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Anti-ergotypic T cells in naïve rats

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Abstract

T regulatory cells play an important role in regulating T cell responses. Anti-ergotypic T cells are a subset of regulatory T cells that proliferate in response to activation markers, ergotopes, expressed on activated, and not on resting syngeneic T cells. Here we report the presence of anti-ergotypic T cells in lymph nodes, spleens and thymuses of naïve rats. The development of anti-ergotypic T cells appeared to be independent of antigen priming, as thymocytes from one-day old rats exhibited significant anti-ergotypic proliferative responses. The anti-ergotypic T cells were found to be of the $CD8^+$ phenotype, and included both $TCR\alpha/\beta^+$ and $TCR\gamma/\delta^+$ T cells. The $TCR\gamma/\delta^+$ anti-ergotypic T cells secreted IFNγ and TNFα in response to activated T cells; the $TCR\alpha/\beta^+$ T cells proliferated but did not secret detectable cytokines. We found that the interaction between the anti-ergotypic T cells and stimulator T cells required cell-to-cell contact between the T cells. Professional APCs were not needed. The response of the $TCR\alpha/\beta^+CD8^+$ anti-ergotypic T cells was MHC-I restricted and B7-CD28 dependent; the response of the $TCR\gamma/\delta^+$ anti-ergotypic T cells was B7-CD28 dependent, but was not inhibited by antibodies to classical MHC-I or MHC-II molecules. The existence of anti-ergotypic T cells in naïve animals suggests that these cells might have a role in the regulation and maintenance of the immune system.

Keywords: T lymphocytes; Regulatory T cells; Autoimmune diseases; Spleen; Lymph nodes and thymus

1. Introduction

Over the last decade, evidence has accumulated regarding different populations of T regulatory (Tr) cells, mainly in the context of the control of autoimmunity [1–6]. Anti-ergotypic T cells are a subset of regulatory T cells that respond to activation markers, ergotopes, expressed on activated, syngeneic T cells. Anti-ergotypic T cells do not respond to non-activated T cells. Anti-ergotypic T cells were first discovered in Lewis rats following T-cell vaccination (TCV), and these T cells could protect rats from experimental autoimmune encephalomyelitis (EAE) following adoptive transfer [7]. Anti-ergotypic T cells could also be isolated from T-cell vaccinated human individuals [8–11]. Thus, anti-ergotypic T cells have been proposed as one of the mechanisms by which TCV induces resistance to autoimmune diseases [12,13].

In the initial studies, anti-ergotypic T cells were defined in cell populations from immunized rats, and relatively little was known about their origin. During previous experiments, while studying the protective effect of an ergotypic DNA vaccine on autoimmune diseases, we noticed the existence of anti-ergotypic T cells also in naïve rats [14]. In the present work, we characterized the

Abbreviations: Tr, T-regulatory; TCV, T-cell vaccination; EAE, experimental autoimmune encephalomyelitis; Mt, mycobacterium tuberculosis; TCGF, T-cell growth factors; ConA, concanavalin A; A2b-S, stimulated A2b; A2b-R, resting A2b; LNC, lymph node cells; AMLR, autologous mixed leukocyte reaction; SPF, specific pathogen free; MS, multiple sclerosis.

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anti-ergotypic T cells in naïve rats: whether they develop in the thymus, their phenotype and cytokine profile, their interaction with stimulator T cells and their MHC and accessory-molecule restrictions.

2. Materials and methods

2.1. Rats

Naïve female Lewis rats were raised and maintained under pathogen-free conditions in the Animal Breeding Center of the Weizmann Institute. Experiments were carried out under the supervision and guidelines of the Animal Welfare Committee. The rats were 8–10 weeks old unless stated otherwise.

2.2. Stimulator T cell clone

For ergotypic stimulation, we used the Lewis rat A2b T cell clone, specific for the p180–188 peptide of the 65 kDa heat shock protein of Mycobacterium tuberculosis (Mt) [15,16]. A2b expresses MHC class-I constitutively, and MHC class-II upon activation (not shown). A2b was grown in stimulation medium composed of DMEM supplemented with 2-ME (5×10^{-5} M), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), non-essential amino acids (1 ml/100 ml) and 1% autologous serum. To activate A2b, $5-7\times10^{6}$ cells were stimulated with 10^{8} syngeneic irradiated thymocytes as APCs together with their target peptide at a concentration of 10 μg/ml in 10 mm plates. The peptide used to stimulate A2b was synthesized by a standard F-moc procedure as previously described [17] and purified by reverse phase HPLC; the sequence is EESNTFGLQLELTEG. After 3 days of stimulation, the A2b cells were transferred without added APCs or specific peptide to rest medium (as above, but containing 10% FCS instead of autologous rat serum and 5% TCGF: T cell growth factors prepared from the supernatant of concanavalin A (ConA) activated spleen cells [18]). Activated A2b cells (A2b-S) were used on day 3 of their stimulation, and resting A2b cells (A2b-R) were used on day 14–16 of their rest cycle, unless stated otherwise.

2.3. T-cell proliferation assay

Single-cell suspensions were prepared from lymph node cells (LNC), spleens or thymuses of naïve Lewis rats by pressing the organs through a fine wire mesh, and the cells were cultured in quadruplicates, 2×10^{5}/200 μl, in round-bottom microtiter wells (NUNC, Roskilde, Denmark). A2b stimulator cells, activated or resting, were irradiated (5000 R) and added to the test cultures in 2-fold dilutions, starting from 10^{5} cells per well. No other APCs were added. Monoclonal antibodies, 10 μg/ml, to MHC class-I, MHC class-II, B7.1, B7.2 or CD28 (Serotec, Oxford, UK) were added where indicated. ConA was used at a concentration of 1.25 μg/ml as a positive control for T cell proliferation. Stimulation medium in the proliferation assays was the same as that used for A2b stimulation (see above). Cultures were incubated for 72 hours at 37 °C in humidified air containing 7% CO_2. Each well was pulsed with 1 μCi of [^{3}H]Thymidine (Amersham, Buckinghamshire, UK) for the last 16 hours. The cultures were then harvested and cpm were determined using a beta counter. The Δcpm was calculated by subtracting the mean cpm of spontaneous proliferation (wells containing responder T cells without stimulator T cells) from the mean cpm of each quadruplicate of T cells proliferating in response to stimulator T cells.

