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Cutting Edge: Autoimmune Disease in Day 3 Thymectomized Mice Is Actively Controlled by Endogenous Disease-Specific Regulatory T Cells

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Female B6AF1 mice thymectomized on day 3 (d3tx) develop autoimmune ovarian disease (AOD) and dacryoadenitis. It has been hypothesized that d3tx breaks tolerance by depleting late ontogeny regulatory T cells (Treg). We now report that Treg greatly expand over effector T cells in d3tx mice and adoptively suppress autoimmune disease in d3tx recipients. In the d3tx donors, Treg from ovarian lymph nodes (LN) preferentially suppress AOD and Treg from lacrimal gland LN preferentially suppress dacryoadenitis, suggesting they are strategically positioned for disease control. Indeed, the autologous disease in d3tx mice is dramatically enhanced by in vivo depletion of endogenous Treg. Moreover, normal 3-day-old mice possess Treg that suppress AOD and autoimmune gastritis as efficiently as adult cells. Thus, d3tx mice possess disease-relevant Treg of presumed neonatal origin. They accumulate in the regional LN and actively inhibit concurrent autoimmune disease; however, they cannot fully prevent autoimmune disease development. The Journal of Immunology, 2008, 180: 4366–4370.

Self tolerance is thought to be maintained by two major mechanisms. Autoreactive thymocytes with high-affinity T cell receptors are largely deleted (1), and peripheral autoreactive T cells that escape deletion are controlled by thymus-derived, natural CD4+CD25+Foxp3+ regulatory T cells (Treg) (2).

The current research on Treg in self-tolerance has stemmed largely from the seminal observation by Nishizuka and Sakakura that day 3 thymectomy (d3tx) leads to severe organ-specific autoimmune disease during adulthood that can be prevented by the infusion of CD4+ T cells from normal donors (3). D3tx mice develop autoimmune disease of the ovary, stomach, thyroid, prostate, lacrimal gland, and testis (4), and in C57BL/6 × A/J)F1 (B6AF1) female mice, autoimmune ovarian disease (AOD) and dacryoadenitis (DA) dominate (5). An intriguing feature of autoimmune disease induction is the requirement for thymectomy on neonatal days 1 to 4. We previously investigated this issue by comparing neonatal vs adult T cells to adoptively induce autoimmune disease in athymic syngeneic recipients. Notably, AOD and autoimmune gastritis (AIG) developed only in the recipients of neonatal CD4+ splenocytes (6). We proposed two mutually exclusive explanations for this observation: 1) d3tx enriches for autoreactive, pathogenic CD4- T cells that escape deletion in the neonatal thymus, and 2) d3tx deprives mice of Treg of late ontogeny (7).

The first hypothesis was supported by the finding of T cell responses to endogenous mouse mammary tumor virus (MMTV) superantigen (8). Thymic expression of endogenous MMTV superantigen in B6AF1 mice appeared late in ontogeny, and the deletion of cognate VB11+CD4+CD8- thymic or peripheral T cells was not observed until neonatal day 10. In the lymph nodes (LN) of adult d3tx B6AF1 mice there was a 10-fold increase in VB11+CD4+ T cells. The findings support the paradigm that T cells that escape thymic deletion in the neonatal period expand following d3tx, and the escapees with high-affinity receptors might elicit autoimmune disease.

The Treg deficiency hypothesis was supported by the findings that autoimmune disease in d3tx mice is suppressed by Treg and that Treg are undetectable in neonatal thymuses or spleens until after day 5 (9, 10). However, this paradigm is not supported by new experimental findings showing that, although Treg were not detectable in 3-day-old spleens, they were detected in 3-day-old LN (11) and suppressed CD25- T cell proliferative responses in vitro (10).

In 2004, Dujardin et al. (12) reported that the percentage of CD25+CD4+ T cells in the spleens of d3tx BALB/c mice was increased over untreated mice (>20 vs <10%). The CD25+ T

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2 E.T.S. and K.M.W. contributed equally to this study.

