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Double-Negative T Regulatory Cells Can Develop Outside the Thymus and Do Not Mature from CD8\(^+\) T Cell Precursors\(^1\)

**Megan S. Ford, Zhu-Xu Zhang, Wenhao Chen, and Li Zhang**

Recent studies have demonstrated that activated peripheral \(\alpha\beta\text{TCR}^+\text{CD}4^+\text{CD}8^-\text{NK}1.1^-\) (double-negative, DN) regulatory T cells (Tregs) from both mice and humans are able to down-regulate immune responses in vitro and in vivo. However, the origin and developmental requirements of functional DN Tregs remain unclear. In this study, we investigated the requirement for CD8 expression as well as the presence of a thymus for the development of functional DN Tregs. We demonstrate that DN Tregs exist in CD8-deficient mice and that stimulation of CD8\(^+\) T cells in vivo with TCR-specific Ag does not convert CD8\(^+\) T cells into DN Tregs. In addition, we found that DN T cells are present in the spleens and lymph nodes of thymectomized mice that are irradiated and reconstituted with T cell-depleted bone marrow cells. Interestingly, DN Tregs that develop in thymectomized mice can suppress syngeneic CD8\(^+\) T cells more effectively than those that develop in sham-thymectomized mice. Taken together, our data suggest that DN Tregs are not derived from CD8\(^+\) T cell precursors and that functional DN Tregs may preferentially develop outside of the thymus. These data suggest that DN Tregs may represent a developmentally and functionally unique cell population. *The Journal of Immunology*, 2006, 177: 2803–2809.

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egulatory T cells (Tregs)\(^5\) have been demonstrated to play a very important role in controlling the development of autoimmune diseases, transplant rejection, malignancy, and infectious diseases (1–3). Several different regulatory cell populations have been identified, including the naturally occurring, and infectious diseases (1–3). Several different regulatory population.

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play in various disease models, further characterization of their developmental pathways may facilitate expansion or reduction of DN Tregs and therefore better control of pathological immune responses.

In this study, we have addressed the question of whether DN Tregs mature from CD8− T cell precursors or represent a separate T cell lineage. We also examined the role of CD8-molecule expression in the development of DN Tregs. Lastly, we investigated the requirement of the thymus in the maturation of functional DN Tregs. Our results indicate that DN Tregs do not develop from CD8+ T cell precursors, nor is CD8 expression required for their development in vivo. Furthermore, we found that functional DN Tregs develop outside the thymus. These results suggest that DN Tregs may mature from a unique and previously uncharacterized lineage of T cells.

Materials and Methods

Mice

C57BL/6 (B6, H-2b), BALB/c (H-2d), and B6.C-H-2b12 (MHC class I-DP, Kpeb), B6 CD8α−/− and BALB/c, and CD8α−/−/− and C57BL/6 Tg(UBC-GFP)30Scha/J mice were purchased from The Jackson Laboratory. B62C transgenic (Tg) mice breeders were provided by Dr. D. Y. Loh (Nippon Roche Research Center, Kamakura, Japan). B6C2 mice express a Tg TCR reactive against MHC-class I-Ld molecules (10), which can be detected by the clonotypic mAb, 1B2. B62C Tg mice were bred with dm2 (Dp, Kc-, and Ld+) mice to produce the (C57 x dm2F1, (H-2b/d, Ld+, and anti-Ld− TCR+ ) that were used in this study. All mice were maintained in the animal facilities at the University Health Network.

Cell surface marker staining

1B2 mAb was produced in our laboratory from the 1B2 hybridoma that was provided by Dr. H. Elson (Massachusetts Institute of Technology, Cambridge, MA). Primary and activated T cells were stained with fluorescence-conjugated mAbs specifically recognizing the CD3, CD4, CD8, Thy-1.2, NK1.1, and the pan-NK marker DX5 (eBioscience). Data were acquired and analyzed on an EPICS XL-MCL flow cytometer (Coulter).

