Continuous control of autoimmune disease by antigen-dependent polyclonal $CD4^+CD25^+$ regulatory T cells in the regional lymph node

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This study investigated the unresolved issue of antigen-dependency and antigen-specificity of autoimmune disease suppression by $CD4^+CD25^+$ T cells (T regs). Based on autoimmune ovarian disease (AOD) in day 3 thymectomized (d3tx) mice and polyclonal T regs expressing the Thy1.1 marker, we determined: (a) the location of recipient T cell suppression, (b) the distribution of AOD-suppressing T regs, and (c) the relative efficacy of male versus female T regs. Expansion of recipient $CD4^+$ T cells, activation/memory marker expression, and IFN-γ production were inhibited persistently in the ovary-draining LNs but not elsewhere. The cellular changes were reversed upon $Thy1.1^+$ T reg depletion, with emergence of potent pathogenic T cells and severe AOD. Similar changes were detected in the regional LNs during autoimmune dacryoadenitis and autoimmune prostatitis suppression. Although the infused $Thy1.1^+$ T regs proliferated and were disseminated in peripheral lymphoid organs, only those retrieved from ovary-draining LNs adoptively suppressed AOD at a suboptimal cell dose. By depriving d3tx recipients of ovarian antigens, we unmasked the supremacy of ovarian antigen-exposed female over male T regs in AOD suppression. Thus, disease suppression by polyclonal T regs depends on endogenous antigen stimulation; this occurs in a location where potent antigen-specific T regs accumulate and continuously negate pathogenic T cell response.

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Abbreviations used: AOD, autoimmune ovarian disease; BrdU, bromodeoxyuridine; CFSE, 5,6-carboxyfluorescein diacetate-succinimidyl ester; d3tx, day 3 thymectomy; EAE, experimental allergic encephalomyelitis; nOX, neonatal ovariectomy; PLP, proteolipid protein; T reg, $CD4^+CD25^+$ regulatory T cell.

There is now general acceptance that natural $CD4^+CD25^+$ T cells that express the functional transcription factor, *Foxp3*, are an important cellular component of the normal immune system (1). They develop in the thymus and are potent suppressors or regulators of effector and helper T cell responses in the setting of organ-specific autoimmunity, allergic responses, allograft rejection, and microbial immunity (2). T regs also modulate antitumor response to favor tumor growth and metastasis; their elimination has led to enhanced immunogenicity of tumor vaccines (3). In studies on common human autoimmune disease, including multiple sclerosis (4), rheumatoid arthritis (5), and autoimmune polyendocrinopathy syndrome (6), the reduction in T reg number or function has been reported. These clinical findings prompted attempts in deploying antigen-specific T regs for treatment of autoimmune disease and allograft rejection (7).

Patients and mice deficient in T reg function that is due to mutation of the *Foxp3* gene develop severe and fatal autoimmunity (8); thus, antigen-specific T regs is a likely mechanism for maintenance of peripheral tolerance that normally prevents pathogenic autoimmune response. In studies of T cells expressing transgenic TCRs against nominal, transgenic, or allogeneic antigens, peripheral T regs respond to cognate antigen (9–12), proliferate extensively in normal and lymphopenic hosts (13–15), and respond to self-peptides presented by dendritic cells with unique properties in tissue-draining LNs (15–18). Naturally processed epitopes recognized by T regs also have been identified (19, 20). Therefore, T regs can recognize and respond to specific antigenic peptides in vivo.

However, the more critical issue of the antigen dependency of natural T regs with respect to their functional acquisition and autoimmune disease suppression has not been resolved. This is a fundamental question because its clarification will allow us to address more fully: (a) T regs as significant participants in physiologic peripheral tolerance, a process that depends on antigen-specific immunoregulation; and (b) application of T regs in antigen-specific immunotherapy.

The second question has been explored in experiments that were based on T reg-expressing transgenic TCRs. They showed that T regs

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that had been expanded previously by the cognate antigen had enhanced capacity in suppressing the relevant autoimmune disease (21–24). Transgenic T regs (BDC2.5) suppressed diabetes that was transferred adoptively by the same transgenic diabetogenic effector T cells (17). Similarly, proteolipid protein (PLP)-specific T regs suppressed experimental allergic encephalomyelitis (EAE) that was induced by PLP immunization (24), and thyroglobulin-specific T regs suppressed thyroiditis that was induced by thyroglobulin immunization (23). Thus, T regs with known specificity suppress autoimmune disease that is induced by pathogenic T cells of shared specificity. In EAE, PLP-specific T regs suppressed EAE that was induced by PLP, but not by myelin oligodendrocyte glycoprotein; this provides more definitive support for antigen-specific suppression. However, these studies also yielded data that implicated antigen-nonspecific suppression. For example, the BDC2.5 transgenic T regs suppressed disease that was transferred adoptively by whole spleen cells from female nonobese diabetic donors (17), and PLP-specific T regs suppressed EAE that was induced by immunization with brain homogenate (24). Moreover, when the PLP-specific T regs were activated in vitro, they suppressed EAE that was induced by PLP or by myelin oligodendrocyte glycoprotein (24). The nonspecific suppression observed could be due to cross-suppression of effector T cells of one specificity by T regs of a second specificity, when both cognate antigens are present in vivo (24, 25). However, it also could be explained by the dual TCRs that are expressed on the nonphysiologic transgenic T cells, due to pairing of a random endogenous Vα with the transgenic Vβ (26–28). Thus, T reg expansion may depend on engagement of the transgenic TCR, whereas disease suppression may depend on the second TCR of unknown specificity.

Studies with polyclonal T regs have addressed the question of acquisition of T reg function in response to physiologic self-antigen. When polyclonal T cells from normal antigen-positive donors were used to suppress autoimmune thyroiditis in adult thymectomized and irradiated rats (29), or autoimmune prostatitis of day 3 thymectomy (d3tx) mice (30, 31), they suppressed disease with greater efficacy than the T cells from antigen-negative donors. Moreover, the T regs from thyroid antigen-negative donors retained the ability to suppress autoimmune diabetes in the same animals. However, when the same strategy was applied to autoimmune ovarian disease (AOD) suppression, the polyclonal T regs from male or female donors suppressed the disease equally (32).

