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Modulation of CD4 co-receptor limits spontaneous autoimmunity when high-affinity transgenic TCR specific for self-antigen is expressed on a genetically resistant background

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Abstract

Myelin proteolipid protein (PLP) 139–151 is an immunodominant peptide that induces experimental autoimmune encephalomyelitis (EAE) in H-2^s SJL/J mice. While PLP 139–151-specific TCR transgenic (tg) 4E3 mice develop fulminant spontaneous disease on the susceptible SJL/J background, spontaneous EAE is dramatically reduced on the H-2^s congenic B10.S background. On this resistant background, we observed a high frequency of positively selected tg CD4−CD8− (DN) thymocytes and peripheral DN tg T cells. Splenic DN tg T cells responded to anti-CD3 stimulation similarly to CD4+ cells, but proliferative and cytokine responses to PLP 139–151 were blunted, implying that CD4 co-receptor down-regulation modulated T cell responses to the self-antigen *in vitro*. Adoptive transfer of tg DN CD3hi cells into RAG-deficient wild-type (WT) recipients induced EAE less efficiently than transfer of CD4+ T tg cells indicating the blunted responses of DN tg T cells to self-antigen *in vivo*. The frequency of tg DN T cells was irrespective of thymic expression of the autoantigen. These data implicate that down-regulation of CD4 co-receptor in the thymus, which is independent from the expression of thymic autoantigen, results in a blunted response to the autoantigen in the periphery and limits the incidence of spontaneous autoimmunity in genetically resistant mice bearing a large autoreactive tg T cell repertoire.

Introduction

The immune system has evolved to combat pathogens, while maintaining tolerance to self-antigens. Although T cells capable of recognizing self-antigens are present in healthy subjects, autoimmune disease does not occur in most individuals. How this tolerance is achieved is fundamental to understanding tolerance and development of autoimmune reactions. During development, thymocytes bearing self-reactive TCRs are clonally deleted in the thymus by negative selection (1). In addition to physical elimination, modulation of TCR expression and functional inactivation (anergy) are believed to inactivate highly autoreactive thymocytes (2, 3). Although central tolerance is important for averting autoimmunity, it does not completely prevent the generation of low-affinity self-reactive T cells, which escape thymic deletion and are exported to the peripheral immune compartment (4–6). Since TCRs are inherently cross-reactive and the activation threshold of memory T cells is lower (7), additional mechanisms in the periphery inhibit the activation of autoreactive T cells to ensure maintenance of self-tolerance. Induction of regulatory/suppressor T cells, ignorance to the self-antigen (8–10), down-regulation of the TCR (11), functional unresponsiveness of T cells (12–16), altered response to cross-reactive ligands (17) and the complete elimination of autoreactive T cell clones from the peripheral repertoire (18, 19) are the mechanisms involved in the maintenance of tolerance against self in the periphery. Modulation of expression of co-receptors on thymocytes or peripheral T cells can contribute to self-tolerance to positively selecting ligands,

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a process that is probably related to the affinity of TCR to the selecting ligand (20–22). We have also shown down-regulation of the CD4 co-receptor on peripheral T cells in response to repeated stimulation with high-affinity heteroclitic ligand *in vitro*, and this co-receptor down-modulation resulted in an increase in the activation threshold of the responding T cells (23).

Myelin proteolipid protein (PLP) is the major protein of central nervous system (CNS) myelin. PLP 139–151 is the dominant epitope that induces experimental autoimmune encephalomyelitis (EAE) in SJL/J (H-2^s) mice. In contrast, B10.S mice are relatively resistant to EAE induction by PLP 139–151 immunization, even though both strains carry the same H-2^s MHC II molecules (24–26). We previously generated different lines of transgenic (tg) mice that express rearranged αβTCR specific to PLP 139–151 (27). When one of the tg lines (4E3) was backcrossed onto the EAE-susceptible SJL/J background, the mice developed spontaneous fulminant disease with such a high frequency that the line could not be maintained (27). We have now fully backcrossed this line onto the EAE-resistant B10.S background and observed a significantly reduced frequency and severity of spontaneous disease. Our data suggest that down-regulation of the CD4 co-receptor appears to be responsible for the increased number of double-negative (DN) tg T cells serving as one of the mechanisms utilized by autoreactive T cells to prevent development of autoimmune disease on the resistant B10.S background. Our data further show that this process may be independent of the level of PLP expression in the thymus of B10.S mice.