Isolation of CD8+ and DN T cells

Spleen and lymph node cells were obtained from naive (2C x dm2F1), B6, or B6 CD8α−/− mice. DN T cells were isolated by depletion of RBC, followed by passage through nylon wool columns to enrich the T cell population. The cells were then treated with depleting mAbs specific for murine CD4 (RL172-4, rat IgM) and CD8 (3.168, rat IgM), followed by the addition of rabbit complement (Cedarlane Laboratories) at 37°C for 45 min, and then washed three times with cold (4°C) 1% FBS/PBS. T cells were depleted by staining with depleting anti-Thy1.2 mAb (Cedarlane Laboratories), along with anti-CD4 (RL172-4, rat IgM) and anti-CD8 (3.168, rat IgM), followed by incubation with low-tox rabbit complement as before. The absence of T cells was confirmed before the transfer of bone marrow cells by staining with anti-CD3 and anti-Thy1.2 mAb and flow cytometry analysis. A total of 5 x 10^6 T cell-depleted bone marrow cells was i.v. injected into sublethally irradiated (700 rad) thymectomized or sham-thymectomized B6 mice. The complete reconstitution of recipient mice was confirmed at the time of sacrifice by flow cytometry analysis for the presence of the 2C or GFP transgene on the lymphocytes and was consistently >98%.

Results

Encountering Ag in vivo does not convert CD8+ T cells into DN T cells

Previous data has shown that CD4+CD25+ T cells can convert into CD4+CD25− T cells after encountering Ag in the periphery (36). Similarly, stimulation with alloantigen has been shown to induce clonal expansion, followed by contraction of CD8+ T cells that is sometimes concomitant with an increase in DN T cells in the peripheral lymphoid organs (12, 15, 18, 30, 37). In this study, we attempted to determine whether the origin of the increased DN T cell population was from CD8+ T cells that had decreased their expression of CD8 molecules or whether they proliferated from a precursor DN T cell population. Naive MHC class I-Ld−specific 1B2 CD8+ T cells were purified from (B6 x dm2F1, H-2b/d, Ld+ ) mice and injected into sublethally irradiated Ld−/− CD8−/− (B6 x BALB/c)F1 (H-2b/d, Ld− ) mice. In this setting, recipients lack CD8+ T cells and do not express the 1B2 TCR; therefore, any 1B2−DN T cells found in the recipient mice must arise from the infused allogeneic 1B2 CD8+ T cells. Mice were sacrificed at various time points, and the presence and phenotype of the infused 1B2+ cells in different lymphoid organs were monitored by flow cytometry. As seen previously (13, 37), the number of 1B2+CD8+ cells increased in the lymph nodes 4 days after adoptive transfer and reduced thereafter (Fig. 1). 1B2+CD8+ T cells were barely detectable at 14 days following the infusion. Importantly, the number of 1B2+ DN T cells did not increase above background levels.
at any of the time points studied (Fig. 1). In addition, no 1B2^+ DN T cells were found in the blood, spleen, or liver of mice that had been infused with 1B2^+ CD8^+ T cells. These data indicate that it is unlikely that CD8^+ T cells can develop into DN T cells following alloantigen stimulation in vivo.

The development of DN T cells does not depend on CD8 molecule expression

We next addressed the question of whether CD8 expression is required for the development of functional DN Tregs. First, we studied whether DN T cells exist in the peripheral lymphoid organs of CD8^+ gene-deficient mice. The lymph nodes and spleens of naïve wild-type as well as CD8^+ gene-deficient B6 and BALB/c mice were stained with mAbs against CD3, CD4, and CD8, and the percentage of DN T cells was compared. As shown in Fig. 2a, both B6 and BALB/c mouse strains showed a relatively equivalent proportion of DN T cells in the spleens of CD8^+/− and CD8^+/+ mice. However, in the lymph nodes, the percentage of DN T cells is higher in B6 CD8^+/− mice compared with wild-type B6 mice (Fig. 2a).

