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This information is current as of May 9, 2021.

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J Immunol 2002; 168:5979-5983; ;
doi: 10.4049/jimmunol.168.12.5979
<http://www.jimmunol.org/content/168/12/5979>

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Cutting Edge: Depletion of CD4⁺CD25⁺ Regulatory T Cells Is Necessary, But Not Sufficient, for Induction of Organ-Specific Autoimmune Disease

Rebecca S. McHugh and Ethan M. Shevach¹

Thymectomy of BALB/c mice on day 3 of life results in the development of autoimmune gastritis (AIG) due to the absence of CD4⁺CD25⁺ regulatory T cells. However, depletion of CD4⁺CD25⁺ T cells by treatment with anti-CD25 rarely resulted in AIG. Depletion was efficient, as transfer of splenocytes from depleted mice induced AIG in *nu/nu* mice. One explanation for this result is that CD4⁺CD25⁻ T cells upon transfer to nude recipients undergo lymphopenia-induced proliferation, providing a signal for T cell activation. Cotransfer of CD25⁺ T cells did not inhibit initial proliferation but did suppress AIG. Surprisingly, immunization with the AIG target Ag, H/K ATPase, in IFA failed to induce disease in normal animals but induced severe AIG in CD25-depleted mice. These results demonstrate that second signals (nonspecific proliferation, TCR activation, or inflammation) are needed for induction of autoimmunity in the absence of CD25⁺ regulatory T cells. *The Journal of Immunology*, 2002, 168: 5979–5983.

Experimental organ-specific autoimmune disease in susceptible strains of mice has been induced by neonatal thymectomy on day 3 of life (d3Tx)² and other protocols that result in removal or delay in the development of regulatory CD4⁺ T cells, primarily CD4⁺CD25⁺ T cells (1, 2). Reconstitution of d3Tx mice with purified CD4⁺CD25⁺, but not CD25⁻, T cells on day 10 of life completely prevents the induction of autoimmunity. Similarly, transfer of CD4⁺CD25⁻ T cells to immunoincompetent recipients results in a spectrum of autoimmune disease that closely resembles that seen after d3Tx; cotransfer of CD4⁺CD25⁺ T cells prevents the development of disease.

The mechanism by which CD4⁺CD25⁺ T cells prevent the development of autoimmune disease remains unknown (3). One difficulty in the interpretation of many of the earlier studies is that most of the protocols, including d3Tx (4), involved induc-

tion of a generalized state of partial lymphocyte depletion or transfer of cells to a lymphopenic host in addition to depletion of CD4⁺CD25⁺ T cells. Indeed, it has been proposed that the lymphopenic state itself may promote the activation of autoreactive effector cells and that reconstitution of a depleted animal with either CD4⁺CD25⁺ or CD4⁺CD25⁻ would prevent disease development (4, 5). A much more direct test of the role of CD4⁺CD25⁺ T cells in regulating the development of autoimmunity *in vivo* would be their selective removal from the intact animal. In the present report, we have attempted to define the requirements for induction of autoimmune disease following depletion of CD4⁺CD25⁺ T cells. We demonstrate that selective depletion of CD4⁺CD25⁺ only rarely results in the development of autoimmunity and that an additional signal is required to activate autoreactive effector CD4⁺CD25⁻ T cells. This signal may be supplied by the induction of proliferation following transfer to a lymphopenic environment or by activation of the effector T cells via strong stimulation of their TCR with specific Ag.

Materials and Methods

Mice, Abs, and reagents

BALB/c females (6–8 wk old), 14-day gestation BALB/c females, BALB/c *nu/nu* females (6–8 wk old), and C.B-17 SCID females (6–8 wk old) were purchased from the National Cancer Institute animal facility (Frederick, MD) and housed under specific pathogen-free conditions. H/K ATPase was obtained by purification of microsomes from rabbit stomach (6). PC61 (anti-CD25) hybridoma was purchased from American Type Culture Collection (Manassas, VA) and ascites was produced in C.B-17 SCID mice. For all injections, an ammonium sulfate cut of PC61 ascites was used. Abs purchased from BD Pharmingen (San Diego, CA) were FITC-anti-CD25 (7D4) and PE-anti-CD25 (PC61). Tri-color (TC)-anti-CD4 was purchased from Caltag Laboratories (Burlingame, CA). Rat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). CFSE was purchased from Molecular Probes (Eugene, OR).

In vivo depletion, immunization with H/K ATPase, and assessment of AIG

BALB/c mice were given 1 mg PC61 every 3 days *i.p.* beginning day 10 of life for 2 wk. Alternatively, mice were given three injections from days 17 to 23 or one injection on day 23 of life only. Depletion of CD25⁺ cells was confirmed by flow cytometry of PBMC using anti-CD4-Tri-color and anti-CD25-FITC (clone 7D4). All flow cytometry was performed on a BD FACScan or FACSCalibur and analyzed using CellQuest software (BD Immunocytometry Systems, San Jose, CA). After depletion, animals were followed until 3 mo of age and were analyzed for the production of anti-parietal cell Abs and gastric pathology (6).