2.4. Transwell system

Transwell experiments were carried out in 24-well plates (Corning Costar, Cambridge, MA), in which 10^{6} LNC were placed in the bottom well and 2.5×10^{5} activated and irradiated A2b stimulators in the upper transwell. Cultures were incubated for 72 hours at 37 °C in humidified air containing 7% CO_2. Each well was pulsed with 4 μCi of [^{3}H]Thymidine (Amersham, Buckinghamshire, UK) for the last 16 hours. A volume of 200 μl of each well was transferred to a 96-well plate for harvesting as mentioned above. The Δcpm of the LNC was calculated as mentioned above.

2.5. Cytokine assays

Supernatants from the T cell proliferation experiments were collected at 72 hours. Rat IFNγ, TNFα, IL-10, IL-4 and IL-2 were quantified by ELISA using Pharmingen's OPTEIA™ kits for each of the cytokines (Pharmingen, San-Diego, CA), according to the manufacturer's instructions. Rat TGFβ was quantified using the TGFβ E_{max}® ImmunoAssay System (Promega, Madison, WI) according to the manufacturer's instructions.

2.6. Separation of thymocytes

For the isolation of double-positive thymic T cells, single-cell suspensions of naïve rat thymocytes were subjected to two rounds of 60%–70% Percoll (Pharmacia) gradient separation. Fractions were analyzed by FACS for CD4 and CD8 expression (Table 1).

2.7. $CD25^+$ cell depletion

$CD25^+$ cells were depleted from naïve LNC using a magnetic bead separation technique. LNC, 10^{8}, were incubated in reaction buffer (PBS without Mg and Ca,

Table 1
Thymocyte separation by 60%/70% Percoll gradient

Responders	Percent of cells			
	$CD4^+CD8^-$	$CD4^-CD8^+$	$CD4^+CD8^+$	$CD4^-CD8^-$
Whole Thymus	5.63	6.97	86.04	1.36
Top Ring	18.24	19.68	58.36	3.72
Bottom Ring	0.08	0.88	99.00	0.03

containing 5% FCS), at 5×10^7 cells/ml. Biotinylated anti-rat CD25 (Serotec, Oxford, UK) was added (6 μl per 10^6 cells) and incubated for 15 minutes at room temperature (RT). The cells were centrifuged and resuspended in reaction buffer with the addition of 10% autologous rat serum (for blocking) for 10 minutes at RT. Anti-biotin tetrameric antibody complexes were added (100 μl/ml) for 15 minutes at RT followed by the addition of 60 μl/ml of magnetic colloid for an additional 15 minutes at RT. The cells were separated by loading on a StemSep 0.3'' column attached to a magnet. The above were all purchased from StemCell Technologies, Vancouver, Canada. FACS analysis was used to measure the reduction of the $CD4^+CD25^+$ T-cell population, from 6 to 8% in naïve $CD4^+$ populations, to $<0.2\%$ in treated T-cell populations.

2.8. Purification of $CD4^+$, $CD8^+$, $TCR\gamma/\delta^+$ and $TCR\alpha/\beta^+$ $CD8^+$ T cells

$CD4^+$, $CD8^+$ and $TCR\gamma/\delta^+$ T cells were purified from naïve LNC or splenocytes by magnetic separation, using MACS anti-Rat specific MicroBeads and the LS type column (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer's instructions. Cells were passed twice on two columns (four purification steps) in order to achieve $>99\%$ purity, checked by flow cytometry. Purified $TCR\alpha/\beta^+$ $CD8^+$ T cells were obtained by first purifying the $TCR\gamma/\delta^+$ from whole splenocytes, followed by the purification of the $CD8^+$ T cells from the $TCR\gamma/\delta^+$-depleted flow through. In this way, we obtained purified $CD8^+$ T cells containing only 0.08% $TCR\gamma/\delta^+$ T cells, compared with 5–6% $TCR\gamma/\delta^+$ cells in the $CD8^+$ T cells that had been purified from whole splenocytes, measured by flow cytometry.

2.9. Flow cytometry

Samples containing approximately 3×10^5 cells were washed twice in 1 ml of FACS buffer (1% FCS and 0.1% NaN_3), followed by a 40-minute incubation at 4 °C with the following mouse anti-rat FITC- or PE-conjugated primary monoclonal antibodies, diluted according to the manufacturer's recommendation. After incubation, cells were washed twice with 1 ml of FACS buffer and resuspended in 0.5 ml PBS. The FL1 and FL2 channels were used to detect FITC and PE fluorescence of the cells, using the FACScan system (Becton Dickinson). The antibodies used were: W3/25 (anti-CD4:FITC), OX-8 (anti-CD8:FITC), OX-39 (anti-CD25:FITC), 3H5 (anti-B7.1:PE), 24F (anti-B7.2:PE), R73 (anti-$TCR\alpha/\beta$:PE) and V65 (anti-$TCR\gamma/\delta$:PE), all of which are mouse anti-rat IgG1-isotype monoclonal antibodies, purchased from Serotec (Oxford, UK).

3. Results

3.1. Naïve T cells proliferate in response to activated syngeneic T cells

We studied the anti-ergotypic responses in the LNC, spleens and thymuses obtained from naïve Lewis rats at the age of 8–10 weeks. LNC were obtained by pooling the popliteal, inguinal, cervical, mesenterial and axillary lymph nodes. The A2b T cell clone, activated or resting, was used as stimulator T cells, after irradiation. We used various numbers of A2b stimulator T cells to monitor the response. As can be seen in Fig. 1, unfractionated LNC (1A) and splenocytes (1B) proliferated strongly to activated T cells, but not to resting T cells. When the popliteal, inguinal, cervical, mesenterial or axillary LNC were studied separately, each population exhibited the same level and pattern of response as did the pooled LNC (not shown). Fig. 1C depicts the effect of the stage of rest on the anti-ergotypic response: A2b cells that had rested only for 6 days showed greater stimulation than did cells that had rested for 12 or 20 days.

The anti-ergotypic response was detected also in the adult thymus, although the thymocyte response was significantly lower than the response detected in the peripheral lymphoid organs (Fig. 1D). We further examined the anti-ergotypic response of thymocytes separated into two fractions: purified immature $CD4^+CD8^+$ double-positive T cells and a fraction enriched for mature single-positive T cells (Table 1). As shown in Figs. 1E,F, the purified $CD4^+CD8^+$ T cells (bottom ring) did not respond to activated T cells, while the thymocytes enriched for mature single-positive T cells (top ring) proliferated in response to activated T cells to the same extent as did the unfractionated peripheral lymphoid organs. Thus, anti-ergotypic T cells were among the mature T cells present in the adult peripheral lymphoid organs, and to a lesser extent in the adult thymus.

