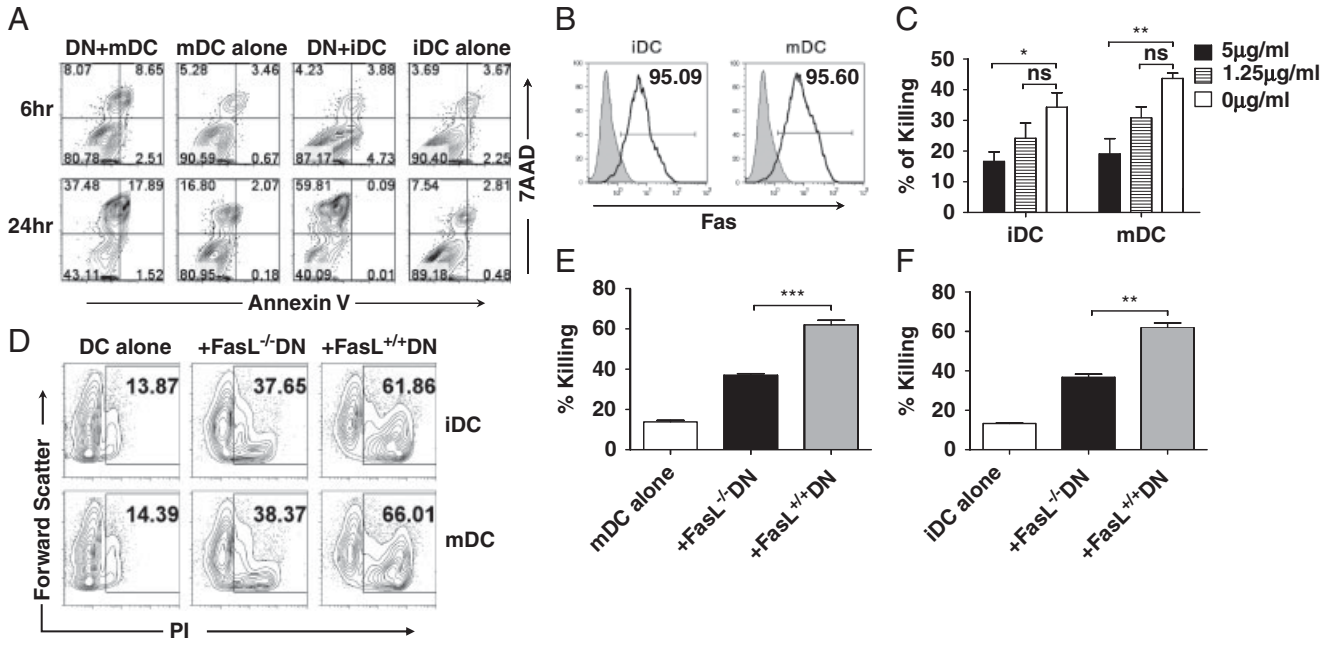

Figure 6. Fas-FasL pathway is involved in DN Treg-mediated killing of Ag-expressing DCs. (A) DN Tregs from $2C_{F1}$ mice were activated as described in Fig. 1. CBy (L^{d+}) mDCs or iDCs were cultured either alone or together with the pre-activated DN Tregs (DN Treg:DC = 5:1). After 6 h (top panels) and 24 h (bottom panels) of culture, cells were stained with anti-CD11c, Annexin V and 7AAD. The expression of Annexin V and 7AAD on $CD11c^+$ cells are shown. (B) iDCs and mDCs were generated as described in the *Materials and methods* section, then stained with PI, anti-CD11c and anti-Fas mAbs, and examined by flow cytometry. The expression of Fas is shown on gated PI^-CD11c^+ cells. (C) $2C_{F1}$ DN Tregs pre-activated by L^{d+} irradiated splenocytes were incubated with Fas-Fc fusion protein at the indicated concentrations for 20 min and then cocultured with L^{d+} iDCs and mDCs at 5:1 ratio for 24 h. The percentages of $7AAD^+$ cells gated on $CD11c^+$ cells are shown. (D–F) Pre-activated DN Tregs from B6 ($FasL^{+/+}$, H-2^b) or Gld mice ($FasL^{-/-}$, H-2^b) were cocultured with iDCs or mDCs from CBy (H-$2^{b/d}$) mice at a 5:1 ratio. The percentages of PI^+ cells are gated on $CD11c^+$ cells after 24 h coculture. (E, F) The bar graphs show the results from three independent experiments. Error bars indicate SEM. ns: not statistically significant; $0.01< {}^*p<0.05$, $0.001< {}^{**}p<0.01$, ${}^{***}p<0.001$, Student's *t*-test.