To gain more insight into antigen-dependency and antigen-specificity of physiologic polyclonal T regs, we studied the classical AOD model of the d3tx mouse. D3tx renders mice T lymphocytopenic and T reg-deficient (33, 34) and, in this setting, the effector T cells spontaneously respond to endogenous ovarian antigens and trigger AOD (35). The disease is inhibited completely by polyclonal T regs that are transferred from normal adults soon after thymectomy (33). Because it is not possible to analyze the function of antigen-specific T regs within a polyclonal population, we determined disease specificity rather than antigen specificity of the T reg function. In addition, the study is based on several novel approaches that are built on current understanding of antigen-specific T cell responses. First, we determined whether T reg suppression, similar to the effector T cell response, is triggered by organ-specific antigens that are processed and presented uniquely in the regional LNs (36, 37). Second, to explore the causal relationship among T reg presence, regional LN changes, and AOD suppression, we studied the effect of in vivo T reg depletion in d3tx mice that are under suppression by T regs. Third, we investigated the lymphoid organ distribution of T regs and, most importantly, reisolated the input T regs from individual lymphoid organs and studied for the first time the distribution of "memory" T regs with enhanced capacity to suppress AOD. Fourth, we revisited our earlier attempt to compare male and female T regs in AOD suppression, but in a setting where the opportunity for the T reg to be educated by endogenous ovarian antigens in the d3tx recipients was no longer permitted. The results of our study uniformly and strongly support the conclusion that the acquisition of T reg function and AOD suppression by polyclonal T regs are highly disease-dependent, and, therefore, antigen-dependent.

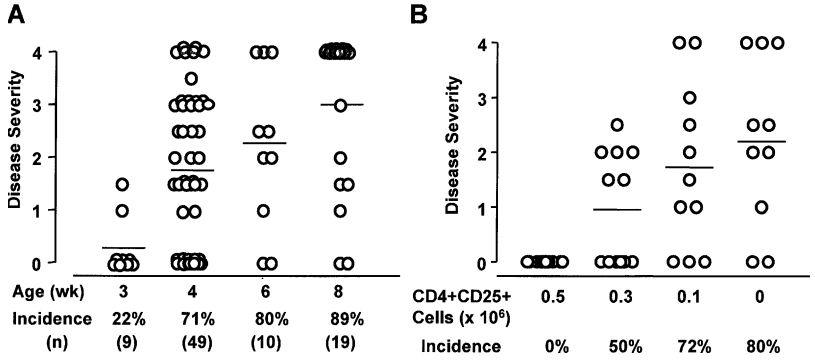

Figure 1. Time course of AOD induction and dose response of suppression by T regs in d3tx B6AF1 mice. (A) AOD in d3tx mice of different ages. (B) AOD in 6-wk-old d3tx recipients of different numbers of Thy1.1$^+$CD25$^+$ T cells from normal female donors.

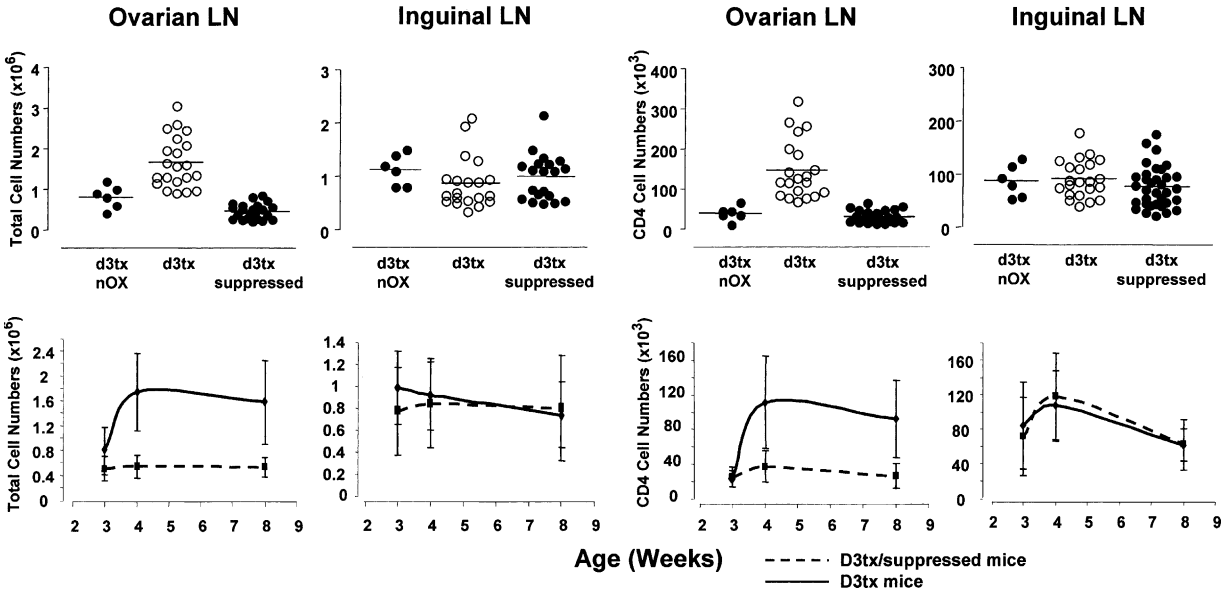

Figure 2. The total cell number and recipient CD4+ T cell number in ovarian and inguinal LNs at 4 wk (top), and from 3 to 8 wk (bottom). Compared with d3tx/nOX control, the total cellularity and CD4+ T cell number in the d3tx mice were increased significantly in ovary-draining LNs, but not inguinal LNs. Compared with d3tx mice, profound reduction in the total cellularity and recipient CD4+ T cell number occurred in ovary-draining LNs but not inguinal LNs of the d3tx/suppressed mice. This was detectable at 4 wk and persisted to 8 wk.

RESULTS

The ovary-draining LN is the site of T cell activation required for AOD induction in d3tx mice

Following d3tx, ovarian inflammation appeared spontaneously in the B6AF1 mice at 3 wk of age, and, by 8 wk, 89% developed severe inflammation and ovarian atrophy (Fig. 1 A). To investigate the requirement of endogenous antigenic stimulation in AOD induction, we compared LN changes between d3tx mice and d3tx mice with neonatal ovariectomy (d3tx/nOX). The d3tx/nOX mice developed severe lymphopenia without evidence of autoimmune response to oocyte antigens or AOD (35), and were used as appropriate control for this study and the subsequent studies on AOD suppression.