3.2. Anti-ergotypic T cells in the newborn thymus

We measured the anti-ergotypic responses in the thymuses and spleens of newborn rats, not older than 16 hours. As shown in Figs. 2A,B, higher anti-ergotypic responses were measured in the newborn thymus, while very low responses could be detected in the newborn spleen. At one week of age, the anti-ergotypic response

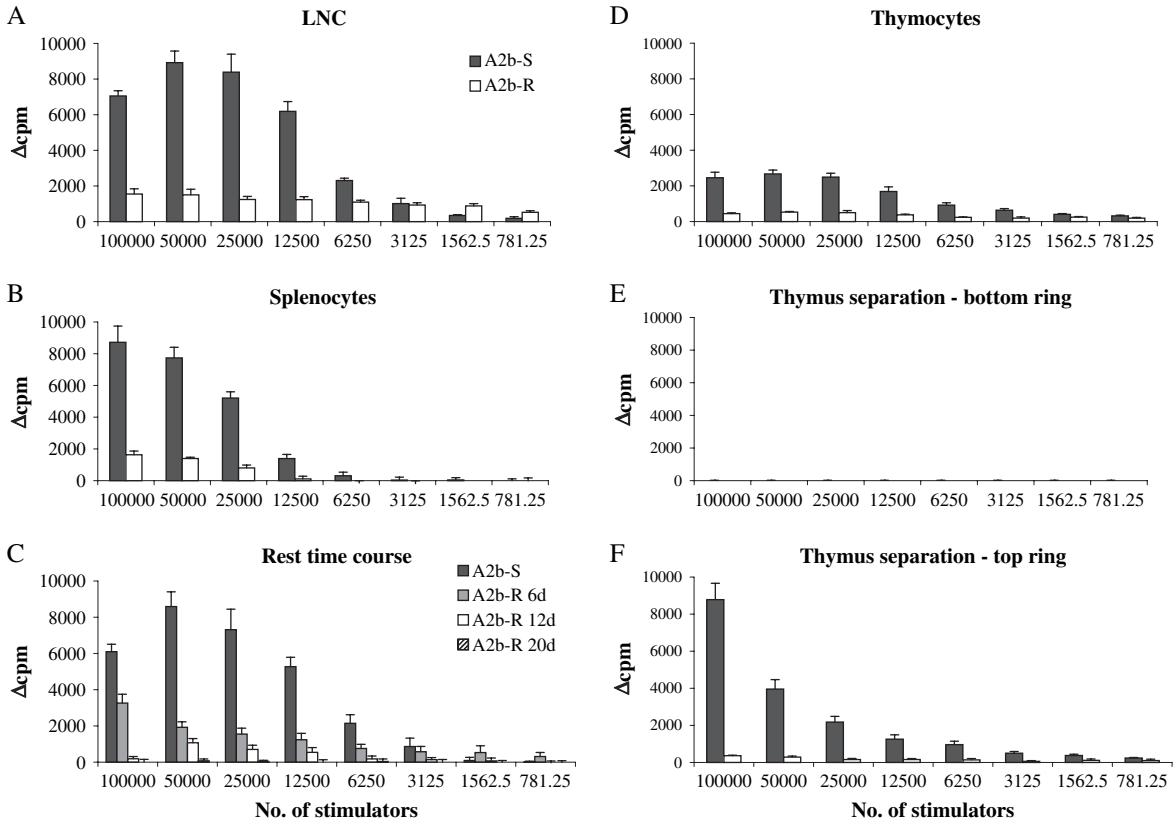

Fig. 1. Naïve lymphocytes proliferate to activated and not to resting T cells. LNC (a), splenocytes (b) and thymocytes (d) from naïve adult rats were measured for their proliferative responses to activated (A2b-S) or resting (A2b-R) irradiated syngeneic T cells, at different stimulator cell concentrations. Stimulators at different stages of rest were also assayed (c). Thymocytes were separated by a 60%/70% Percoll gradient to obtain purified immature $CD4^+CD8^+$ T cells (e; bottom ring) and a fraction enriched for mature thymocytes (f; top ring). Proliferative responses are presented as the Δcpm ± SEM of quadruplicate cultures.

in the thymus decreased, while the response in the spleen increased (Figs. 2C,D). Table 2 compares the spontaneous response and the response to ConA of thymocytes and splenocytes obtained from 1-day old rats to that of 12-week old rats. The response to ConA was the same in thymocytes from both age groups, but the splenocytes from the newborn rats hardly proliferated to ConA. The low response to ConA could be explained by the relatively low number of mature T cells in the periphery of newborns. However, three and four-week old rats exhibited anti-ergotypic responses equal to those measured in 12 week old adults. Thymocytes from 12-week old rats were less responsive to activated T cells than were newborn thymocytes (Fig. 2F). Thus, anti-ergotypic T cells are detectable in the thymus of newborn rats before they appear in the spleen, suggesting that anti-ergotypic T cells probably can differentiate and mature as single-positive T cells in the thymus.

3.3. Anti-ergotypic T cells in naïve rats are of the $CD8^+$ phenotype

To characterize the anti-ergotypic T cells in the naïve rat, we purified the $CD4^+$ or $CD8^+$ cells from the whole LNC population and assayed them for the presence of anti-ergotypic responses. As shown in Fig. 3B, purified $CD4^+$ T cells did not proliferate in response to activated T cells. In contrast, purified $CD8^+$ T cells proliferated strongly to activated T cells (3C). Moreover, the response produced by 2×10^5 $CD8^+$ T cells was twice as strong as that produced by the same number of unfractionated LNC (compare Figs. 3A,C).