To further determine the phenotype of the DN T cells that developed in CD8^+/− vs CD8^+/+ mice, the lymphocyte populations were stained to differentiate the DN T cell population from a subpopulation of NK1.1^+ T cells that also expresses CD3 but lacks expression of CD4 and CD8. B6 CD8^+/− and CD8^−/− mice spleen and lymph node cells were triple-stained with mAbs specifically recognizing CD3, CD4, CD8, and NK1.1. As shown in Fig. 2b, the majority of the DN T cells found in either the spleen or the lymph node of CD8^+/− and CD8^−/− mice did not express NK1.1. Lymphocytes from BALB/c CD8^+/+ and CD8^−/− mice were also stained with mAbs specific for TCR, CD4, CD8, and the NK cell marker DX5 and showed a similar percentage of DN T cells in the spleen and lymph nodes (data not shown). These data demonstrate that the CD8 molecule is not required for the development of cells bearing the DN Treg phenotype in vivo.

DN Tregs from both CD8^+/− and wild-type mice can be activated and expanded in vivo

Donor lymphocyte infusion (DLI) has been shown to expand DN Tregs in CD8 wild-type animals. DLI-activated DN Tregs can inhibit T cell proliferation in vitro and can inhibit graft rejection when adoptively transferred into recipient mice in an Ag-specific fashion in vivo (10, 12, 38). To determine whether DN Tregs can also expand in response to DLI in the absence of CD8^+ cells, allogeneic lymphocytes were prepared from the spleens of one MHC class I-mismatched B6^{bm1} mice and infused into B6 CD8^+/− or B6 CD8^+/+ control mice. Seven days later, lymphocytes from the thymus, spleen, lymph nodes, and blood of DLI-treated and untreated control mice were stained using mAbs specific for TCR-β, CD4, CD8, and NK1.1. At this time point, donor bm1 cells had been eliminated from the periphery because CFSE-labeled donor cells could not be found in the spleen or lymph nodes of recipient mice (data not shown). However, we found that the percentage of αβTCR^+ NK^− DN T cells significantly increased following DLI in both CD8^+/+ and CD8^−/− mice (Fig. 3a, p < 0.002 and 0.005, respectively).

We next determined the function of the in vivo-expanded DN T cells from either CD8^+/+ or CD8^−/− mice. DN Tregs were purified from DLI-treated mice, cultured in vitro for 4 days (Fig. 3b), and used as effectors. As seen previously, DN Tregs from CD8^+/+ mice were cytotoxic when co-cultured with activated syngeneic CD8^+ T cells in a dose-dependent manner (Fig. 3c). In addition, DN Tregs from DLI-treated CD8^+/− mice could kill activated syngeneic CD8^+ T cells to a similar extent as those taken from CD8^+/+ mice (Fig. 3c). These results demonstrate that in vivo-activated DN Tregs from CD8^+/− mice are able to inhibit immune responses as effectively as those obtained from wild-type mice.
DN T cells develop in the absence of a thymus. T cell-depleted B6C (a and b) or B6GFP (c) bone marrow cells were i.v. injected into sublethally irradiated B6 mice that had been thymectomized or sham-thymectomized as a control. a and b. Spleen cells were stained with anti-1B2-TCR, CD4, and CD8 mAbs 50 days after reconstitution. a, Representative flow plot showing splenic T cells in B6 mice reconstituted with 2C bone marrow in the presence (left panel) or absence (right panel) of a thymus. Cells are gated on the 1B2-TCR population. b, Total numbers of splenic 1B2-CD8+ (left panel) or 1B2-2N (right panel) T cells that develop in the presence ([ ]) or absence ([ ]) of a thymus at 50 days following bone marrow reconstitution. c, GFP+ spleen cells were stained with anti-TCR-β, -CD4, -CD8, and -NK1.1 mAbs 50 days after the reconstitution. Total numbers of splenic CD4+ and CD8+ (left panel) or DN (right panel) T cells that develop in the presence ([ ]) or absence ([ ]) of a thymus at 50 days following bone marrow reconstitution. Value of \( p = 0.0003 \) for the number of CD4+ and CD8+ T cells within the spleens of sham vs thymectomized mice.