At 4 wk, there was a significant increase in the number of total and CD4+ T cells in the ovary-draining LNs of d3tx mice, and the CD4+ T cells expressed significantly higher levels of CD69 and CD44hi markers (Fig. 2; Table I). These changes were not observed in the nondraining LNs. Therefore, the autoimmune T cell response to endogenous antigen occurs in the ovary-draining LNs, a finding that is consistent with other models of spontaneous autoimmune disease (36, 38).

Table I. Activation and memory T cell markers on recipient CD4+ T cells from ovary-draining LNs and nondraining LNs from d3tx mice, d3tx/nOX mice, and d3tx/suppressed mice

		Number ($\times 10^3$) of recipient CD4+ cells with marker ± SEM (% positive)		
Cell marker	Treatment of mice (*n*)	Ovarian LN	Axillary LN	Inguinal LN
CD44hi	d3tx/nOX (6)	24 ± 5 (58)	61 ± 7 (70)	49 ± 6 (57)
	d3tx (9)	103 ± 19 (74)	61 ± 8 (72)	49 ± 7 (60)
	d3tx/supp (9)	19 ± 5 (60)	51 ± 8 (63)	47 ± 8 (55)
CD69	d3tx/nOX (6)	5 ± 1 (13)	10 ± 5 (11)	13 ± 2 (15)
	d3tx (8)	43 ± 11 (25)	11 ± 1 (14)	13 ± 2 (18)
	d3tx/supp (8)	6 ± 2 (17)	11 ± 3 (12)	13 ± 3 (15)
CD62Lhi	d3tx/nOX (6)	16 ± 3 (43)	31 ± 3 (43)	37 ± 7 (45)
	d3tx (8)	12 ± 2 (25)	36 ± 8 (40)	21 ± 3 (29)
	d3tx/supp (8–12)	26 ± 6 (46)	43 ± 6 (46)	26 ± 2 (35)
CD25	d3tx (8)	28 ± 5 (28)	27 ± 6 (28)	22 ± 6 (35)
	d3tx/supp (8)	7 ± 1 (23)	22 ± 3 (25)	15 ± 2 (22)

D3tx/nOX, d3tx mice with neonatal ovarian ablation; D3tx/supp, d3tx mice suppressed with CD4+CD25+ T cells.

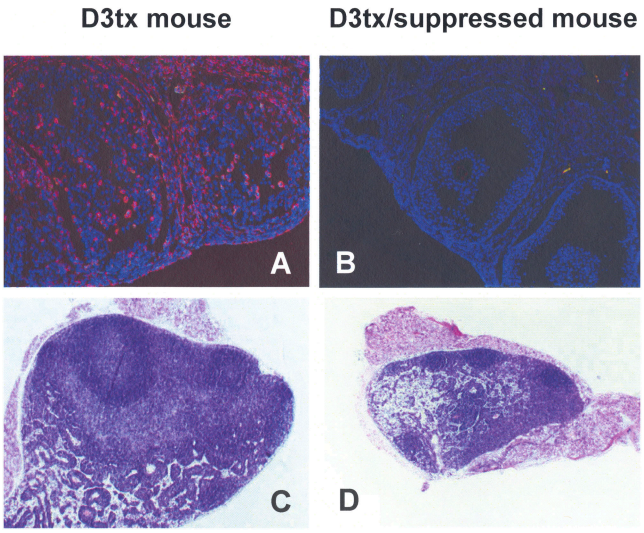

Figure 3. Ovarian immunohistology and ovarian LN histology of d3tx mice and the d3tx/suppressed mice. At 4 wk, the ovary of a d3tx mouse has heavy T cell infiltration (A), whereas the ovary of a d3tx/suppressed mouse is completely devoid of T cells (B) (Texas red–labeled anti-CD5 antibody plus DAPI). Findings are representative of 10 mice for each group. The ovary-draining LN of the d3tx mice is enlarged, and contains a germinal center and a broad T cell zone (C), whereas the ovary-draining LN of the d3tx/suppressed mice has an empty T cell zone and is devoid of germinal centers (D) (hematoxylin and eosin).

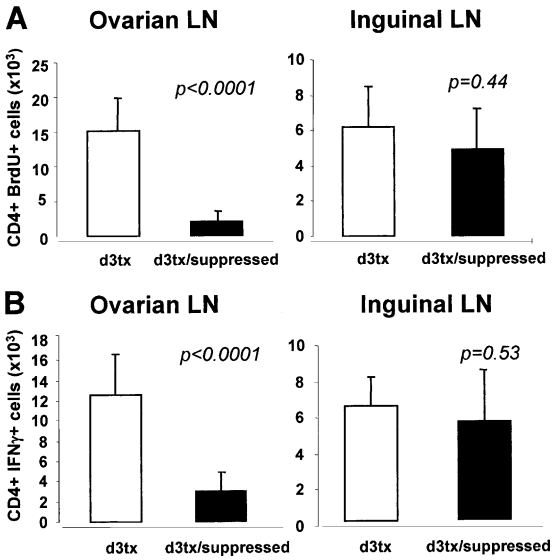

Figure 4. Proliferation (A) and IFN-γ production (B) of Thy1.1-negative recipient CD4$^+$ T cells in the ovary-draining LNs of d3tx versus d3tx/suppressed mice. BrdU uptake in 12 h by cells in d3tx mice greatly exceeds that of d3tx/suppressed mice; the difference is not found in the inguinal LN. IFN-γ–producing CD4$^+$ T cell numbers of d3tx mice exceed those of d3tx/suppressed mice in ovarian LNs but not in inguinal LNs.

Profound suppression of recipient T cell response occurs in the ovary-draining LNs of d3tx mice with complete AOD prevention by T regs

AOD was suppressed completely in d3tx mice that received 0.5 million Thy1.1$^+$ T regs that were pooled from the LNs and the spleens of normal adult mice (d3tx/suppressed mice). Partial suppression was noted in the recipients of 0.3 million cells; those that received 0.1 million cells showed no evidence of disease suppression (Fig. 1 B). The ovaries of d3tx/suppressed mice were free of pathology and devoid of infiltrating T cells (Fig. 3, A and B). The mice did not have oocyte autoantibodies (35), and their splenic T cells adoptively transferred only mild and infrequent AOD to neonatal recipients. To locate where the autoimmune response was suppressed, we compared the phenotype of the recipient Thy1.1-negative CD4$^+$ T cells in LNs and spleen between d3tx mice and d3tx mice that had received 0.5 million Thy1.1$^+$ T regs.