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cell population expressed Forkhead box p3 (Foxp3) mRNA, suppressed in vitro polyclonal anti-CD3 stimulated CD25+ T cell responses, and prevented colitis in scid recipients of CD25− T effector cells. Thus there is a fractional increase in functional Treg in the CD4+ T cell compartment of d3tx mice (12). However, Dujardin et al. (12) did not directly examine the capacity of Treg to suppress autoimmune diseases known to occur in d3tx mice (AOD and AIG), thereby leaving open the question of whether Treg deficiency is the mechanism underlying d3Tx-induced autoimmune disease (9). Also, colitis is not relevant to d3tx because it does not occur in d3tx mice (4). Because colitis does not affect germfree animals, even its autoimmune nature is in question (13).

Thymus-derived natural Treg exhibit a broad range of potent activities, suppressing adaptive and innate immune responses that include CD4+ and CD8+ T cells, B cells and innate cells (2), with the outcome being the maintenance of a quiescent state of immune responsiveness (14). Notwithstanding, whether Ag-specific Treg participate in physiological tolerance remains an open question, and the occurrence of autoimmune disease in d3tx mice is frequently cited as evidence supporting this possibility.

To further explore the role(s) of Treg in tolerance, it is therefore critical to fully elucidate whether Treg deficiency is causal to d3tx-induced autoimmune disease. Toward this end, we have addressed the following: 1) the status of Treg in d3tx mice; 2) the LN distribution of functional, organ-specific Treg; 3) the capacity of endogenous Treg to suppress autoimmune diseases induced by d3tx; and 4) the ability of Treg from normal 3-day-old neonatal mice to suppress the same autoimmune diseases.

Materials and Methods

Mice and surgery
B6AF1 mice were produced by mating C57BL/6N and A/J adults from the National Cancer Institute (Bethesda, MD); Foxp3−/− mice on a mixed (C57BL6 × 129) background, a gift of Dr. A. Rudensky (University of Washington, Seattle, WA), were crossed twice onto BALB/cByJ. To produce A/J null mice, Fox1nu was transferred from B6.Cg-Fox1nu/J onto the A/J background through 10 generations of backcrossing and selection. Fox1nu heterozygotes were identified using a single-strand conformation polymorphism (CAGGAGGAGTGTCCATCAAGTGCC-3′ and 5′-H11032/H11032/H11032/H11032) of Fox1nu (C57BL/6, F1, 129) background, a gift of Dr. A. Rudensky (University of Washington, Seattle, WA), were crossed twice onto BALB/cByJ. To produce A/J null mice, Fox1nu was transferred from B6.Cg-Fox1nu/J onto the A/J background through 10 generations of backcrossing and selection. Fox1nu heterozygotes were identified using a single-strand conformation polymorphism of the denatured sequence amplified by the 5′-CAGGAGGAGTGTCCATCAAGTGCC-3′ and 5′-AGAGGTGTCCATCAAGTGCC-3′ primers. Thymus-derived natural Treg exhibit a broad range of potent activities, suppressing adaptive and innate immune responses that include CD4+ and CD8+ T cells, B cells and innate cells (2), with the outcome being the maintenance of a quiescent state of immune responsiveness (14). Notwithstanding, whether Ag-specific Treg participate in physiological tolerance remains an open question, and the occurrence of autoimmune disease in d3tx mice is frequently cited as evidence supporting this possibility.

Antibodies and flow cytometric analysis
The Abs used for flow cytometry and cell isolation were FITC-conjugated anti-CD4 (clone L3T4), PE-conjugated anti-CD8 (clone 53-6.7), anti-CD25 (clone 7D4), PE-Cy5.5-conjugated anti-CD25 (clone PG61), allopurinol-conjugated anti-Foxp3 (clone FJK-166), and allophycocyanin-conjugated anti-CD4 (clone L3T4) (BD Pharmingen and eBioscience). Intracellular Foxp3 was stained with the Foxp3 intracellular staining kit from eBioscience.

Depletion of Treg from d3tx mice
D3tx mice received i.p. injections of 50, 100, 100, and 200 μg of anti-CD25 mAb (PC61) or rat IgG (Thermo Scientific) control Ab on days 5, 7, 14, and 21, respectively.