Taken together, these data suggest that the development and function of DN Tregs can occur in the absence of CD8+ cells and that DN Tregs may develop from a cell lineage that is independent of CD8 expression.

**DN T cells can develop extrathympically**

The thymus has been shown to be important for the development of both CD4+CD25+ (39) and NK Tregs (9). To determine whether the thymus plays a role in the development of DN Tregs, we conducted reconstitution studies. Groups of normal B6 and thymectomized B6 mice were sublethally irradiated and reconstituted with T cell-depleted bone marrow cells from B62C mice, which express the anti-L4 Tg (1B2-TCR). Fifty days following bone marrow reconstitution, the donor-derived 1B2+CD8+ and 1B2+DN T cells in the spleen and lymph nodes of the recipients were monitored by staining lymphocytes with the 1B2 mAb in combination with CD4 and CD8 mAbs. The percentage of 1B2+DN and 1B2+CD8 T cells that had accumulated in the spleens of nonthymectomized mice was similar to that found previously in bone marrow donor B62C mice (40), indicating that reconstitution had occurred (Fig. 4a). As expected, CD8+ T cells were reduced in the spleen from 36.7% in nonthymectomized mice to 3.1% of the T cell population in thymectomized mice, demonstrating the successful removal of the thymus (Fig. 4a). Likewise, the total number of CD8+ T cells in the spleen was also reduced in the thymectomized compared with nonthymectomized mice (Fig. 4b, left panel). Interestingly, DN T cells comprised over 90% of the splenic 1B2+ T cells in thymectomized mice and over 60% of the splenic 1B2+ T cells in nonthymectomized mice (Fig. 4a). Despite the higher percentage of DN T cells in thymectomized mice, the total number of DN T cells was similar in the spleens of thymectomized and nonthymectomized mice (Fig. 4b, right panel).

As 2C mice have an abnormally high number of CD8+ T cell (40) (Fig. 4a), to control for possible skewed T cell development because of the expression of the Tg TCR (41), we repeated this experiment using non-TCR Tg animals. B6 mice that express the GFP molecule under the control of the ubiquitin promoter were used as bone marrow donors. Recipient B6 mice were either thymectomized or sham-thymectomized and left to recover for 3 wk. Both groups of mice were then sublethally irradiated and reconstituted with T cell-depleted bone marrow cells from syngeneic B6GFP mice. Fifty days following reconstitution, the donor-derived T cells in the spleens of the recipients were identified by the
expression of GFP, in combination with TCR-β, NK1.1, CD4, and CD8 expression. As seen in the 2C Tg cell recipients, there was a significant decrease in the total numbers of CD4+ and CD8+ T cells in the spleens of thymectomized mice (Fig. 4c, left panel). However, donor-derived DN T cells developed in the spleens (Fig. 4c, right panel) and lymph nodes (data not shown) of the reconstituted mice in the presence or absence of a thymus. These data demonstrate that, unlike CD4+ and CD8+ T cells, which depend on the thymus for their development, a significant population of DN T cells can develop in the absence of a thymus.

Extrathymically derived DN T cells possess enhanced regulatory function

Both murine and human DN Tregs can suppress immune responses mediated by syngeneic T cells that are activated by the same Ags as those that activate the DN Tregs (11, 14). To further study the relative ability of DN T cells that had developed in the presence or absence of a thymus to regulate immune responses, GFP+DN T cells were purified from the spleens and lymph nodes of mice that had been thymectomized or sham-thymectomized, then B6GFP bone marrow-reconstituted. DN T cells were then used as putative suppressor cells for naive syngeneic T cell proliferation toward alloantigen stimulation. We found that DN T cells that had developed in either the presence or absence of a thymus could suppress syngeneic T cell proliferation in a dose-dependent fashion (Fig. 5a). Interestingly, the DN T cells that had developed in the absence of a thymus had a more potent ability to suppress T cell proliferation than those that had developed in the presence of a thymus.