Methods

Mice

Generation of 4E3 (lines 1736 and 1740) and 5B6 tg mice that express PLP 139–151-specific tg αβTCRs have been previously described (27). RAG-2-deficient mice were backcrossed onto the B10.S background for >10 generations. 4E3 and RAG-deficient tg mice were generated by crossing the tg mice to the RAG2-deficient B10.S mice. PLP-deficient mice (28) were obtained from K. Armin Nave (University of Heidelberg, Heidelberg, Germany) and were backcrossed onto the B10.S background. All the mice were housed under specific pathogen-free conditions at the animal facility at the Harvard Institutes of Medicine, Brigham and Women's Hospital, Boston, MA, according to the animal protocol guidelines of standing committee of animals, Harvard Medical School. Eight- to 12-week old female mice were used in all the experiments.

Antigens

The PLP 139–151 peptide (HSLGKWLGHPDKF) was synthesized using F-moc chemistry (QCB BioSource International, Hopkinton, MA, USA). The peptide was HPLC purified and its identity was confirmed by mass spectroscopy. The peptide was dissolved in sterile water before use.

Antibodies

Anti-CD4, anti-CD8, anti-CD3, anti-TCRβ, anti-CD24 and anti-CD69 mAbs were purchased from PharMingen (San Diego, CA, USA). The cells were stained in PBS with 2% BSA (Sigma, St Louis, MO, USA) and acquired by FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

Cell sorting

To analyze *in vitro* T cell responses and to transfer T cell subsets into RAG2-deficient B10.S hosts, splenocytes or thymocytes from 16 tg female mice were pooled. None of the tg donor mice had developed spontaneous EAE. CD3+ T cells were enriched by negative selection using a Mouse T cell Enrichment Column (R&D Systems, Minneapolis, MN, USA) and labeled with anti-CD3–FITC or anti-TCRβ–FITC, anti-CD4–APC and anti-CD8–PercP. Cells were sorted by FACSVantage SE (Becton Dickinson). The purity of sorted T cells was 98–99%.

5-Bromo-2-deoxyuridine incorporation

Recent thymic emigrants were detected as described previously (29), with a modification. Briefly, mice were given sterile drinking water containing 0.8 mg/ml 5-bromo-2-deoxyuridine (BrdU) (Sigma Chemical Co., St Louis, MO, USA), which was changed daily. After 10 days, the mice were sacrificed and splenocyte suspensions were prepared. Cells were surface stained with anti-TCRβ, anti-CD4 and anti-CD8, then washed and 2×10^6 cells were re-suspended in BD PharMingen™ Stain Buffer. Cells were stained using a BrdU Flow Kit (PharMingen) according to the manufacturer and acquired by flow cytometry as described above. The data were analyzed by FlowJo software.

In vitro proliferation assay

Splenocytes (4×10^5) were cultured in 96-well plates together with three different concentrations of PLP 139–151 peptide (1, 10 and 100 mg/ml). Sorted T cell fractions (3×10^4) were stimulated with PLP 139–151 peptide in the presence of 5×10^5 irradiated splenocytes as APC or with plate-bound anti-CD3 (5 μg) and soluble anti-CD28 (2 mg/ml) mAbs. Cultures were pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear, MA, USA) after 48 h, and plates were harvested and the filters were counted using a Wallac scintillation counter 16 h later. The proliferative responses were determined based on [^{3}H]thymidine incorporation as mean counts per minute in triplicate wells.