understanding of the mechanisms by which DN Tregs mediate Ag-specific immune suppression and may shed light on the development of novel therapies for the treatment of immunological disorders.

Materials and methods

Mice

C57BL/6 (B6, H-2^b), BALB/c (H-2^d), CD45.1^+ (H-2^b), BALB/c-$H2^{dm2}$ (Dm2, a BALB/c L^d-loss mutant, H-2^d, L^0) and Gld (H-2^b, FasL loss mutant) mice were purchased from The Jackson Laboratory. CBy (H-$2^{b/d}$, L^{d+}) mice were created by crossing B6 and BALB/c mice. Breeder 2C anti-L^d TCR transgenic mice (on B6 background, expressing the anti-L^d transgenic TCR, which can be detected by 1B2 mAb) were kindly provided by Dr. D. H. Loh (Nippon Research Centre, Japan). Dm2 mice were bred with 2C mice to create $2C_{F1}$ mice ($1B2^+$, H-$2^{b/d}$, L^{d-}) or with B6 mice to create $(B6 \times Dm2)_{F1}$ (H-$2^{b/d}$, L^{d-}) mice. Breeder $CTLA4^{-/-}$ mice on a B6 background were kindly provided by Dr. Tak W. Mak. $Rag2^{-/-}Pfp^{-/-}$ double mutant mice (H-2^b) were obtained from the Taconic Farm (Germantown, New York). All mice were housed in specific pathogen-free conditions at the University Health Network (UHN). All experiments were approved by the UHN animal care committee.

Antibodies and reagents

The hybridoma that produces 1B2 mAb, which specifically binds to the 2C-TCR, was provided by Dr. D.H. Loh. Other mAbs including anti-CD3, anti-CD4, anti-CD8, anti-CD28, anti-CD40, anti-CD80, anti-CD86, anti-CTLA4, anti-Fas, anti-MHCII, anti-TCRß and the reagents used to identify dead cells including Annexin V, PI, 7-amino-actinomycin D (7AAD) were purchased from eBioscience. Anti-NK1.1 and anti-CD11c mAbs were purchased from BD PharMingen (BD Biosciences). LPS was purchased from Sigma. SIY (SIYRYYGL) and AAA (AARYYGL) peptides were synthesized by Sigma-Genosys. Recombinant Fas–Fc (Fas/TNFRSF6/Fc Chimera) and TGF-β1 were purchased from R&D Systems.

DN Treg and $CD8^+$ T-cell culture and purification

$2C_{F1}$ DN Tregs and $CD8^+$ T cells were activated and purified as described previously [31]. The purity and viability of $1B2^+CD8^+$ and $CD4^-CD8^-NK1.1^-1B2^+$ cells were >90%, and these cells

were used in various assays. To obtain DN Tregs from B6, Gld or CTLA4$^{-/-}$ mice, CD4$^+$ and CD8$^+$ T cells were depleted from spleen and lymph node cells by incubating the cells with anti-CD4 and anti-CD8 mAbs, followed by incubation with MACS beads and passed through an autoMACS column according to the manufacturer's instruction. Remaining cells were cultured with irradiated CBy splenocytes for 4–6 days in the presence of 50 U/mL rIL-2 and 30 U/mL rIL-4 in α-MEM complete medium (CM) including 10% FCS, Penicillin (100 U/mL, Sigma), Streptomycin (100 μg/mL, Sigma), L-glutamine (2 mM, Sigma) and 2-ME (50 μM, Sigma). Cells were then stained with anti-CD4, anti-CD8, anti-NK1.1 and anti-TCRβ mAbs. The CD4$^-$CD8$^-$NK1.1$^-$TCRβ$^+$ population was sorted using a BD Facs-Aria cell sorter. The purity and viability of B6, Gld and CTLA4$^{-/-}$ DN Tregs used as effector cells in various assays were >95%.