DN Tregs have been shown previously to suppress syngeneic T cell proliferation via directly killing activated T cells. The ability of DN Tregs that had developed in the absence of a thymus to kill activated syngeneic T cells was compared with those that had developed in the presence of a thymus. 1B2+ DN T cells were purified from the thymectomized or nonthymectomized then B62C bone marrow-reconstituted B6 mice, stimulated with irradiated allogeneic splenocytes, and used as putative effector cells. Naïve syngeneic 1B2+CD8+ T cells were also purified from B62C mice, similarly stimulated, and used as target cells in cytotoxicity assays. As shown in Fig. 5a, 1B2+ DN T cells from both thymectomized and nonthymectomized B6 mice could kill activated syngeneic CD8+ T cells. Interestingly, similar to their ability to suppress T cell proliferation, the DN Tregs obtained from thymectomized mice had an increased ability to kill activated T cells when compared with those obtained from mice that had developed in the presence of a thymus (Fig. 5b). Collectively, these results demonstrate that the maturation and gain of regulatory function of DN Tregs does not require trafficking through the thymic microenvironment.

Discussion

Recent studies into the developmental requirements of Tregs have been focused largely on the CD4+CD25+ Treg subset. The development of naturally occurring CD4+CD25+ Tregs, which have been shown to be potent inhibitors of autoimmune diseases, requires thymic maturation (7, 39, 42). Neonatal thymectomy significantly reduces the number of CD4+CD25+ Tregs and promotes the development of autoimmune diseases (43). In addition, data have shown that some CD4+CD25+ Tregs undergo a positive selection process in the thymus that involves interaction with self-peptides presented in the context of MHC class II (44–46). On the other hand, studies have also shown that adult thymectomy does not prevent the development of CD4+CD25+ Tregs from CD4+CD25− cells (47). This suggests that, although the thymus may be required for the initial development of some CD4+CD25+

Tregs, the gain of Treg function can also happen in the absence of the thymus. In addition to CD4+CD25+ Tregs, several other subtypes of Tregs have also been shown to play an important role in regulating immune responses (1, 3–5). However, there have been conflicting data regarding the requirement for the thymus during their development. Recently, several studies have demonstrated that DN Tregs from both mice and humans can inhibit immune responses in an Ag-specific fashion (3, 10–12, 48), indicating that DN Tregs represent another important subset of Tregs. Similar to naturally occurring CD4+CD25+ Tregs, DN Tregs exist in naive animals and can readily suppress immune responses both in vitro (14, 17) and in vivo (10, 12, 16) upon activation with alloantigens. Whereas the ontogeny and maturation pathways for NK+ DN T cells have been well studied, the origin and developmental requirements for functional NK+ DN Tregs remain elusive.

Several reports have shown that after stimulation of splenocytes with Ags in vitro (28, 29) or injection of Ags into TCR Tg mice (14, 18, 30, 31, 37), there was a decrease of CD8+ T cells concomitant with an increase of DN T cells. Therefore, this suggested that DN T cells may be derived from activated CD8+ T cells as a consequence of down-regulation of CD8 during immune responses (30). However, in the previous experiments, both the responder
cells used in cultures as well as the TCR Tg mice that received Ag stimulation in vivo contained a pre-existing population of DN T cells. Thus, it is possible that the increased number of DN T cells in those studies was due to direct activation and expansion of pre-existing DN T cells. To distinguish these two possibilities and directly address the question of whether CD8⁻ T cells can covert to DN Tregs, instead of using a mixed whole splenic population, we used purified Ag-specific CD8⁻ T cells in this study. We found neither a down-regulation of CD8 expression nor an increase in the number of Ag-specific DN T cells when purified L⁵⁻specific CD8⁻ T cells were stimulated in vitro with L⁹⁺ allogeneic cells (data not shown) or adoptively transferred into alloantigen-expressing recipients (Fig. 1). Our findings are consistent with some reports from others that demonstrate that CD8⁺ T cells do not convert to DN T cell after Ag stimulation (49, 50). These data suggest that Ag exposure does not cause down-regulation of CD8 expression but rather the increase in DN T cells is the result of direct activation and proliferation of a pre-existing DN T cell population. This notion is further supported by the finding that CD8 RNA was undetectable by either northern blot or RT-PCR analysis in DN Tregs that have been cloned from alloantigen-recipient mice (10).