Cytokine measurement by ELISA

Supernatants obtained from splenic and fractionated T cell cultures stimulated with PLP 139–151 peptide were collected after 48 h of activation and stored at −70°C until analysis. Secretion of IL-2, IL-4, IL-10 and IFN-γ was measured by ELISA. Briefly, cytokine antibodies for IL-2, IL-4, IL-10 and IFN-γ were coated onto 96-well plates at a concentration of 1 mg/ml overnight. The plates were washed and treated with blocking solution (Kierkegaard and Perry, Gaithersburg, MD, USA), followed by incubation of culture supernatants overnight at 4°C. The plates were subsequently washed and incubated with their corresponding biotinylated cytokine-detecting mAb (1 mg/ml) for 2 h (IL-2, JES6-A112 and

JES6-5H4; IL-4, 11B11 and BVD6-24G2; IL-10, JES5-16E3 and SXC-1 and IFN-γ, R4-6A2 and XMG1.2). The plates were developed after adding avidin peroxidase and its substrate and read on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Adoptive transfer to induce EAE

T cell subsets (CD3+, CD4+CD3hi and DN CD3hi) were isolated either by enrichment of CD3+ T from spleens and thymus of tg mice using mouse T cell enrichment columns by negative selection (R&D Systems) or by flow cytometric sorting. Cells (5×10^6) were injected in PBS into RAG2-deficient B10.S mice. The recipient mice were monitored for appearance of clinical signs of EAE and were scored on a 0–5 scale (1: limp tail, 2: hindlimb paresis, 3: complete hindlimb paralysis, 4: forelimb paresis and 5: moribund).

Histopathology

Mice were sacrificed when they reached a score of 4 or higher or when they began to recover from disease as indicated by no further increase in clinical score. Mice that did not develop EAE were sacrificed between days 28 and 35 after immunization. Brain and spinal cords were removed and fixed in 10% phosphate-buffered formalin. For histological assessment of inflammation, the brain and spinal cord were embedded in paraffin and sections stained with Luxol fast blue–hematoxylin and eosin stain. Meningeal and parenchymal inflammatory foci were counted by an observer blinded to the clinical status of the animals.

Results

4E3 tg mice expressing a PLP 139–151-specific TCR do not develop fulminant EAE on the resistant B10.S background

We previously generated tg mice that express different rearranged αβTCRs specific for PLP 139–151, i.e. 5B6 and 4E3 mice. The 4E3 TCR has a higher affinity for the PLP 139–151/IA^s complex than the 5B6 TCR (27). When these tg mice were backcrossed onto the EAE-susceptible SJL/J background, 4E3 mice (line 1740) developed spontaneous EAE with a higher frequency than 5B6 tg mice. The high incidence of fulminant disease resulted in the loss of this line on the SJL/J background (Table 1). Another 4E3 line (1736) also could not be maintained on the EAE-susceptible SJL/J background (27). However, when 4E3 mice were backcrossed onto the EAE-resistant B10.S background, the frequency of spontaneous disease was significantly lower reaching a steady-state level of 25% by the age of 6 months (Table 1).

Table 1. Frequency of spontaneous EAE in 4E3 TCR tg mice, line 1740

Generation	SJL/J		B10.S	
	Incidence[a]	Severity[b]	Incidence	Severity
N4	1/8 (13%)	3	3/7 (40%)	2
N5	9/15 (60%)	2.2	2/6 (30%)	1
N6	NA[c]	NA	4/17 (24%)	2

4E3 TCR tg mice on SJL/J and B10.S back-cross generations were monitored regularly for signs of EAE for at least 6 months.
[a]Number of tg mice with spontaneous EAE per total number of tg mice in each generation are shown and frequencies of disease are given in percentages (%). [b]Severity is shown as mean peak disease severity. [c]NA, not applicable, the line could not be maintained.

4E3 tg B10.S mice demonstrate a high proportion of CD4−CD8− (DN) CD3hi cells in the thymus and spleen