There was a profound reduction in the total cellularity in the ovary-draining LNs at 4 wk (Fig. 2, top); this persisted for at least 8 wk (Fig. 2, bottom). Morphologically, the ovary-draining LNs of d3tx/suppressed mice were exceedingly small, with empty-looking T cell zones and no germinal centers (Fig. 3, C and D). These changes were not observed in the spleen or the nondraining LNs, including the inguinal and axillary LNs (unpublished data).

There also was significant reduction in the recipient CD4$^+$ T cell numbers within the ovary-draining LNs (Fig. 2). The number in d3tx mice increased 400% from weeks 3 to 4, and remained elevated at week 8 (Fig. 2, bottom), whereas the number of recipient CD4$^+$ cells in the ovary-draining LNs of the d3tx/suppressed mice remained very low for the entire 8-wk period (Fig. 2, bottom). In parallel, there was a profound reduction in recipient CD4$^+$ T cell division as documented by bromodeoxyuridine (BrdU) incorporation ($P < 0.0001$) (Fig. 4 A, left). This was not observed in the nondraining LNs or the spleen (Figs. 4 A, right).

In the ovary-draining LNs of the d3tx/suppressed mice, phenotypic changes that are characteristic of T cell activation and memory T cells (CD25$^+$, CD44^{hi}, CD62L^{lo}, and CD69$^+$) were reduced greatly (Table I). In addition, significantly fewer recipient CD4$^+$ T cells in the ovary-draining LNs produced IFN-γ (Fig. 4 B). Again, these changes were not detectable in the nondraining LNs or the spleen (Fig. 4 B). Thus, suppression of recipient T cell response of the d3tx mice occurs exclusively in the ovary-draining LNs.

Suppression of the recipient T cell response is not unique to AOD or the ovary-draining LNs

To address whether regional LN suppression was unique to the ovary-draining LNs in mice with AOD, we studied the recipients' T cell response in (a) the LNs draining the lacrimal glands in d3tx B6AF1 male mice with autoimmune dacryoadenitis, and (b) the LNs draining the prostate in d3tx B6AF1 male mice with autoimmune prostatitis. Similar results were found in both extraovarian autoimmune diseases. As shown in Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20041033/DC1), the recipient CD4$^+$ T

cell number and the number of $CD4^+$ T cells expressing the activation/memory phenotypic markers (CD69, CD44 and $CD62L^{low}$), were reduced significantly in the d3tx/suppressed mice in which the extraovarian autoimmune disease was suppressed completely by 0.5 million T regs.

Polyclonal Thy1.1+ T regs are distributed widely, but AOD-suppressing T regs are enriched in the ovary-draining LNs

To accrue additional evidence for the response of T regs to endogenous antigens in regional LNs, we determined the distribution of total and AOD-suppressing $Thy1.1^+$ T regs in d3tx/suppressed mice. 5,6-carboxyfluorescein diacetate-succinimidyl ester (CFSE)-labeled $Thy1.1^+$ polyclonal T regs proliferated extensively in the d3tx/suppressed mice. By day 23, they made up ~25% of $CD4^+$ T cells in the LNs and the spleen (Fig. 5; not depicted), and >90% had divided at least eight times. We next reisolated the polyclonal Thy 1.1^+ T regs from the ovary or the nondraining LNs of d3tx/suppressed mice, and studied the LN-specific input T regs for suppression of AOD and dacryoadenitis in d3tx mice. This approach was based on the assumption that if ovarian antigen-specific memory T regs existed, they should have enhanced regulatory capacity for suppression of AOD, but not dacryoadenitis.

0.1 million $Thy1.1^+$ T cells at ~90% purity were reisolated from the ovary-draining LNs from 10 d3tx/suppressed donors (Fig. 5 C), and were transferred into a single d3tx recipient. Remarkably, with the suboptimal 0.1 million $Thy1.1^+$ T regs from the ovary-draining LNs, a complete suppression of AOD and oocyte autoantibody response was achieved (Fig. 5 D). In contrast, 0.1 million $Thy1.1^+$ T regs that were reisolated from nondraining LNs did not affect the development of AOD or autoantibody response, although they completely suppressed dacryoadenitis (Fig. 5, C and D). Also important was the failure of the T regs from ovary-draining LNs to suppress dacryoadenitis (Fig. 5 D). Thus, in the ovary-draining LN there is an accumulation of T regs with enhanced AOD-suppressing capacity and a concomitant reduction of dacryoadenitis-specific T regs.

If the T regs with enhanced AOD-suppressing property that accumulated in the regional LNs were responsible for AOD suppression and for negating host T cell responses in this location, these changes should reverse following depletion of the infused T regs from the d3tx/suppressed host.

Thy1.1+ T reg depletion in vivo is followed by reversal of ovary-draining LN suppression and emergence of severe AOD

Infusion of Thy1.1 antibody completely eliminated all $Thy1.1^+$ T reg cells from the 3-wk-old, d3tx/suppressed mice (Fig. 6 B). 4 wk later, the recipient $CD4^+$ T cells in the ovary-draining LNs increased significantly in number as well as in the expression of T cell activation and memory T cell markers (Fig. 6 A). These changes were observed only in the ovary-draining LNs (Fig. 6 A), and not in the nondraining LNs or the spleen (not depicted). Remarkably, severe AOD also emerged in essentially all $Thy1.1^+$ T cell-depleted mice; disease incidence and severity matched that of 7-wk-old d3tx female mice (Fig. 6 A).