3.4. Naive $TCR\gamma/\delta^+$ T cells are enriched in anti-ergotypic T cells

Anti-ergotypic T cells in humans were shown to be of both the $TCR\alpha/\beta^+$ and the $TCR\gamma/\delta^+$ phenotypes [9,10]. To characterize the TCR type of the anti-ergotypic T cells in naïve rats, we purified the $TCR\gamma/\delta^+$ T cells from naïve splenocytes; $TCR\alpha/\beta^+CD8^+$ T cells were purified from the flow through of the $TCR\gamma/\delta^+$ purification. Flow cytometry analysis of the purified $TCR\gamma/\delta^+$ T cells showed that 80–83% were of the $CD8^+$ phenotype (not shown). As can be seen in Fig. 4B, depletion of the $TCR\gamma/\delta^+$ from the $CD8^+$ T cells resulted in a 2-fold decrease in the response of the purified $TCR\alpha/\beta^+CD8^+$ T cells to activated T cells

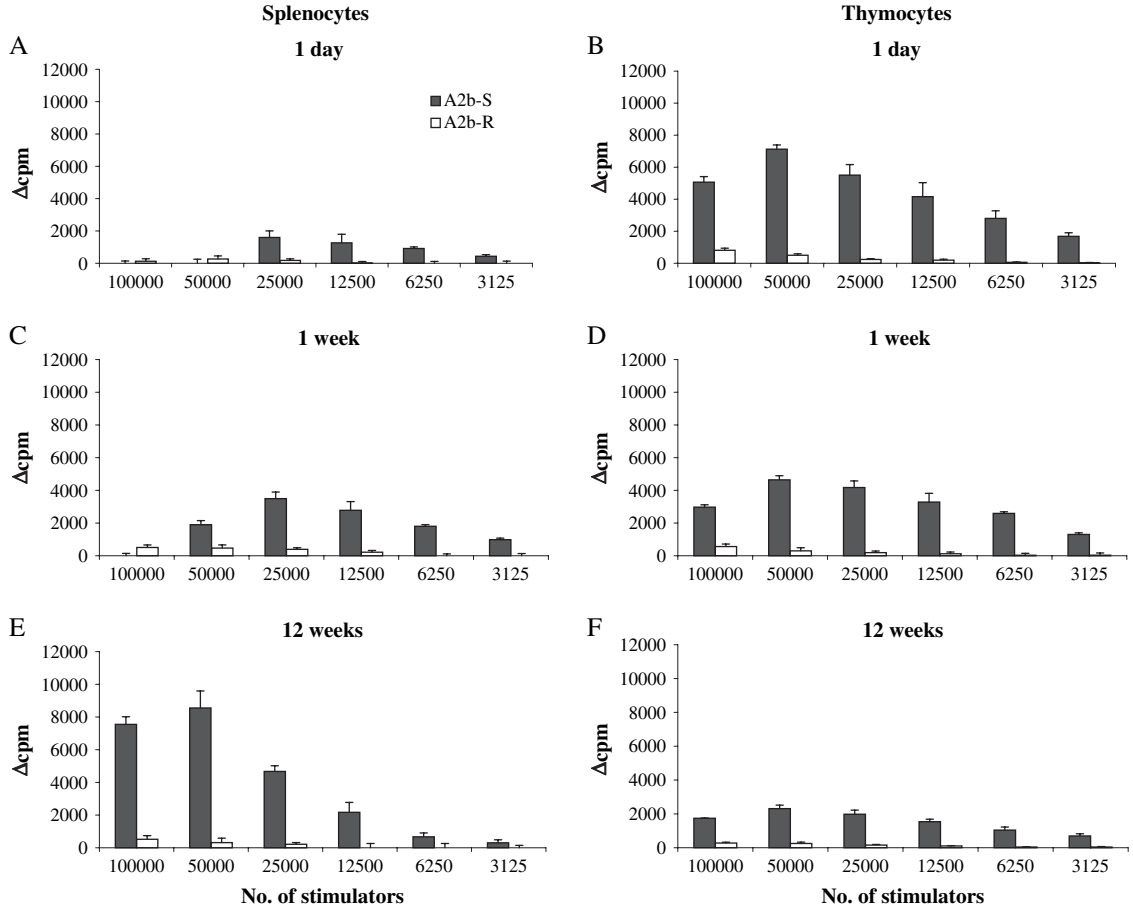

Fig. 2. Anti-ergotypic T cells are present in the newborn thymus. Splenocytes and thymocytes obtained from rats at their first day of life (a, b), one week old rats (c, d) and 12 week old rats (e, f) were measured for their proliferative responses to activated (A2b-S) or resting (A2b-R) irradiated syngeneic T cells, at different stimulator cell concentrations. Proliferative responses are presented as the Δcpm±SEM of quadruplicate cultures.

(compare 4B to 4A). This lower response, nevertheless, was still significant. However, the response of purified TCRγ/δ$^+$ T cells to activated syngeneic T cells was four times stronger than the response of an equal number of TCRα/β$^+$CD8$^+$ T cells (Fig. 4C). Thus, we can conclude that the CD8$^+$ anti-ergotypic T cells in the naïve rat can be divided into TCRα/β$^+$ and TCRγ/δ$^+$ T cells, both of which respond to activated T cells. Nevertheless, the major part of the response can be related to the TCRγ/δ$^+$ T cells.

Table 2
ConA reactivity at 1 day and 12 weeks: spleen and thymus

Cells	Age	T-cell proliferation (cpm)	
		Spontaneous	ConA
Spleen	1 day	956±86	1021±243
	12 weeks	1483±160	56796±5917
Thymus	1 day	132±28	9875±1116
	12 weeks	54±8	9616±1420

3.5. The naïve anti-ergotypic population does not include CD25$^+$ T cells

To learn whether naïve anti-ergotypic T cells express the CD25 marker, we studied whether depleting the CD25$^+$ cells affected the anti-ergotypic response. We depleted the CD25$^+$ cells from naïve LNC, and compared the anti-ergotypic response of the CD25-depleted LNC to non-depleted LNC. As shown in Fig. 5, depletion of CD25$^+$ cells did not affect the naïve anti-ergotypic response: the CD25-depleted LNC (5B) proliferated to the same extent to activated T cells as did the whole LNC (5A). Thus, the bulk of the anti-ergotypic T cells were not among the naïve CD25$^+$ population. Likewise, the anti-ergotypic T-cell response did not seem to be enhanced by removing the CD25$^+$ regulators.