PC61 treated or nontreated BALB/c mice were immunized *s.c.* in the hind flank with 50 μ g of H/K ATPase containing rabbit microsomes emulsified in CFA or IFA in a volume of 50 μ l. These animals were then assayed 6–10 wk later for anti-parietal cell Abs and gastric pathology.

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Received for publication March 18, 2002. Accepted for publication April 22, 2002.

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² Abbreviations used in this paper: d3Tx, thymectomy on day 3 of life; AIG, autoimmune gastritis.

Purification of cells and transfer to recipient animals

Splenocytes ($10\text{--}20 \times 10^6$) from depleted and nondepleted animals were harvested and transferred i.v. into 6- to 8-wk-old BALB/c wild-type and *nu/nu* females. These animals were analyzed for anti-parietal cell Abs and gastric pathology at 5–6 wk posttransfer.

CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were purified and in vitro suppression assays were performed as described previously (7). For some experiments cells were labeled with CFSE at a final concentration of 1 μM in PBS for 8 min at room temperature. Labeled CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells (5×10^6) were injected i.v. alone, or labeled CD4⁺CD25⁻ T cells were injected in combination with unlabeled CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells (5×10^6) into BALB/c *nu/nu* animals. Labeled CD4⁺CD25⁻ cells were also injected into BALB/c hosts as nonproliferating controls. Cell division was measured by loss of CFSE on live-gated CD4⁺ cells at various time points posttransfer by flow cytometry using a BD FACSCalibur and analyzed using CellQuest software.

Results

In vivo depletion of CD4⁺CD25⁺ T cells does not result in the development of AIG

The anti-CD25 mAb, PC61, is capable of depleting CD25⁺ cells in vivo (8). We initially injected 1 mg of PC61 every other day for 2 wk beginning on day 10 of life. One day after the last injection (day 24 of life), CD25⁺ T cells were markedly depleted from peripheral blood (Fig. 1A, 10.5 to 0.8%). Identical results were observed when mice were injected once on day 23 of life or every other day between days 17 and 23 (data not shown). The depleted mice were followed until they were 3 mo of age. A low (10%) but consistent incidence of spontaneous autoimmune gastritis (AIG) was observed as measured by the presence of autoantibodies to gastric parietal cells or gastric pathology (Fig. 1B). In contrast, when BALB/c mice are subjected to d3Tx, the incidence of AIG is

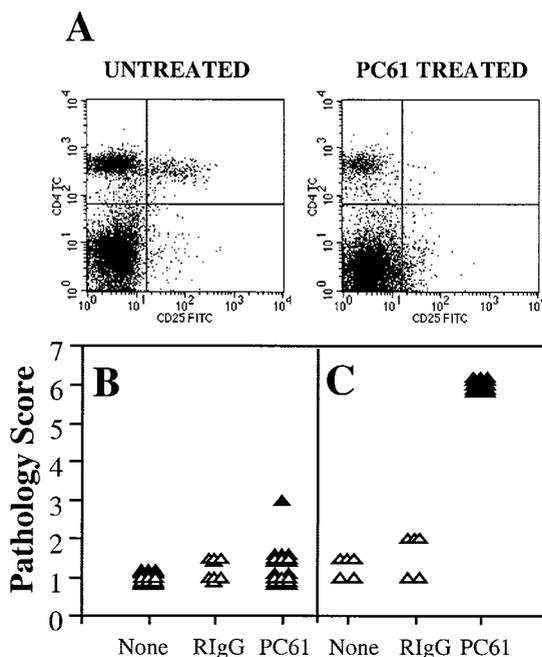


FIGURE 1. In vivo treatment with PC61 depletes CD25⁺ cells but does not induce AIG. **A**, BALB/c mice (day 10) were injected every other day for 2 wk with PC61 ascites. One day following the final injection, PBMC were analyzed for the presence of CD4⁺CD25⁺ cells by flow cytometry. **B**, BALB/c mice treated with PC61 or control rat IgG, as in **A**, or left untreated were monitored for the production of anti-parietal cell Abs and gastric pathology at 3 mo of age. \blacktriangle , Animals positive for anti-parietal cell Abs. **C**, Splenocytes from BALB/c mice, treated as in **B**, were harvested 1 day after final Ab injection and transferred to BALB/c *nu/nu* mice. Recipient mice were analyzed 5–6 wk posttransfer for AIG.