Although suppression of the recipient $CD4^+$ T cell response and AOD development depend on the persistent ac-

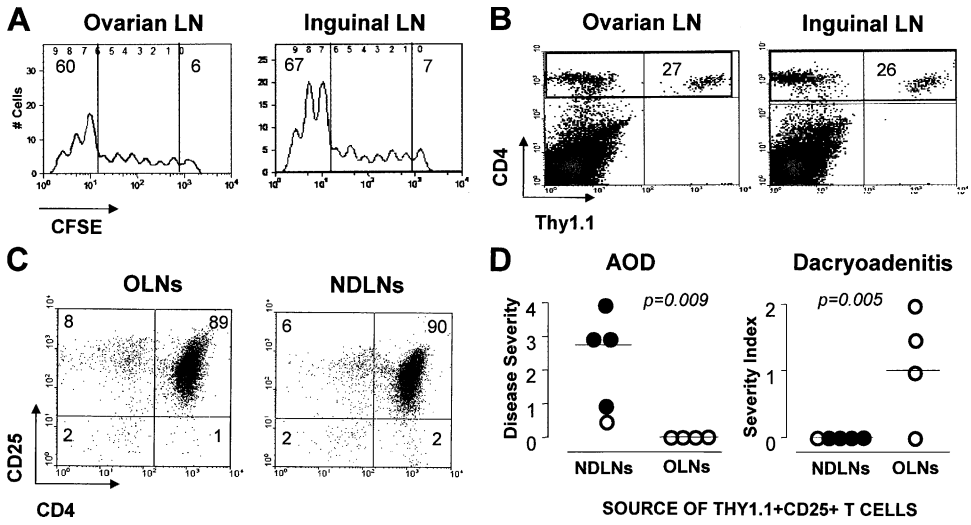

Figure 5. Proliferation, distribution, and function of the polyclonal T reg cells in d3tx/suppressed mice. T regs proliferated and were distributed equally in ovarian LNs and nondraining LNs (A, B). However, the $Thy1.1^+$ T regs (C) that were reisolated from the ovarian LNs (OLNs) but not the nondraining LNs (NDLNs) suppress AOD and oocyte antibody response in d3tx mice at 0.1 million cells per recipient (D). Conversely, dacryoadenitis was suppressed by T regs that were reisolated from NDLNs but not by T regs from OLNs (D). In D, closed circles denote mice with serum oocyte antibody. The NDLNs included the inguinal, axillary, brachial, and cervical LNs. T reg proliferation was determined by injecting 0.5 million CFSE-labeled $Thy1.1^+$ T regs into 5-d-old d3tx B6AF1 mice, and studying them 23 d later. In A, donor cells (gated on $CD4^+Thy1.1^+$ cells) were analyzed for percentages that are undivided, have divided one to six times, or more than six times. The CFSE line graph represents data pooled from four to eight mice.

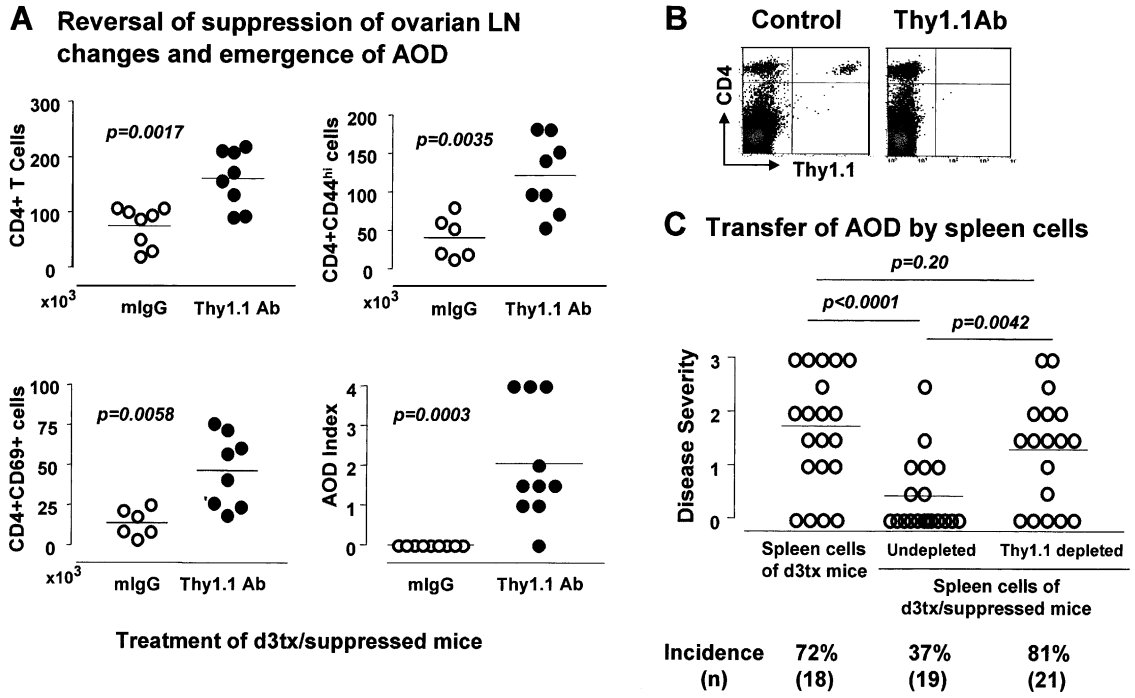

Figure 6. Reversal of AOD suppression by in vivo and in vitro depletion of Thy.1 T reg cells. (A) Reversibility of ovarian LN suppression and AOD. (B) Thy1.1$^+$ T regs in d3tx/suppressed mice were eliminated by a single injection of Thy1.1 mAb at 3 wk, and were studied at 7 wk. (C) To detect functional splenic effector T cells and T regs in the d3tx/suppressed mice, spleen cells were transferred to 5 d-old recipients with or without ex vivo depletion of the infused Thy1.1$^+$ T regs. AOD in cell recipients was determined 12 d later. AOD in recipients of spleen cells from d3tx mice served as control.

tion of the Thy1.1$^+$ T regs in the ovary-draining LNs, suppression was not evident in the spleen, despite the coexistence of polyclonal T regs and effector T cells. Therefore, we investigated the function of regulatory and effector T cells in the spleens of the d3tx suppressed mice by evaluating their capacity to transfer AOD adoptively.

The spleen contains T regs and effector T cells relevant to AOD but exhibits no evidence of T cell suppression in the d3tx/suppressed mice

Compared with d3tx donors, the total splenocytes from the d3tx/suppressed mice transferred much milder and less frequent ovarian inflammation to young naive recipients (Fig. 6 C). This could be due to deficiency in effector T cells in the spleen or to suppression of the donor effector T cells by the Thy1.1$^+$ T regs in the neonatal recipients. To differentiate the two possibilities, the Thy1.1$^+$ T cells were depleted from the splenocytes ex vivo before cell transfer. As shown in Fig. 6 C, splenocytes depleted of Thy1.1$^+$ T regs transferred AOD with comparable severity and frequency to the AOD that was transferred by total splenocytes of d3tx donors. Therefore, despite the colocalization of functional splenic effector T cells and T regs, T cell suppression was not evident in the spleen, presumably because ovarian antigens are not accessible to the spleen. However, when the cells were cotransferred to the neonatal recipients where they are accessible to ovarian antigens, AOD suppression ensues.