Histopathology grading
Tissues fixed in Bouin’s solution were stained with H&E and evaluated as blinded samples. AOD grading was as previously described (5), with additional scoring of the degree of ovarian atrophy (loss of primordial oocytes and loss of growing/mature oocytes), graded from 1 to 4. Total AOD scores were determined as follows: ovarian inflammation + (1.5 × growing/mature follicle atrophy) + (2 × primordial follicle atrophy). Lacrimal gland and gastric inflammation were graded from 1 to 4 as follows: grade 1, focal infiltrate; grade 4, lacrimal atrophy or gastric hypertrophy and parietal cell loss; grades 2 and 3, increasing inflammation between grades 1 to 4.

Immunofluorescence microscopy and cell quantification
The infiltrating leukocytes in diseased ovaries were identified by immunofluorescence on ethanol-fixed frozen sections using the TSA biotin system (PerkinElmer) with anti-CD4 (clone L3T4) and biotinylated anti-Foxp3 (clone FJK-166) Abs. Foxp3 was detected on sections permeabilized with 0.3% Triton X-100 (Sigma-Aldrich). CD4+ and Foxp3+ cells were counted on serial frozen sections of 3- to 4-wk-old d3tx ovaries, and the percentage of Foxp3+ CD4+ cells was computed.

Results and Discussion

Foxp3+ CD4+ T cells are increased in the LN and target organ of d3tx mice
D3tx mice developed persistent T cell lymphopenia after the first week of life (Fig. 1A). Simultaneously, the Treg fraction of CD4+ T cells rapidly expanded from 1 to 3 wk to reach a plateau above that of control B6AF1 mice (Fig. 1B). Twenty-five percent of CD4+ T cells in d3tx B6AF1 mice were Foxp3+ as compared with 5–10% in controls (Fig. 1C). This expansion is likely due to the propensity of Treg to proliferate in lymphopenic environments (16). Treg from the LN of d3tx mice suppressed the proliferation of CD25- T cells at a comparable dose response as Treg from untreated donors (data not shown).

The enrichment of Treg in d3tx mice was not confined to the LN, as they were also found in the target organ with early inflammation. At the onset of AOD (3–4 wk) (15), an average of 13% (n = 11) of the ovarian CD4+ T cells were Foxp3+, with some exceeding 35% (data not shown).

Our findings confirm the observations of Dujardin et al. (12); in d3tx mice, functional Treg, now identified as Foxp3+ CD4+ T cells, are persistently increased relative to the CD25− effector T cells. To extend the study, we investigated the in vivo function of Treg in d3tx mice, in particular their capacity to influence autoimmune diseases that develop in d3tx mice (5).

Disease-specific Treg suppress d3tx-induced autoimmune disease and accumulate in the regional LN of d3tx mice
Ag-specific polyclonal Treg cannot be identified due to low clonal frequency. We can, however, detect disease-specific Treg...
pressed by 0.1 the lacrimal gland LN. Notably, AOD was completely sup-
pressed from the nonovarian LN (Fig. 2). Conversely, 0.1 Treg from ovarian LN (renal LN) suppressed AOD completely, but not DA. Conversely, the Treg obtained from the lacrimal gland LN (cervical LN) suppressed DA but not AOD. Because effector T cell suppression also occurred exclusively in the draining LN, the disease-specific Treg from normal donors must exert their effect in the regional LN of relevant target organs, recapitulating the distribution of donor Treg in day 3 thymectomized (d3tx) mice at the peak of disease can adoptively suppress AOD and/or DA. CD25 Treg were ob-
gained from the ovarian LN but not by Treg enriched from lacrimal gland-draining LN (NDLN). B, DA is more effectively suppressed by Treg from NDLN than Treg from ODLN. CD4+CD25+ Treg (0.1 × 10⁶) from ODLN or NDLN, pooled from 6- to 8-wk-old d3tx mice, were transferred to 5-day-old d3tx recipients and pathology was evaluated 8 wk later. p values were determined by Student’s t test.

populations that preferentially suppress one autoimmune disease over another in the same d3tx host. This approach was used previously to investigate d3tx mice under suppression by normal Thy1.1- Treg (5). At the suboptimal dose of 0.1 × 10⁶ cells, Thy1.1- donor Treg retrieved from ovarian LN (renal LN) suppressed AOD completely, but not DA. Conversely, the Treg obtained from the lacrimal gland LN (cervical LN) suppressed DA but not AOD. Because effector T cell suppression also occurred exclusively in the draining LN, the disease-specific Treg from normal donors must exert their effect in the regional LN of recipient d3tx mice during disease suppression (5).