There was no apparent difference in the size of thymus, spleen and lymph nodes or in the absolute number of cells in these organs between 4E3 mice and wild-type (WT) littermates, indicating that there was no gross clonal deletion of tg T cells (data not shown). The percentage of CD3hi cells and CD4/CD8 ratios were increased in the thymus of 4E3 tg mice as compared with WT littermates, indicating positive selection of tg CD4+ T cells. However, we found a 10- to 15-fold increase in the percentage of DN cells among mature (CD3hi) T cells compared with WT littermates (tg 4E3: 33.9 ± 3.25%, WT: 2.25 ± 0.36%, $P < 0.01$), while the percentage of CD4 single-positive (SP) thymocytes was proportionally decreased (Fig. 1A). There was, however, no apparent difference in the percentage of either CD3hi or CD3low double-positive (DP) T cells between tg and control mice (Fig. 1A). Moreover, while the majority of DN T cells in 4E3 mice expressed high CD3 (Fig. 1A and B), the majority of DP cells was CD3low similar to WT mice (Fig. 1A). This increase in percentage of DN CD3hi thymocytes with the proportional decrease of CD4+ T cells was specific to 4E3 tg mice, since in another tg line (5B6), the percentages of DN CD3hi cells were similar to those in WT littermates (tg 4E3: 33.9 ± 3.25%, tg 5B6: 3.4 ± 0.41%, WT: 2.25 ± 0.36%, $P < 0.01$), while the proportions of tg CD4 SP thymocytes were increased (Fig. 1A and B). Although we observed a slight increase in the percentage of DN T cells also on the SJL/J background in 4E3 mice, this did not reach the magnitude of that observed on the B10.S background (data not shown) (27). Since none of the 4E3 TCR tg lines could be propagated on the SJL/J background, the rest of this paper deals with the analysis of 4E3 TCR tg line on the resistant background.

The high expression of CD3 on DN cells indicated that these thymocytes had been positively selected and that they do not represent thymocytes that are arrested at an early stage of development due to transgenesis. In addition, ~16% of DN CD3hi cells expressed the CD69 molecule in 4E3 tg mice (16.08 ± 0.7), while CD69 was undetectable on DN cells of WT littermates (Fig. 1C). These data suggest that in 4E3 tg B10.S mice, the DN CD3hi population contains positively selected mature T cells (30).

DN TCRβ+ cells in the spleen of 4E3 tg mice are recent thymic emigrants

If DN CD3hi thymocytes are positively selected, they should emigrate from the thymus. Indeed, the percentage of DN TCRβ+ T cells was increased up to 3-fold in the spleens of 4E3 tg mice compared with WT littermates (mean 5.8 ± 0.26 versus 1.5 ± 0.16%) (Fig. 2).

To determine if the peripheral tg DN T cells were generated in the thymus or by extrathymic maturation in the