AOD is suppressed more effectively by female T regs than male T regs when their encounter with recipient ovarian antigens is avoided for 2.5 wk after cell transfer

Our previous study showed that splenocytes from ovarian antigen-positive females and ovarian antigen-negative males suppressed d3tx-induced AOD with equal efficacy; this was taken as evidence against ovarian antigen-dependency of AOD suppression by T regs (32). However, the interpretation is not consistent with the cumulative evidence provided by the present study. To reevaluate the old data, we reasoned that if T reg function was critically antigen-dependent, then the male T regs would respond to ovarian antigens of the neonatal d3tx recipients, gain AOD-suppressing capacity, and suppress like female T regs. Therefore, we studied AOD suppression in d3tx female recipients devoid of ovarian antigens for the first 3 wk of life.

Neonatal mice were ovariectomized within 24 h of birth and were thymectomized on day 3. On day 5–7, they received 0.3 million CD4$^+$CD25$^+$ T cells from male or female donors. At 3 wk of age they were engrafted with an age-matched ovary, which provided antigenic stimulus for effector T cell response and also served as the target of AOD. In d3tx recipients that were devoid of neonatal ovarian antigen expression, AOD was suppressed completely by T regs from female donors (Fig. 7, A and C), whereas AOD was not suppressed by T regs from male donors (Fig. 7, A and D). The ovarian disease in the recipients of T regs from male donors

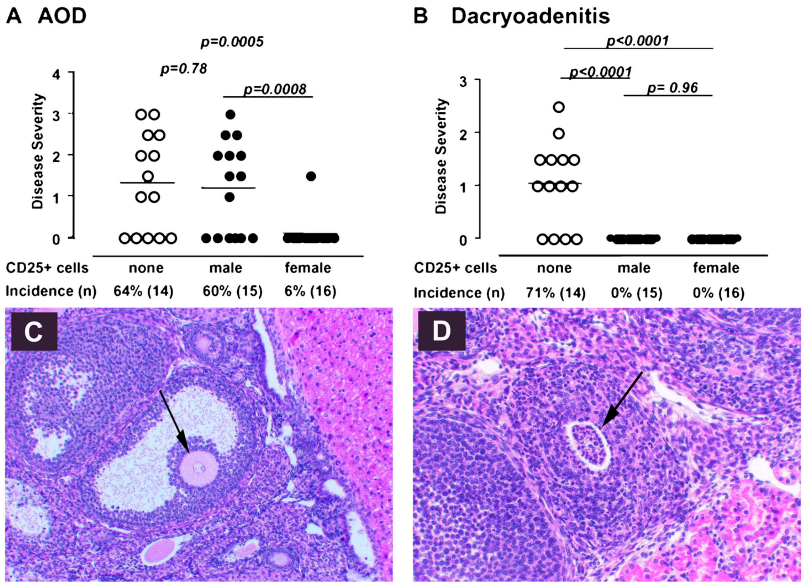

Figure 7. Ovarian antigen exposure in neonatal mice capacitates AOD-specific T reg function. In this experiment, each neonatal recipient received 0.1 million Thy1.1$^+$ cells retrieved from the LNs of d3tx/suppressed mice. (A) AOD does not develop in the d3tx/nOX recipients of female T regs, whereas the d3tx/nOX recipients of male T regs developed AOD with the same incidence and severity as the AOD in control d3tx mice. (B) In contrast to AOD, dacryoadenitis in these d3tx/nOX recipients is inhibited completely by male or female T regs. (C) The ovarian graft of d3tx/nOX recipients of female T regs is normal histologically (arrow points to normal oocyte). (D) The ovarian graft of d3tx/nOX recipients of male T regs is infiltrated heavily by inflammatory cells, some replacing the oocyte (arrow; hematoxylin and eosin).

did not differ from the disease of the d3tx mice in frequency and severity (Fig. 7 A). As an important tissue-specificity control, the T regs from the same male and female donors suppressed dacryoadenitis in the same d3tx/nOX recipients with equal efficacy (Fig. 7 B). We conclude that T regs from the ovarian antigen-positive female donors are intrinsically more competent in AOD suppression than the T regs from antigen-negative male donors. However, when the male T regs have the opportunity to respond to ovarian antigens from the ovary-intact female recipients, they gain AOD-suppressing function and suppress AOD as efficiently as female T regs (32).

DISCUSSION

We have investigated the mechanism of AOD suppression in d3tx mice by polyclonal Thy1.1$^+$ natural T regs from normal donors, and accrued results that strongly support the conclusion that the polyclonal T reg action in this setting is antigen-dependent and disease-specific. (a) Stimulation and suppression of the pathogenic T cell response of d3tx mice occurred exclusively in the ovary-draining LNs in AOD suppression. (b) The LN changes were not unique to AOD because the host T cell suppression was confined to the lacrimal gland–draining LNs in autoimmune dacryoadenitis suppression, and to the prostate-draining LNs in autoimmune prostatitis suppression. (c) Suppression of host T cell response and AOD suppression were reversed upon depletion of the infused Thy1.1$^+$ T regs. (d) In contrast, suppression of the recipients' T cell response was not detectable in the non-draining LNs or the spleen even though effector T cells and T regs relevant to AOD coexisted in these locations. (e) In d3tx mice suppressed by Thy1.1$^+$ T regs, the ovary-draining LN was enriched in Thy1.1$^+$ T regs that exhibited greatly enhanced capacity to suppress AOD, but did not suppress the autoimmune dacryoadenitis that also develops in d3tx B6AF1 mice. (f) T regs from normal females had a greater capacity than male T regs to suppress AOD; however this was demonstrable only in d3tx recipients that were deprived of ovarian antigens in the neonatal period. Thus, a short exposure to ovarian antigens in neonatal recipients is sufficient for male T regs to gain AOD-suppressing capacity to the level of female T regs.