In addition, our study demonstrates that the percentage and total number of DN T cells does not differ significantly between wild-type and CD8α⁻/⁻ mice (Fig. 2). This suggests that CD8α expression is not required for the development of DN T cells in the peripheral lymphoid organs. Furthermore, since the DN T cells from CD8α⁻/⁻ mice can suppress allogeneic immune responses as effectively as those obtained from wild-type mice (Fig. 3), it suggests that the regulatory subset of DN T cells do not develop from CD8⁺ T cell precursors.

Although the thymus has been shown to support the maturation of natural occurring CD4⁺CD25⁺ Tregs (39) and NKT cells (9), whether this organ is also required for the development of DN Tregs has not been investigated previously. We found that DN Tregs could develop in sublethally irradiated thymectomized B6 mice following reconstitution with bone marrow cells from both B6C3H as well as B6GFP mice (Fig. 4). These results clearly demonstrate that the development of at least some subsets of DN Tregs does not require the thymus. Whereas the total number of splenic DN T cells reached a similar level in both thymectomized and nonthymectomized mice (Fig. 4), this discrepancy could be due to Tg expression of the TCR inducing aberrant development of DN T cells within nonthymic tissues. The fact that the number of DN T cells within the lymphatic tissues of 2C mice is significantly higher than that found in B6 mice suggests that 2C TCR expression may preferentially promote the development of DN T cells. Importantly, although mice reconstituted with 2C bone marrow had increased overall development of DN T cells, the 1B2⁺ DN T cells showed similar functional characteristics to GFP⁺ DN T cells. Both 1B2⁺ DN T cells and GFP⁺ DN T cells that had developed in the absence of a thymus could inhibit immune responses to a greater extent than those that had developed in the presence of a thymus (Fig. 5). This finding suggests the possibility that the nonregulatory DN T cell subset develops and matures through the thymus, whereas regulatory DN T cells may preferentially develop outside the thymus. Further studies are needed to identify cellular markers to differentiate between regulatory DN T cells and nonregulatory DN T cells.

Currently, the specific location for the maturation of DN Tregs remains unclear. Studies have suggested that some DN T cells may develop in the bone marrow (51–53), appendix (32), female genital tract (20), NALT (33) and liver (34, 35). In addition, the levels of DN T cells in the female genital tract or the NALT of nude mice was found to be similar to that found in wild-type mice, indicating that development of DN T cells within these tissues does not require the thymus (20, 33, 51). Strober’s group (54) has also shown that precursors to functional CD4⁺ and CD8⁻ T cells are found in the bone marrow, not the thymus, and that functional T cells could develop from these precursors in athymic nude mice. Similarly, Blais et al. (55) showed that development of CD4⁺ and CD8⁻ T cells could occur in the lymph node of nude mice. However, although these extrathymically developed T cells could recognize and expand in response to viral Ag, they were unable to clear the infection at the dose where thymically developed T cells were effective at clearing the virus (55). In the present study, we found that the DN T cells obtained from thymectomized mice are more potent in both suppression and killing of syngeneic CD8⁺ T cells when compared with the DN T cells obtained from nonthymectomized mice (Fig. 5). These findings suggest that T cells that develop extrathymically may have different functions from those that develop through the thymus.

Taken together, our data demonstrate that peripheral DN Tregs are not derived from CD8⁺ T cells as a consequence of down-regulation of CD8 upon Ag stimulation and do not require CD8α expression to develop in vivo. In addition, we show that the development of functional DN Tregs does not require the thymus, indicating that selection events that occur in the thymus are not essential for the development of DN Tregs. These data suggest that regulatory DN T cells may represent a unique cell lineage that can develop extrathymically through a pathway that is independent of CD8α expression. Further investigation into the molecular mechanisms involved in the extrathymic selection and maturation of DN Tregs in vivo may provide the means to manipulate their functions and improve immunotherapy.

Disclosures
The authors have no financial conflict of interest.

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