3.6. Cytokine secretion by proliferating anti-ergotypic T cells

We tested the supernatants of the anti-ergotypic proliferation reactions for the following secreted

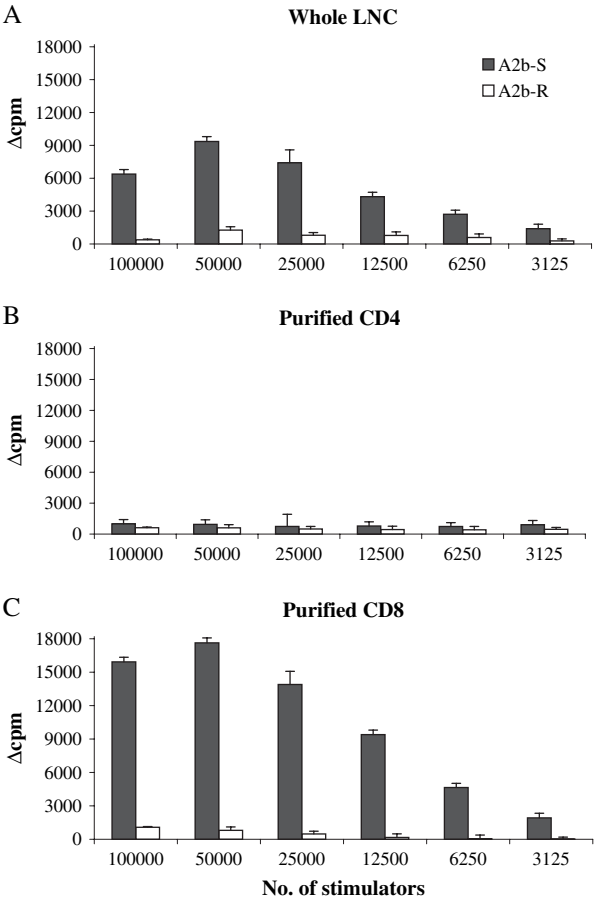

Fig. 3. Anti-ergotypic T cells in naïve rats are of the CD8 subclass. CD4 (b) and CD8 (c) T cells were purified from whole LNC (a) taken from naïve adult rats, and measured for their proliferative responses to activated (A2b-S) or resting (A2b-R) irradiated syngeneic T cells, at different stimulator cell concentrations. Proliferative responses are presented as the Δcpm ± SEM of quadruplicate cultures.

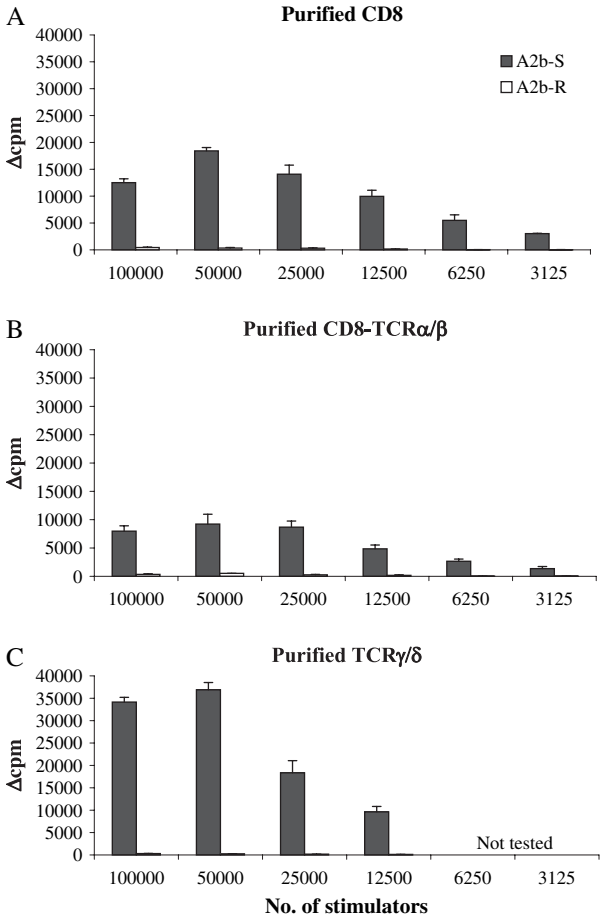

Fig. 4. Anti-ergotypic T cells in naïve rats include both TCRγ/δ$^+$ and TCRα/β$^+$ T cells. TCRα/β$^+$CD8$^+$ (b) and TCRγ/δ$^+$ (c) T cells were purified from whole splenocytes (a) taken from naïve adult rats, and measured for their proliferative responses to activated (A2b-S) or resting (A2b-R) irradiated syngeneic T cells, at different stimulator cell concentrations. Proliferative responses are presented as the Δcpm ± SEM of quadruplicate cultures.

cytokines: IFNγ, TNFα, TGFβ, IL-2, IL-4 and IL-10. None of these cytokines could be detected in the supernatants of whole LNC, whole splenocytes, purified CD8$^+$ T cells, or purified TCRα/β$^+$CD8$^+$ T cells. However, purified TCRγ/δ$^+$ T cells, which strongly proliferated to activated T cells, secreted IFNγ and TNFα (Fig. 6). Low levels of IL-2 could also be detected (not shown), but most of the IL-2 was probably utilized by the proliferating cells. IL-10, IL-4 and TGFβ were not detectable. No cytokine release was detected in response to resting T cells (not shown).

3.7. The anti-ergotypic response depends on cell-to-cell contact, but not on professional APCs

The purification of the CD8$^+$ T cells from the naïve LNC eliminated the professional APCs from the responder population. As shown in Table 3, purified CD8$^+$ T cells, in contrast to the unfractionated LNC, did not respond to ConA, a response known to be APC dependent [19,20]. The addition of irradiated thymocytes as APCs restored the response to ConA by the purified CD8$^+$ cells. Nevertheless, the purified CD8$^+$ T cells strongly responded to activated T cells (see Figs. 3 and 4). Thus, we can conclude that the anti-ergotypic response exhibited by the purified CD8$^+$ T cells is independent of professional APCs. In fact, the addition of APCs to the proliferation reactions resulted in a significant (50%) decrease in the response to activated T cells (not shown).