It is generally agreed that in spontaneous autoimmunity, the regional LN is the location of autoimmune response induction (36–39). The obligatory role of the regional LN response in autoimmune diabetes was demonstrated by the experiment wherein excision of the pancreatic LN of young nonobese diabetic mice prevented development of diabetes mellitus (36). In AOD of d3tx mice, neonatal ovarian ablation also abrogated ovarian autoimmune response and prevented AOD development in ovarian grafts at specific time points (35); as shown here, ablation of ovarian antigens also abrogated T cell activation in the d3tx/nOX mice. Thus, changes in the regional LN in spontaneous autoimmune disease are indicative of cellular events of the antigen-specific immune responses. We have now extended this paradigm and demonstrated that the regional LN also is the site of suppression of autoimmune diseases by polyclonal T regs. Even

more remarkable was the finding that Thy1.1$^+$ T regs with greatly enhanced capacity to suppress AOD also accumulate in the ovary-draining LNs. Based on these observations, we conclude that the regional LN is where polyclonal antigen-specific T regs respond to self-antigens, gain disease-suppressing function, suppress pathogenic T cell response, and prevent autoimmune disease.

Suppression of the host CD4$^+$ T cells in the regional LNs affected all levels of T cell response, including profound reduction in DNA synthesis and proliferation of CD4$^+$ T cells, changes in host CD4$^+$ T cell phenotype to indicate reduction of activated and memory T cells, and reduction in proinflammatory IFN-γ production. The regional LN response was not unique to the ovary or to the ovary-draining LNs, because similar changes were observed in autoimmune dacryoadenitis and autoimmune prostatitis. This finding strongly argues that the ovarian LN changes represent the response to ovarian antigen, rather than the response to other unique ovarian factors. The suppression of the host T cell response in the regional LN is critically dependent on the continuous presence of the T reg. When the Thy1.1$^+$ T regs were eliminated by antibody in vivo, host T cell suppression in the regional LNs reversed and was followed by the emergence of severe AOD. Thus, the presence of T regs in the regional LN is linked causally to suppression of AOD. This finding also indicates that T reg action is cytostatic, rather than cytolytic (40–42); that the capacity of T regs to suppress AOD has not been transferred to recipient T cells through infectious tolerance (43–45); and that there has been no significant conversion of recipient CD4$^+$CD25$^-$ T cells to T regs during disease suppression (46, 47). Conversely, we consider the finding to be consistent with the requirement of colocalization and cell–cell interaction of T regs and effector T cells within the draining LNs where ovarian antigens are presented to both T cell subsets (48). Depletion of the infused Thy1.1$^+$ T regs allows the effector T cells to respond to ovarian antigens presented in the regional LNs and mediate AOD. The reversibility of T reg suppression was documented in EAE of Rag knockout mice that expressed transgenic T cell receptor for myelin basic peptide (49), allograft rejection (45), and inflammatory bowel disease (50).

The new finding of the superactive Thy1.1$^+$ T regs in ovary-draining LNs of the d3tx/suppressed mice is of considerable interest. These cells are estimated to be at least five times more potent than the T regs from nondraining LNs when we take into consideration the dose response of AOD suppression by T regs from normal B6AF1 donors, shown in Fig. 1. The finding is reminiscent of the response of TCR transgenic T regs to pancreas-specific cognate antigens (16, 17, 21, 51), and strongly supports the response of polyclonal T regs to ovarian antigens in this location. It also should be noted that although they exhibited superactive AOD-suppressing capacity, the polyclonal T regs from ovary-draining LNs are defective in dacryoadenitis-suppressing function when compared with the T regs in nondraining LNs. Perhaps the nonfunctioning T regs are excluded from the ovary-draining LN when it is dedicated to AOD suppression.

In contrary to the ovary-draining LNs, the nondraining LNs and the spleen showed no evidence of host T cell suppression. This was not due to the absence of functional effector or regulatory T cells because both activities were demonstrable when the cells were transferred adoptively, singly or together, into the naive neonatal recipients. As a more tenable explanation, we believe that suppression did not occur in the nondraining LNs or the spleen of the d3tx/suppressed host because ovarian antigens were not presented in those locations; however, the function of the T cells was manifested when they were stimulated by ovarian antigens of the cell recipients after adoptive transfer. This possibility is supported by the fact that immunogenic ovarian antigens are present in neonatal mice and have been documented to stimulate pathogenic T cells responsible to neonatal AOD (35, 52–54).

If endogenous antigens can prime antigen-specific T reg function, then the polyclonal T regs from normal female donors should suppress AOD more efficiently than the polyclonal T regs from normal male donors. However, our earlier study showed that both populations suppressed d3tx-induced AOD equally (32). We have now documented that the equal AOD suppression by male and female T regs is explicable by the response of male T regs to recipient ovarian antigens; this finding strongly supports, rather than refutes, the antigen-dependency of polyclonal T reg function.

In conclusion, this study provides strong evidence that endogenous antigens are major players in spontaneous autoimmunity. They stimulate pathogenic T cells in disease induction. They also stimulate antigen-specific polyclonal T regs to gain disease-suppressing capacity quickly and to accumulate in the regional LNs, the site of ovarian antigenic stimulation. In AOD, the ovarian antigen orchestrates important cellular events in the ovary-draining LNs during disease suppression, and allows the accumulation of highly potent AOD-specific polyclonal T regs that continuously negate pathogenic T cell response and inhibit AOD.

MATERIALS AND METHODS

Mice and surgery. C57BL/6 × AJ (B6AF1) mice were produced from C57BL/6 and AJ adults from the National Cancer Institute. B6.PL-Thy1a/Cy (Thy1.1$^+$) female mice came from The Jackson Laboratory. All mice were kept in a pathogen-free facility; experiments were approved and performed in accordance with the guidelines of the Animal Care and Use Committee of University of Virginia. Thymectomy was performed by suction under hypothermia anesthesia (35); complete thymectomy was verified histologically and mice with residual thymus excluded. Bilateral nOX was performed through vertical posterior incision under hypothermia; mice with residual ovaries were excluded. Ovary was implanted by insertion of a 3-wk-old ovary under kidney capsule through posterior incision.

Media, reagents, and antibodies. Cells were harvested in complete RPMI 1640 medium (Biowhittaker) supplemented with 10% heat-inactivated FCS, 2-mercaptoethanol (5×10^{-5} M), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), Hepes (10 mM), nonessential amino acids (0.1 mM), and sodium pyruvate (1 mM) (Invitrogen). The fol-

lowing antibodies and isotype controls (BD Biosciences) were used for flow cytometric analysis and cell isolation: biotin-conjugated anti-CD25 (7D4), anti-CD16/CD32 (2.4G2), anti-CD90.1 (HIS51), FITC-conjugated anti-CD4 (H129.19), CyC-conjugated anti-CD4 (L3T4), PE-conjugated anti-CD4 (L3T4), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD25 (7D4), anti-CD69 (H1.2F3), anti-CD90.1 (HIS51) rat IgG2a (R35-95), rat IgG2b (R35-38), rat IgM (R4-22), hamster IgG (A19-3), anti–IFN-γ (XMG1.2), anti-CD25 (PC61), anti-CD90.1 (OX-7), rat IgG1 (R3-34), rat IgG2a (R35-95), and rat IgG2a (R35-95).