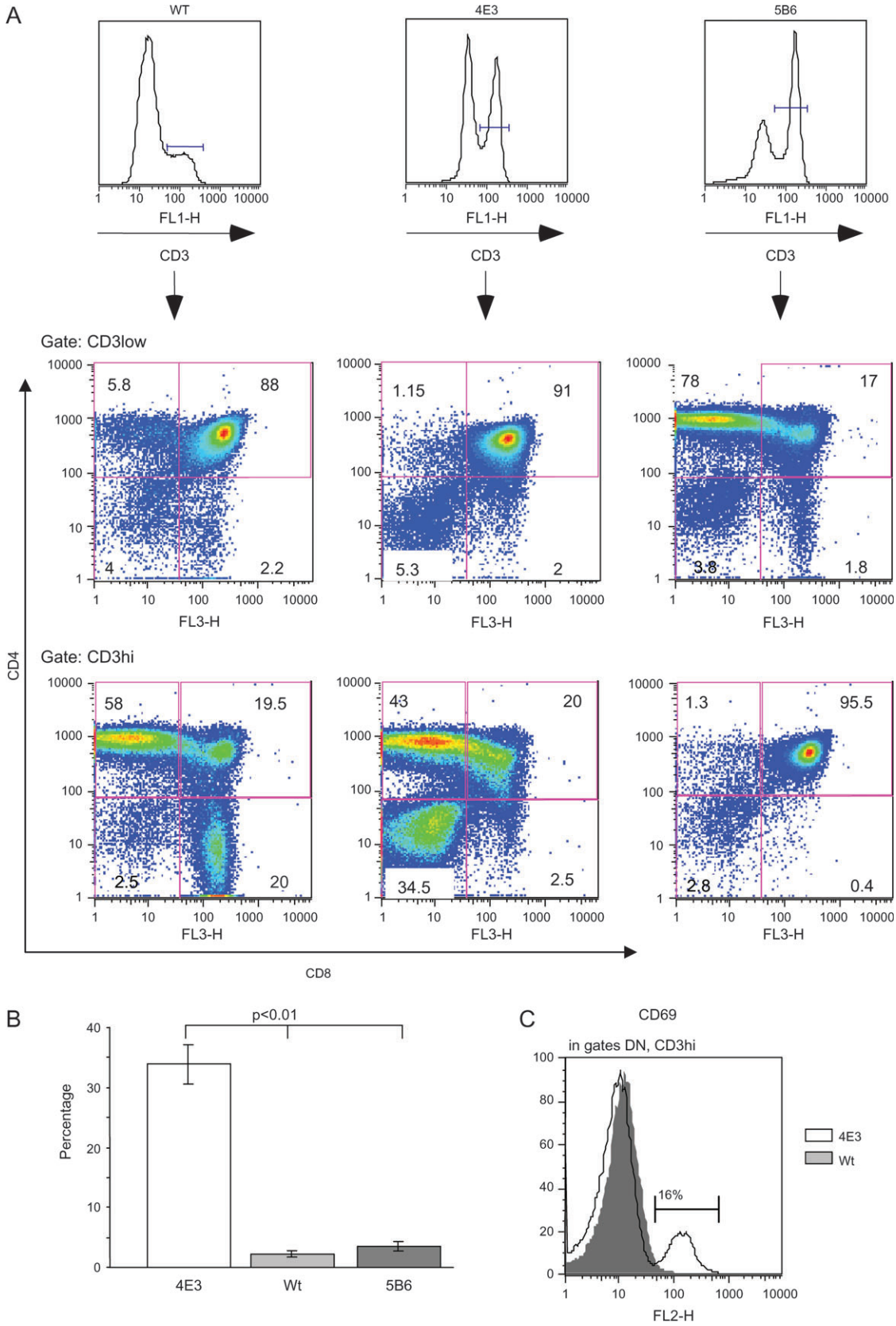

Fig. 1. The percentage of DN CD3hi cells is increased in the thymus of PLP 139–151-specific TCR tg 4E3 mice. (A) Surface expression of CD4 and CD8 was examined on CD3hi thymocytes in female PLP 139–151-specific 4E3 tg and 5B6 tg mice and WT littermates. Representative data are shown. (B) Surface expression of CD4 and CD8 was examined on CD3hi thymocytes as shown in (A) in 26 female PLP 139–151-specific 4E3 tg mice, 11 WT littermates and 5B6 tg mice. Percentage of DN CD3hi cells is shown. (C) The percentage of CD69+ DN thymocytes is shown in a tg 4E3 mouse and WT littermate. Representative data of six separate experiments are shown.