Herein we determined the existence of disease-specific Treg in 6- to 8-wk-old d3Tx B6AF1 mice and studied their capacity to adoptively suppress AOD and/or DA. CD25 Treg were ob-
gained from the ovarian LN or were pooled from cervical, inguinal, and axillary LN, of which ~50% of the Treg came from the lacrimal gland LN. Notably, AOD was completely sup-
pressed by 0.1 Treg from the ovarian LN but not by Treg from the nonovarian LN (Fig. 2A). Conversely, 0.1 lacrimal gland, LN-enriched Treg did not suppress AOD but re-
duced DA severity (Fig. 2B). Therefore, the Treg obtained from d3tx mice at the peak of disease can adoptively suppress AOD and DA. Moreover, the endogenous disease-specific Treg of d3tx mice are enriched in the draining LN of relevant target organs, recapitulating the distribution of donor Treg in d3tx recipients under disease suppression (5).

Depletion of endogenous Treg from d3tx mice significantly enhances the incidence and severity of subsequent autoimmune disease.

In the preceding study we showed that the Treg in d3tx mice can traffic and respond to self-Ags in appropriate locations and, when transferred to other d3tx mice, adoptively prevent autoimmune disease. Although the experiment has defined the LN distribution of disease-specific Treg in d3tx mice and docu-
mented their ability to prevent autoimmune disease in new neo-
natal d3tx recipients, it does not elucidate whether the Treg can suppress disease in the autologous d3tx host. We therefore under-
took a more direct approach to examine the endogenous Treg in the d3tx host itself.

Treg from d3tx mice were depleted during the first 3 wk of life using anti-CD25 Ab, and disease outcome was evaluated at 6 wk. The timing of Ab treatment coincided with the duration of pathogenic T cell priming by endogenous ovarian Ags (15).

In vivo depletion of endogenous CD25+ cells greatly enhances the autologous autoimmune disease of d3tx mice. A and B, The incidence and severity of AOD (A) and DA (B) in d3tx mice treated with anti-CD25 Ab or control rat IgG. In CD25 Ab-treated mice (D and F), heavy and diffuse tissue inflammation is associated with the loss of oocytes (D) or the loss of lacrimal glandular acini (F). The ovary (C) and lacrimal gland (E) of rat IgG-treated d3tx mice had focal lymphocyte infiltrate (arrows) with intact oocytes or glandular acini (H&E staining; original magnification ×100). p values were determined by the Mann-Whitney U test.

D3tx mice treated with anti-CD25 Ab had a dramatic increase in mean AOD severity (p = 0.011) (Fig. 3A, C, and D). Treg depletion also increased the prevalence of DA from 58 to 100% (p = 0.007) and enhanced the mean DA severity (p < 0.0001) (Fig. 3, B, E, and F). Therefore, the endogenous Treg in d3tx mice are actively restraining the full pathogenic autoimmune response and d3tx mice develop an attenuated pathology.

Three-day-old neonatal mice possess functional CD4+CD25+Foxp3+ Treg capable of suppressing autoimmune disease in d3tx mice.

Pivotal support for the Treg-depletion hypothesis of d3tx disease was provided by the reported late ontogeny of Treg develop-
ment (9, 10), but the supporting data are controversial (10, 12). More importantly, there has not been an attempt to inves-
tigate the in vivo function of neonatal Treg, specifically their capacity to suppress the autoimmune disease of the d3tx mice.