~60%. To rule out the possibility that depletion was not complete and that the low number of surviving CD4⁺CD25⁺ cells were capable of controlling the induction of autoimmunity, we transferred splenocytes from 24-day-old PC61-depleted animals to BALB/c *nu/nu* recipients. All (100%) of the PC61-depleted splenocyte recipients developed severe destructive gastritis (Fig. 1C). As a positive control for suppression of the development of disease in recipients, we cotransferred CD4⁺CD25⁺ T cells from normal adult mice with these splenocytes into *nu/nu* recipients; none of the recipients of the cotransferred cells developed AIG (data not shown).

Control of autoimmunity in CD25-depleted animals does not result from repopulation of CD25⁺ T cell pool

As we did not thymectomize the animals following depletion of the CD25⁺ T cells, it remained possible that CD25⁺ cells could be re-emerging from the thymus and repopulating the periphery, controlling the induction of autoimmunity. Indeed, analysis of the percentage of CD4⁺CD25⁺ population at different time points following depletion did reveal a slow, partial recovery of the percentage of CD25⁺ T cells in splenocytes, as well as peripheral blood with 3–4% of the CD4 pool expressing CD25 at 6 wk post-depletion (Fig. 2A). To determine whether this low number of CD25⁺ cells is capable of suppressing the induction of AIG, we transferred splenocytes from 24-day-old depleted animals to *nu/nu* recipients at various time points following depletion and monitored

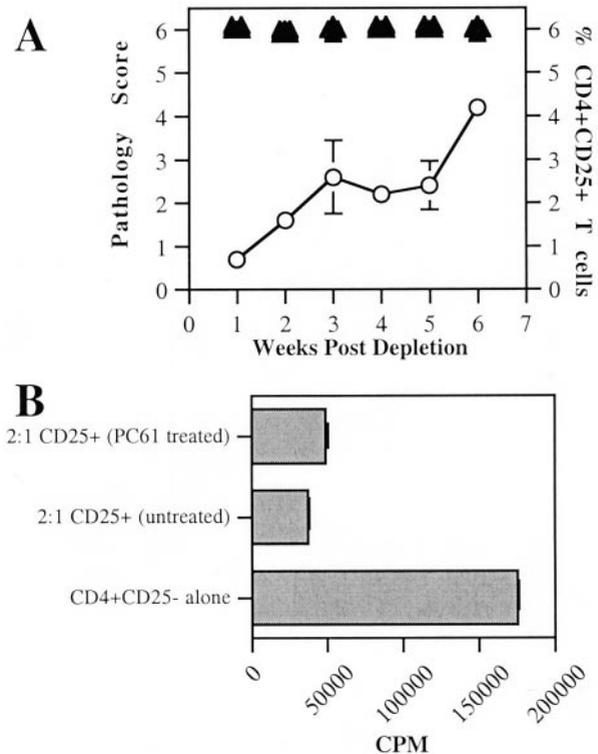


FIGURE 2. CD4⁺CD25⁺ cells do repopulate following depletion but do not control AIG upon transfer to BALB/c *nu/nu* mice. **A**, BALB/c mice (day 17) were treated with PC61 every other day for 1 wk. Splenocytes were harvested at various time points following depletion and transferred to BALB/c *nu/nu* animals. At the time of transfer, the percentage of CD4⁺CD25⁺ T cells was measured in the donor splenocytes. Animals were analyzed for AIG 5–6 wk posttransfer. **B**, CD4⁺CD25⁺ T cells were purified from animals 6 wk postdepletion or from untreated BALB/c mice. These cells were used at a 2:1 responder to suppressor cell ratio in an in vitro suppression assay.

Discussion

The general approach used to analyze the role of regulatory T cell function *in vivo* is to compare the incidence of autoimmune disease in the presence of T cell populations that have been depleted of regulatory T cells with the incidence of disease in T cell populations that contain regulatory T cells. These studies are performed by transfer of the T cell populations to a T cell-deficient recipient, most frequently an immunoincompetent *nu/nu*, SCID, or recombination-activating gene^{-/-} mouse. A major difficulty interpreting the prevention of disease by cotransfer of regulatory T cells is that T lymphocytes undergo a rapid, vigorous proliferative response when transferred to such a recipient. Therefore, it has been proposed that a major component of regulatory T cell function is to nonspecifically inhibit this lymphopenia-induced expansion.