We tested whether the anti-ergotypic response was dependent on contact between the stimulators and the responders, or could be due to cytokines or other molecules secreted by the stimulator T cells. We used a transwell system that blocked contact between the responding LNC and the activated T-cell stimulators. The transwell apparatus still allowed the transfer of cytokines and other molecules to the responders. After

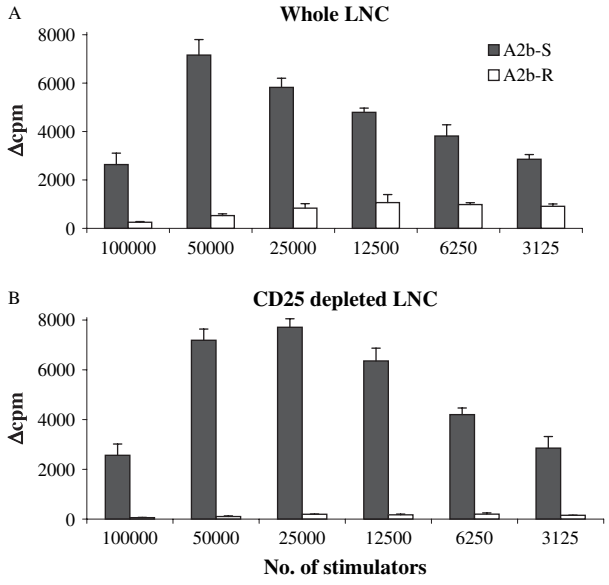

Fig. 5. The anti-ergotypic response is independent of the $CD4^+CD25^+$ population. Whole LNC (a) from naïve adult rats were depleted of the $CD25^+$ cells (b) and measured for their proliferative responses to activated (A2b-S) or resting (A2b-R) irradiated syngeneic T cells, at different stimulator cell concentrations. Proliferative responses are presented as the Δcpm ± SEM of quadruplicate cultures.

three days of incubation, LNC from the bottom well were assayed for their thymidine incorporation. As shown in Fig. 7, contact between the anti-ergotypic T cell population and activated stimulators triggered a significant anti-ergotypic proliferation (treatment 5). In contrast, when the stimulators were separated from the LNC responders (treatment 4), the response was equal to the spontaneous proliferation, in which there were no stimulators in the transwell (treatment 1). Thus, the anti-ergotypic T-T cell response appeared to be contact-dependent and professional-APC independent, which suggests that activated T cells can serve as APCs in this response.

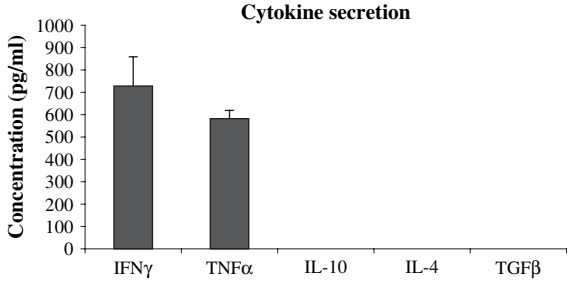

Fig. 6. Cytokine secretion by anti-ergotypic T cells in the naïve rat. The medium of the $TCR\gamma/\delta^+$ anti-ergotypic T cell, proliferating to activated T cells, was taken after 72 hours in culture and analyzed by ELISA for cytokine secretion. The results are presented as pg/ml for IFNγ and TNFα. IL-4, IL-10 and TGFβ were undetectable.

Table 3
Purified $CD8^+$ T cells are free of APC

Responders	T-cell proliferation (cpm)		
	Spontaneous	ConA	ConA + APC[a]
Whole LNC	633 ± 60	37102 ± 4177	52263 ± 1919
Purified CD8	20 ± 2	58 ± 21	42336 ± 3137

[a] 10^6 irradiated thymocytes were used as APCs.

3.8. MHC restriction and B7-CD28 dependency

We tested whether the response of anti-ergotypic T cells is MHC-restricted. We found that the addition of anti-MHC-I antibody to the anti-ergotypic proliferation of purified $CD8^+TCR\alpha/\beta^+$ T cells inhibited the response by 95% (Fig. 8A). In contrast, anti-MHC-II I-A antibody (the A2b clone is I-A restricted), had only a slight effect on the proliferation. Anti-MHC-II I-E antibody did not have any effect on the response. In contrast, the strong anti-ergotypic proliferation of the purified $TCR\gamma/\delta^+$ T cells was not affected by either of the anti-MHC monoclonals (not shown).

To function as ergotypic stimulators, activated T cells might also need to express costimulatory molecules. Indeed, human and murine activated T cells have been reported to express B7 costimulatory molecules [21–25]. Therefore, we assayed our rat T-cell clones for the expression of the B7 molecules upon activation. Fig. 8B shows upregulation of the expression of both B7.1 and B7.2 on the A2b T-cell clone, used as the ergotypic stimulator in our experiments. The activated A2b

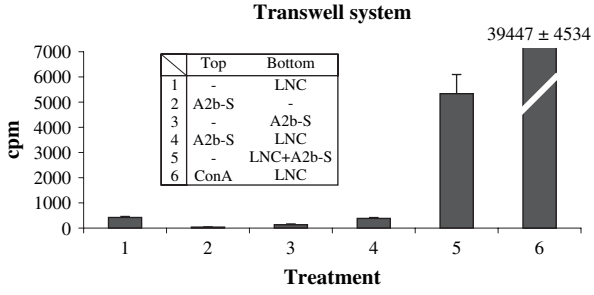

Fig. 7. The anti-ergotypic T-T cell interaction is contact dependent. LNC from naïve adult rats were measured for their proliferative responses to activated A2b T cells in a transwell system. The LNC were added to the bottom well and irradiated activated T cells to the transwell (treatment 4), avoiding the contact between them. Activated T cells were also incubated together with the responding LNC (treatment 5). Treatment 1 is the spontaneous proliferation of the LNC alone. In treatment 2, non-irradiated activated T cells were added to the transwell only to ensure they do not migrate to the bottom well. Irradiated activated T cells were added alone to the bottom well to check that they do not proliferate themselves (treatment 3). In treatment 6, LNC were added to the bottom well and ConA was added to the transwell as a control for T cell proliferation. Proliferations were checked after 72 hours in the bottom wells. Proliferative responses are presented as the cpm ± SEM of quadruplicate cultures.