Generation of DCs from BM

BM-derived DCs were generated according to Lutz et al. [32]. On days 8 and 9, non-adherent cells were collected and used as iDCs. To induce DC maturation, 0.1 μg/mL LPS was added into the culture. The non-adherent cells were harvested the next day and were used as mDCs. Cell surface marker expression was analyzed by flow cytometry (Cytomics^{TM} FC 500, Beckman Coulter). Over 90% of the DCs used in experiments were CD11c$^+$ cells. Viability of DCs was >95%, as determined by staining with PI.

CFSE proliferation assay

CD8$^+$ T cells were purified from 2C$_{F1}$ spleen and lymph node cells using autoMACS columns (Miltenyi Biotech, Auburn, CA) after incubation with anti-CD8 mAb and MACS beads (Miltenyi Biotech) according to the manufacturer's instructions. The purity and viability of CD8$^+$ T cells isolated using this method were >95%. The purified CD8$^+$ T cells were then labeled with 1 μM CFSE (Molecular Probes, Oregon) at 37°C for 10 min. The CFSE-labeled T cells were washed three times with complete medium before coculture with DCs. After 3-4 days of coculture, CFSE signal was measured by flow cytometry.

In vivo killing assay

Rag2$^{-/-}$Pfp$^{-/-}$ mice were intravenously injected with 5×10^6 purified CD11c$^+$ BM-derived DCs, which had been cultured for 7 days from (B6 × Dm2)$_{F1}$ (L^{d-}) or CBy (L^{d+}) mice as described above. Two hours later, the mice were injected with either PBS or 5×10^6 DN Tregs purified from 2C$_{F1}$ (L^{d-}) mice. The DN Tregs had been pre-activated by stimulating with irradiated L^{d+} CBy splenocytes in vitro. Four days later, the recipient spleen cells were isolated and stained with anti-H-2K^d, anti-CD11c, anti-1B2 mAbs and PI. The percentages of H-2K^{d+} cells were determined by flow cytometry and analyzed by gating on PI$^-$1B2$^-$cells.

Cytotoxicity assays

For the flow cytometry-based killing assay, activated DN Tregs were purified and co-incubated with 5×10^4 syngeneic (B6 × Dm2)$_{F1}$ or allogeneic CBy BM-derived DCs at varying ratios for 24 h. Cells were then stained with anti-CD11c and anti-1B2 mAbs, followed by labeling with Annexin V, 7AAD or PI according to the manufacturer's directions. CD11c$^+$ or CD11c$^+$ 1B2$^-$cells were gated and the percentages of Annexin V$^+$ and/or 7AAD$^+$/PI$^+$ cells were determined by flow cytometry.

For the chromium release assay, activated DN Tregs were cultured with ^{51}Cr-labeled target cells for 18–20 h. Target cells were also cultured alone and with 1% acetic acid to assess spontaneous release and maximum release, respectively. Eighteen to twenty hours later, plates were spun down at 450 × *g* for 5 min and 90 μL of supernatant was taken from each well and added to a scintillant-coated Lumaplate-96 (Perkin Elmer Life and Analytical Science). Cytotoxicity was assayed by counting cpm on a TOPCOUNT plate reader (Perkin Elmer Life and Analytical Science) once the supernatants had completely dried. Percentage killing was calculated by the equation: 100(experimental release–spontaneous release)/(max releasespontaneous release).

Statistical analysis

All statistical analyses were performed using Prism 4.0 software (GraphPad Software, San Diego CA). Error bars indicate SEM. Statistic analyses were done using the Student's *t*-test. ns, not significant; $0.01 < {}^{*}p < 0.05$, $0.001 < {}^{**}p < 0.01$, ${}^{***}p < 0.001$ and $p < 0.05$ was considered significant.

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Abbreviations: **GVHD:** graft versus host disease · **iDC:** immature DC · **mDC:** mature DC · **7AAD:** 7-amino-actinomycin D

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