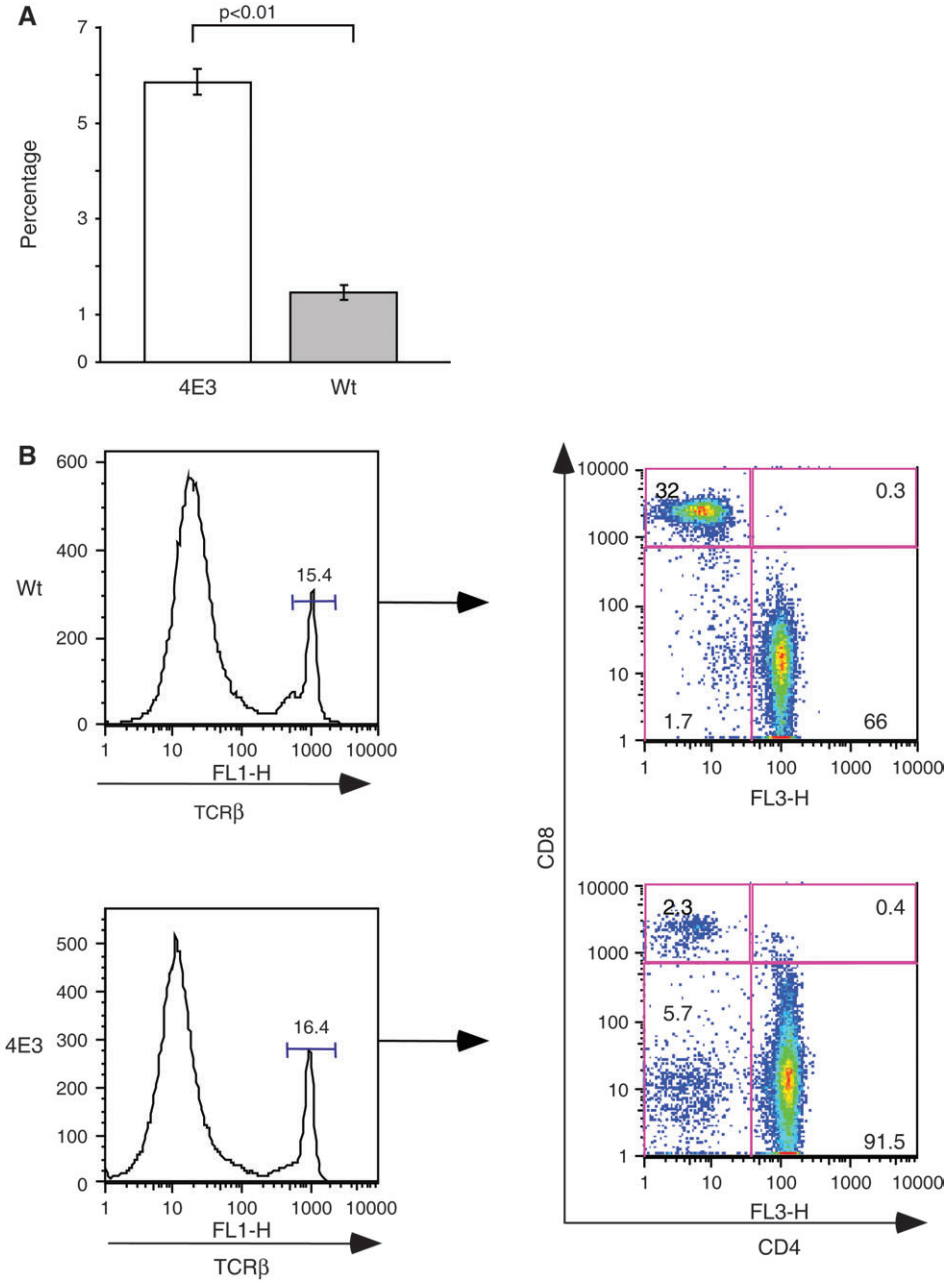

Fig. 2. The percentage of DN TCRβ+ T cells is increased in the spleen of 4E3 TCR tg mice. (A) Surface expression of CD4 and CD8 was examined on TCRβ+ splenocytes in 14 female 4E3 tg mice and WT littermates. (B) Representative data are shown.

periphery, 4E3 mice and WT littermates were given daily BrdU in the drinking water for 10 days. At the end of the labeling period, splenocytes were isolated and stained with anti-TCRβ, anti-CD4, anti-CD8 and anti-BrdU mAbs. It has been shown that recent thymic emigrants are BrdUlow, in contrast to the majority of peripheral T cells which are BrdU-negative resting cells and a small proportion of peripheral T cells which are BrdUhi cycling cells (29). The majority of DN TCRβ+ cells (mean 74.2 ± 6.8%) was BrdUlow in 4E3 mice, suggesting that the majority of DN TCRβ+ cells in the periphery was thymically derived and few if any proliferate in the periphery (Fig. 3A). When the proportion of DN tg T cells was examined among total thymic immigrants, 36% of the BrdUlow TCRβ+ cells were DN, in contrast to 5% in the WT mice (36.1 ± 4.5 versus 5 ± 1.22%, $P < 0.01$). These data indicate that DN thymocytes were exported to the periphery in high numbers in 4E3 tg B10.S mice (Fig. 3B).

DN CD3hi and TCRβ+ thymocytes and splenocytes are true tg TCRαβ T cells

Currently, a clonotypic antibody is not available to detect the expressed 4E3 tg TCR or its alpha and beta chains. To