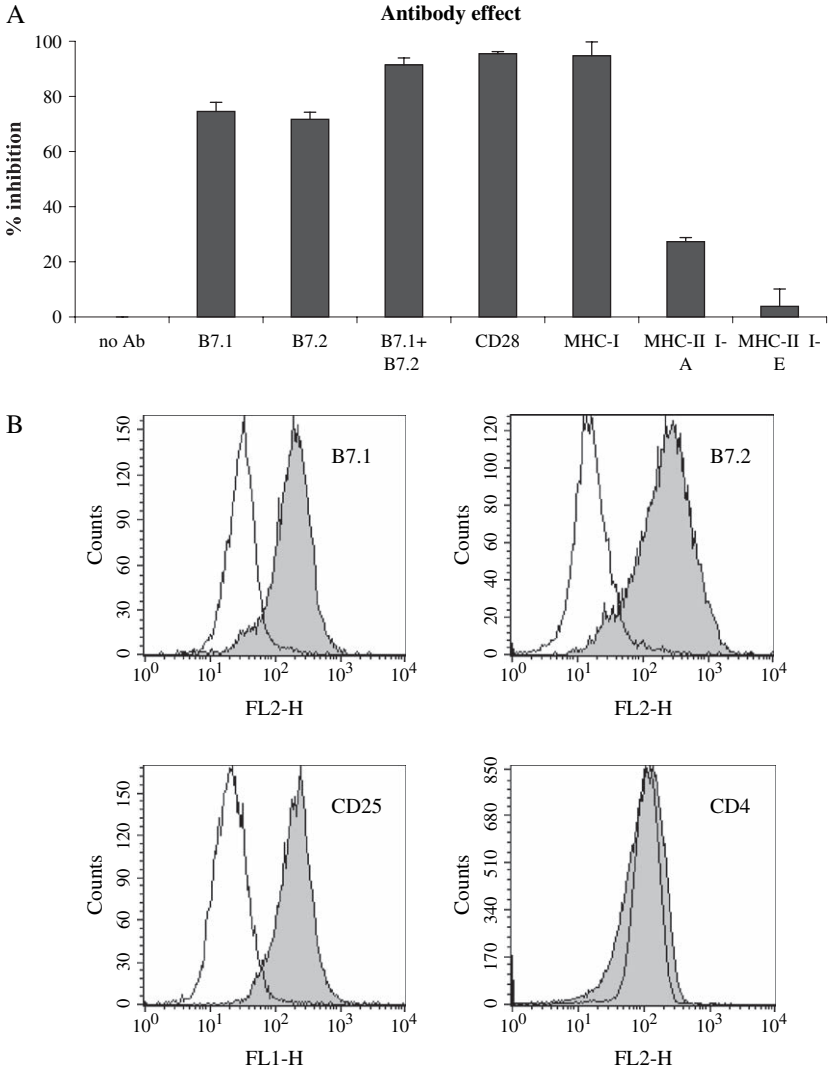

Fig. 8. The anti-ergotypic response is MHC-I restricted and B7-CD28 dependent. a. The following monoclonal antibodies were assayed for their ability to block the proliferative response of 10^5 purified $CD8^+TCR\alpha/\beta^+$ or $TCR\gamma/\delta^+$ T cells to 2.5×10^4 activated and irradiated A2b T cells: anti-B7.1, B7.2, CD28, MHC-I, MHC-II I-A and MHC-II I-E. Results are presented as the percent of proliferation inhibition $\pm$ SEM of triplicate cultures. b. Activated (gray) and resting (white) A2b T cells were analyzed by flow cytometry for B7.1 and B7.2 expression. CD25 expression is shown as a control for activated T cells, and CD4 as a control for a constitutively expressed cell marker.

stimulator T cells also expressed CD25. A2b T cells express CD4 constitutively.

To examine the functional involvement of the B7 molecules in the anti-ergotypic T cell interaction, we tested whether the addition of anti-B7.1 or anti-B7.2 antibodies would block anti-ergotypic proliferation. As shown in Fig. 8A, 2 μg of each of the antibodies alone inhibited about 75% of the proliferation of the purified $CD8^+TCR\alpha/\beta^+$ T cells to activated T cells; the addition of both antibodies together, 1 μg each, inhibited 92% of the proliferation. Anti-CD28 monoclonal antibody also blocked 95% of the proliferation (Fig. 8A). The same pattern of inhibition was detected when we tested the proliferation of the purified $TCR\gamma/\delta^+$ T cells to activated T cells. Thus, activated T cells can serve as APCs in the anti-ergotypic T-cell interaction. Stimulation of the $TCR\alpha/\beta^+CD8^+$ anti-ergotypic T cells was MHC-I restricted and B7-CD28 dependent; the response of the $TCR\gamma/\delta^+$ anti-ergotypic T cells was not MHC-I restricted, although it was still B7-CD28 dependent.

4. Discussion

T cells responding to activated and not to resting T cells were first described in the beginning of the 1980s in studies of the autologous mixed lymphocyte reaction (AMLR) in humans [26–28]. Anti-ergotypic T cells responding to activated syngeneic T cells were described

in the context of experimental autoimmune disease only at the end of the 1980s, associated with the mechanism of protection induced by TCV [12,13]. Anti-ergotypic T cells were shown to protect rats from EAE [7].

The initial studies of anti-ergotypic T cells were done in immunized rats. In the present experiments, we studied anti-ergotypic responses in naïve rats, and characterized the phenotypes of these cells. We made the following observations: a. Anti-ergotypic T cells were detected in naïve LNC, splenocyte and thymocyte populations; b. Anti-ergotypic T cells could be detected first in the newborn thymus, and only later in life in the spleen; c. The responders were of the $CD8^+$ subclass, and the $TCR\gamma/\delta^+$ T cells were relatively stronger responders than were the $TCR\alpha/\beta^+$ T cells; d. The $TCR\gamma/\delta^+$ anti-ergotypic T cells secreted IFNγ and TNFα, but the $TCR\alpha/\beta^+$ T cells did not; e. The anti-ergotypic response was contact-dependent, and independent of professional APCs. Hence, activated T cells can serve as APCs; f. The response of the $TCR\alpha/\beta^+$ anti-ergotypic T cells was MHC-I restricted, while the $TCR\gamma/\delta^+$ response was MHC independent.

The development of different regulatory T cell populations is either dependent or independent of overt antigen priming: Type 1 regulatory (Tr1) T cells were described to arise by the activation of $CD4^+$ T cells with specific antigen in the presence of IL-10 [5,29]; Th3 regulatory T cells were isolated following the induction of oral tolerance to an antigen [30–32]. Thus, these regulatory populations seem to develop following antigen activation. In contrast, $CD4^+CD25^+$ Tr cells, which exhibit immunoregulatory functions in vitro and in vivo [33], develop in the thymus and can be isolated from naïve animals [34–36].

The finding of anti-ergotypic T cells in newborn rats and in naïve rats grown in a specific pathogen free (SPF) facility suggests that, like the $CD4^+CD25^+$ Tr cells, anti-ergotypic T cells do not need priming by exogenous antigen for their development. It is conceivable that anti-ergotypic T cells are primed in the thymus by T cells that are activated during their differentiation and express activation markers.