LN dissection, cell purification, and CFSE labeling. LNs that drain the ovary (renal), prostate (iliac or lumbar), and lacrimal gland (superficial cervical), as well as the axillary, brachial, and inguinal (nondraining) LNs were dissected from 8–10-wk-old Thy1.1$^+$ B6AF1 mice and dissociated into single-cell suspension. CD4$^+$CD25$^+$ T cells were purified as described (55). Enriched on T cell enrichment columns (R&D Systems), T cells were stained with biotin-conjugated anti-CD25 antibody (7D4) or anti-Thy1.1 antibody, followed by PE-conjugated streptavidin (Rockland Immunochemicals) and anti-PE microbeads (Miltenyi Biotec), and isolated on the autoMACS system (Miltenyi Biotec). Cell purity ranged from 90 to 95%. To suppress disease, the CD4$^+$CD25$^+$ T cells—in 50 μl of HBSS—were injected i.p. into 5-d-old d3tx pups. CD4$^+$CD25$^+$ T cells were labeled with CFSE (Invitrogen) by incubation for 10 min at 37°C in 10μM CFSE in HBSS, washed in complete RPMI 1640 and then HBSS, and injected i.p. in 5-d-old d3tx pups.

Histology and disease grading. Tissues were fixed in Bouin's fixative, embedded in paraffin, and 5-μm serial sections were stained with hematoxylin and eosin, and examined as unknown samples. Ovarian pathology was evaluated in 50 step sections, and graded on a scale of 1–4 (35). Grade 1 inflammation consists of 1–2 foci of inflammatory cells, including inflammatory infiltration restricted to the hilar region. Grades 2 and 3 include incremental extent of ovarian inflammation, involving follicles and interstitial space, but no ovarian atrophy. Grade 4 pathology has ovarian atrophy with loss of mature, growing, and/or primordial oocytes. Dacryoadenitis and prostatitis were graded from 1 to 4 as follows. Grade 1 disease was focal mononuclear infiltration. Grade 4 lesions had diffuse inflammation with atrophy and loss of glandular and ductal structure. Grades 2 and 3 represent increasing inflammation between grades 1 and 4.

Immunofluorescence microscopy. Antibody to ovarian oocytes was detected by indirect immunofluorescence, and the intensity was graded from 1–3. Serum diluted 1:50 in PBS, was incubated with frozen sections of adult mouse ovaries prefixed in 95% ethanol and blocked by goat serum, followed by incubation with FITC-conjugated goat anti–mouse IgG (Jackson ImmunoResearch Laboratories). Inflammatory cells in normal and diseased ovaries were identified by immunofluorescence using the TSA Biotin System (PerkinElmer), with antibody to CD5 (53–7.313), CD4 (GK1.5), CD8 (53–6.7) T cells, macrophage (F4/80), B cell (B220), and MHC class II (M5/114.15-2) for activated macrophages and dendritic cells, as described previously (56).

Cell counting. LN cells, dissociated by syringe plunger, were incubated in HBSS containing collagenase D (Roche Applied Sciences; 400 U/ml) and collagenase VII (Sigma-Aldrich; 100 U/ml) for 20 min at 37°C. The cells were filtered through nylon and washed in RPMI 1640. Cell number was determined with hemacytometer or automatic counter (Hemavet 850, CDC Technologies Inc.), with comparable results.

Flow cytometric analysis and intracellular cytokine staining. LN cell suspension (10^6 per well) was incubated with anti-CD16/CD32 antibody to block IgG-Fc receptors, which was followed by 30-min incubation with FITC-, PE-, and/or CyC-conjugated monoclonal antibodies. Isotype control IgG was included. Cells were studied on a FACScan and analyzed by Cell Quest software (BD Biosciences), the FlowJo software (Tree Star, Inc.), or by WINMDI (http://facs.scripps.edu/software.html). For intracellular cytokines, cells treated with phorbol-12-myristate-13-acetate, ionomycin, and brefeldin A (BD Biosciences) for 6 h were stained with anti-CD4-CyC, fixed and permeabilized in formaldehyde/saponin (Fix/Perm Buffer, BD Biosciences), washed, and stained with PE-conjugated antibody to cytokine or control antibody. Cells were washed in buffer containing saponin before study.

BrdU labeling. D3tx B6AF1 mice were injected i.p. with 1 mg of BrdU in PBS on day 25 and studied 12 h later, or they were fed BrdU, 0.8 mg/ml, in drinking water starting on day 21 for 4 d. Cells from individual LNs were analyzed for BrdU incorporation using BrdU Flow Kits (BD Biosciences). In brief, LN cells, stained with PE-conjugated anti-CD4 antibody, fixed, and permeabilized, were treated with DNase (Sigma-Aldrich), stained with FITC-labeled anti-BrdU antibody (BD Biosciences), and analyzed by flow cytometry.

In vivo and in vitro cell depletion. D3tx/suppressed B6AF1 mice received one i.v. injection of 100 μg mouse antibody to Thy1.1 [CD90.1 (HIS51)] at 3 wk, and the completeness of cell depletion was verified by flow cytometry at 7 wk. For ex vivo Thy1.1$^+$ cell depletion, splenocyte suspension was incubated with FITC-conjugated anti-Thy1.1 (CD90.1) (HIS51) (BD Biosciences), followed by anti-FITC microbeads (Miltenyi Biotec) and separation on the autoMACS system (Miltenyi Biotec).

Adoptive transfer of AOD. 10^7 total splenocytes, or those depleted of Thy1.1 cells, were injected i.p. into 3–5-d-old naive pups. Ovarian pathology of cell recipients was determined 12 d later.

Online supplemental material. Fig. S1 shows that the recipient CD4$^+$ T cell number and the number of CD4$^+$ T cells expressing the activation/memory markers were reduced significantly in the prostate and lacrimal gland draining but not the inguinal LNs. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20041033/DC1.

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