In an attempt to more accurately define a role for CD4⁺CD25⁺ suppressor T cells in the control of organ-specific autoimmune disease in the absence of lymphopenia-induced proliferation, we selectively depleted them with Ab. Depleted animals only rarely developed AIG, the most common manifestation of a deficiency of regulatory T cell function in BALB/c mice. This result must be compared with that of Taguchi and Takahashi (8), who observed a significant increase in organ-specific autoimmune disease using a very similar protocol. This difference may be secondary to the different animal strains used, as our studies involved the induction of AIG in BALB/c mice, whereas Taguchi and Takahashi (8) used (B6 × A/J)F₁ animals, which are more susceptible to diseases of the reproductive system following d3Tx. Curiously, CD25-depleted (B6 × A/J)F₁ mice developed gastritis, normally not observed in that strain after d3Tx, while they failed to develop diseases of the reproductive system. Importantly, our data are consistent with studies (14, 15) in which depletion of CD25⁺ cells to induce tumor immunity did not enhance susceptibility to autoimmune disease. One trivial explanation for our failure to observe autoimmune disease following depletion of CD25⁺ T cells in euthymic animals is that the depletion was inadequate or that new thymic emigrants rapidly repopulated the CD4⁺CD25⁺ pool. This explanation is highly unlikely, because transfer of T cells from CD25-depleted animals to *nu/nu* recipients for up to 6 wk following depletion resulted in the induction of autoimmune disease in 100% of recipients, even when partial reconstitution of the CD25⁺ population was observed.

The disparity between the ability to transfer disease to a lymphopenic animal, while the lymphocyte-sufficient, CD25-depleted donor does not develop autoimmune disease, forced us to reexamine the contribution of lymphopenia for the induction of organ-specific autoimmunity. Annacker et al. (16) have proposed that a primary function of CD4⁺CD25⁺ T cells is to regulate the homeostatic proliferation of peripheral T cells, most likely by the secretion of IL-10. However, the concept of homeostatic proliferation must be clearly defined. In the lymphopenic animal, transferred T cells are stimulated through TCR/MHC interactions to proliferate and fill the “empty” space to reach T cell homeostasis (17). Unseparated CD4⁺, CD4⁺CD25⁻ (CD45RB^{high}), and CD4⁺CD25⁺ T cells are capable of proliferating in a lymphopenic host with proliferation beginning on days 3–4 posttransfer and reaching as many as eight divisions by days 21–28 (12, 16). We did not observe a suppressive effect of cotransfer of CD4⁺CD25⁺ T cells on this early phase of proliferation of CD4⁺CD25⁻ T cells, particularly when compared with cotransfer of an equivalent number of CD4⁺CD25⁻ T cells. It is likely that the modest reduction of the proliferation of CD4⁺CD25⁻ T cells observed by Annacker et al. (16) by cotransfer of CD4⁺CD25⁺ T cells was secondary to an overall increase in the number of injected

T cells (12). Cotransfer of CD4⁺CD25⁻ T cells was not examined by Annacker et al. (16).

In contrast to the lack of an effect of CD4⁺CD25⁺ T cells on the early phase of the expansion of CD4⁺CD25⁻ T cells, regulatory T cells have been reported to exert a profound effect on the accumulation or steady state numbers of CD4⁺CD25⁻ T cells when the recipient animals are studied 2–6 mo after cell transfer (16, 18). This suppressive effect of cotransfer of CD4⁺CD25⁺ T cells on the late accumulation of CD4⁺CD25⁻ T cells must be interpreted with caution. Recipients of CD4⁺CD25⁻ T cells frequently develop autoimmune diseases (1, 19) and therefore have undergone Ag-specific stimulation and expansion in addition to lymphopenia-driven T cell proliferation. CD4⁺CD25⁺ T cells are known to inhibit the induction of these autoimmune diseases. Indeed, the requirement for IL-10 to control the late expansion of the CD4⁺CD25⁻ in BALB/c recombination-activating gene 2^{-/-} mice reported by Annacker et al. (16) is most consistent with the requirement for IL-10 production by CD4⁺CD25⁺ T cells to prevent the induction of inflammatory bowel disease that develops in these recipients (20, 21).

Collectively, our data are most consistent with a model in which CD4⁺CD25⁺ T cells play no role in the control of lymphopenia-induced proliferation, but that the proliferative response functions as a second signal that is required for the differentiation of CD4⁺CD25⁻ autoreactive effector cells. Our ability to readily induce AIG in CD25-depleted, but not normal, animals by stimulation with the target Ag in IFA also suggests that strong TCR stimulation can provide the necessary second signal. We have not yet tested what other environmental inflammatory insults might be able to precipitate induction of autoimmunity in the CD25-depleted animal. Last, these results have important implications with regard to the therapeutic manipulation of CD4⁺CD25⁺ T cells *in vivo*. Depletion of CD4⁺CD25⁺ T cells greatly enhances the ability of the host to respond to immunization with a weak tumor vaccine (14, 15, 22). Depletion of CD4⁺CD25⁺ T cells might also be useful in the enhancement of immune responses to weak vaccines to infectious agents or to promote sterilizing immunity in the setting of chronic indolent infections. Although young adult euthymic mice appear to repopulate the CD4⁺CD25⁺ T cell pool within 2 mo following depletion, extrapolation of this finding to humans should be undertaken with caution in the absence of data on the capacity of the adult human thymus to produce CD4⁺CD25⁺ T cells.

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