The anti-ergotypic T cells we describe in naïve rats are much like with these reported in humans: anti-ergotypic T cell clones, both of the $TCR\gamma/\delta^+$ and $TCR\alpha/\beta^+CD8^+$ phenotypes, were isolated from peripheral bloods of multiple sclerosis (MS), myasthenia gravis (MG) and healthy individuals. All these T cell clones proliferated strongly to activated T cells, irrespective of their antigen specificity [8–10]. The proliferative responses of $TCR\gamma/\delta^+$ T-cell clones in humans were not stronger than the responses of the $TCR\alpha/\beta^+CD8^+$ anti-ergotypic clones [9]. Our findings that the purified population of $TCR\gamma/\delta^+$ T cells proliferated more strongly than did the $TCR\alpha/\beta^+CD8^+$ population suggests that the $TCR\gamma/\delta^+$ population may contain a higher concentration of anti-ergotypic T cells. However, precursor frequency studies need to be done to establish this point.

As we show here, the anti-ergotypic T cells present in naïve rats appear only in the $CD8^+$ subclass. We could, however, detect $CD4^+$ anti-ergotypic T cells in rats vaccinated with DNA encoding CD25 [14]. Vaccination with CD25 appeared to strengthen the effectiveness of anti-ergotypic T-cell regulation of adjuvant arthritis. Thus it is conceivable that vaccination can enlist $CD4^+$ T cells into the anti-ergotypic regulatory pool of T cells.

Correale and colleagues isolated from healthy human individuals anti-ergotypic T cell clones of both $TCR\gamma/\delta^+$ and $TCR\alpha/\beta^+CD8^+$ phenotypes. Similar to our findings in naïve rats, the human $TCR\gamma/\delta^+$ anti-ergotypic T cells secreted IFNγ and TNFα [9]. However, the human $TCR\alpha/\beta^+CD8^+$ anti-ergotypic T cells secreted IFNγ, TNFα and TGFβ; we, in contrast, could not detect any cytokine secretion by the naïve rat $TCR\alpha/\beta^+CD8^+$ anti-ergotypic T cells. Finally, the MHC restriction of the human anti-ergotypic T cells found by Correale and coworkers is similar to the MHC restriction we characterized in the Lewis rat: anti-MHC class-I monoclonal antibody blocked the proliferation of the $TCR\alpha/\beta^+CD8^+$ anti-ergotypic T cells and not that of the $TCR\gamma/\delta^+$ anti-ergotypic T cells. These findings are consistent with the fact that most $TCR\gamma/\delta^+$ T cells recognize their antigen in an MHC-independent manner [37,38].

Although recognition between the $TCR\gamma/\delta^+$ anti-ergotypic T cells and the stimulator T cells is not MHC restricted, we found that B7-CD28 costimulatory signaling is necessary also in the case of $TCR\gamma/\delta^+$ anti-ergotypic T cells. The mechanism by which $TCR\gamma/\delta^+$ T cells are activated, and their biological role are poorly understood generally. $TCR\gamma/\delta^+$ T cells might have a regulatory function in organ-specific autoimmune diseases, possibly by regulating $TCR\alpha/\beta^+$ autoreactive T cells [38]: this was shown in murine experimental models such as NOD and MRL.lpr mice [39,40]. Further work has to be done to elucidate the relationship between these regulatory $TCR\gamma/\delta^+$ cells and the anti-ergotypic T cells we describe here.

It is important to note that in contrast to human anti-ergotypic T cells, which can be cloned and grown in vitro [8–10], rat anti-ergotypic T cells could not. We and others in the past [41] could not propagate such lines from rats. Anti-ergotypic T cells expand rapidly following activation in vitro with irradiated activated T cells, but when T-cell growth-factors are added after 3 days, the cells grow for another 24 hours and then aggregate and die. Perhaps anti-ergotypic T cells ultimately suppress themselves as they too become activated. In any case, our present work had to be done using primary proliferation experiments.

An interesting question is whether anti-ergotypic T cells might have a regulatory role in maintaining

immune homeostasis in naïve animals. The immune system does express a basal level of activity even without overt immunization or disease. The TCRγ/δ^+ anti-ergotypic T cells in naïve rats secreted IFNγ and TNFα, which are considered pro-inflammatory and not inhibitory cytokines. However, activated human $CD8^+$ T cells were found to inhibit T cell responses by the secretion of IFNγ [42]. Thus, the mechanism of their possible regulatory role is not yet clear. $CD4^+CD25^+$ regulators isolated from naïve animals were shown to suppress the proliferation of other T cells [2,6]. Human anti-ergotypic T cell clones were also shown to inhibit proliferation of syngeneic T cells in vitro, but these human clones were obtained from MS patients following TCV [11].

Activated anti-ergotypic T cells have been shown to inhibit EAE [7] and adjuvant arthritis [14]. Unfortunately, however, the possible regulatory function of the anti-ergotypic T cells found in naïve subjects is not readily accessible to study using experimental systems presently available: since anti-ergotypic T cells are heterogeneous in TCR markers and target antigens, it is not presently possible to delete them specifically from naïve animals. Likewise, since naïve anti-ergotypic T cells have not been successfully raised as specific lines, it is not presently possible to adoptively transfer them. Technically then, we cannot yet add or subtract naïve anti-ergotypic T cells and see the effects in vivo. Nevertheless, naïve animals do manifest a basic resistance to both active induction and adoptive transfer of experimental autoimmune diseases, and this is compatible with the functional operation of anti-ergotypic, or other regulatory cells in these animals. For example, lines and clones of activated EAE effector T cells usually cause EAE in rats only when injected at doses of a million or more cells [43]. Lower doses of such line cells, not sufficient to cause EAE, can still vaccinate the recipient rats and render them resistant to future EAE [44]. Thus there is a natural barrier to adoptive EAE that can be overcome using a high dose of pathogenic T cells. A natural barrier to autoimmune disease is also evident in adjuvant arthritis: Adoptive transfer of adjuvant arthritis to naïve recipients by otherwise arthritogenic T cells requires that the recipients be irradiated [15]. The detection of anti-ergotypic T cells in naïve rats thus invites investigation, methodology permitting, of the possible role of these cells in the natural barrier to activated autoimmune effector T cells.

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