Foxp3+ Treg, hardly detectable in the spleen, were readily detected in the LN and the thymus of 3-day-old B6AF1 mice. When the day of birth was counted as day 0, ~5% of the CD4+ T cells in 3-day-old LN were Foxp3+ (Fig. 4A) (70% of the CD25+ T cells are Foxp3+; data not shown), and ~3% of the CD4+CD8- thymocytes were Foxp3+ (Fig. 4A). Similar data were obtained in the 3-day-old Foxp3-gfp knockin mice (Fig. 4A). This result is comparable to the report on 4-day-old Foxp3-gfp knockin mouse thymocytes by Fontenot et al. (17) if we consider the fact that they counted the day of birth as day 1 and showed a significant rise in the percentage of Foxp3+CD4+CD8- thymocytes from day 3 (0.74%) to day 4 (2.24%).
The percentages of LN Treg in B6AF1 mice increased from day 3 to day 5 (Fig. 4B), reaching the normal plateau of 5–10% (Fig. 1B). Neonatal LN Treg expressed adult Treg markers (CTLA4, GITR, and high CD62L) and also suppressed in vitro CD25+ T cell proliferation induced by CD3 Ab with comparable efficiency as adult Treg (data not shown).

To study in vivo function of the limited Treg obtainable from neonatal mice, we used a highly sensitive model of AOD and AIG induced by the transfer of 10^5 CD25+ effector T cells of adult euthymic B6AF1 donors into adult B6AF1 nu/nu recipients of 0.1 × 10^6 adult CD25+ CD4+ T cells are completely suppressed by 0.1 × 10^5 Treg from adult or 3–5 day-old donors. CD4+ CD25+ and CD4+ CD25− T cells were isolated by magnetic beads to >80% purity. Histopathology was determined 10–12 wk after cell transfer.

Our study provides direct evidence for the existence of functional Treg in d3tx mice with progressive autoimmune disease. The Foxp3+ Treg expand quickly to reach an increased Treg:effector cell ratio that persists in the face of d3tx-induced disease progression. These endogenous Treg are fully functional as they prevent diseases in other d3tx mice that are representative of the d3tx-induced autoimmune syndrome, including AOD, DA, and AIG. Most importantly, we documented that the d3tx mice possess functional disease-specific Treg for both AOD and DA and that they significantly restrain the diseases of the autologous d3tx host. The Treg that preferentially accumulate in the regional LN of the relevant target organs thus are strategically positioned to control the autoimmune disease progression in the d3tx mice (5). Finally, we document for the first time the capacity of neonatal Treg to suppress autoimmune disease. Together, our study provides conclusive evidence that the Treg in d3tx mice can down-modulate concurrent autoimmune disease and, in the presence of putative highly pathogenic effector T cells, the coexisting Treg strongly influence autoimmune disease outcome. Still, the endogenous Treg in d3tx mice, in and of themselves, are not sufficient to fully control the disease.

Why then do the endogenous Treg in d3tx mice fail to fully control their own autoimmune diseases? Recent studies indicated that Treg suppress effector T cell response through interaction with dendritic cells (18). Although d3tx mice have increased Treg in relation to effector T cells, their profound lymphopenia is expected to reduce the T cell to dendritic cell ratio, and this would in turn reduce the efficiency of Treg to compete against effector T cells for dendritic cell interaction (18). Thus, lymphopenia can render Treg less functional. However, lymphopenia is not the sole explanation, because comparable T lymphopenia and Treg enrichment also occur in the disease-free day 7 thymectomized B6AF1 mice (K. M. Wheeler and K. K. S. Tung, unpublished observation). We therefore consider as an additional explanation the enhancement of effector T cell function (7, 8).

The Aire (autoimmune regulator/autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) transcription factor controls the ectopic thymic expression of peripheral autoantigens required for the central deletion of autoreactive T cells (19–21). In Aire knockout mice, thymic expression of many ectopic Ags is reduced and the thymic deletion of potentially pathogenic T cells is also significantly curtailed (21, 22). We are impressed by the almost identical organ distribution, histopathology, and autoantibody specificity associated with the diseases in d3tx mice and the Aire knockout mice (19, 20, 23) and how they are different from the findings in Treg-deficient Scurfy mice (24). The concordances of autoimmunity raise the intriguing possibility that d3tx mice and Aire knockout mice may share a common mechanism. If this is true, then the expression of Aire function might be ontogenetically regulated.

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Disclosures
The authors have no financial conflict of interest.

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