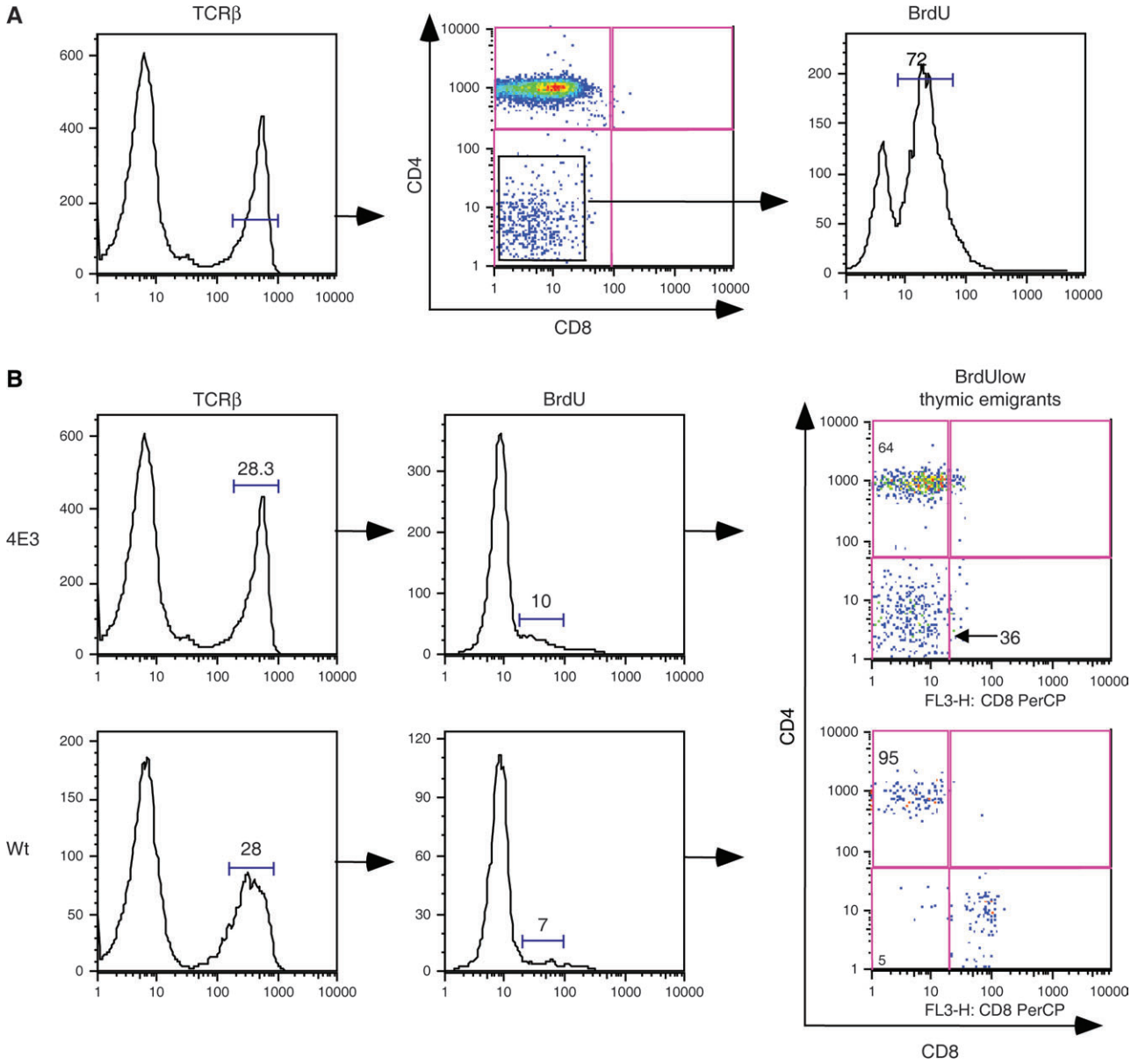

Fig. 3. Recent thymic emigrants in 4E3 TCR tg mice. (A) The percentage of recent thymic emigrants (BrdUlow) is shown in the DN TCRβ+ subset in a 4E3 tg mouse. Representative data of two different experiments with five mice in each are shown. (B) The percentage of TCRβ+ DN and CD4+ cells is shown in the subset of recent thymic emigrants (BrdUlow) in the spleen of a 4E3 tg mouse and WT littermate. The numbers in dot plots indicate the percentage of DN and CD4 cells in the $CD8^-$ recent thymic emigrant populations (BrdUlow). Representative data of two different experiments with five mice in each are shown.

preclude TCRγδ and endogenous TCRαβ gene rearrangements, we generated 4E3 TCR tg mice on a RAG2-deficient background by crossing the TCR tg mice with RAG-2-deficient B10.S mice. Cellular analysis in 4E3 and 4E3 RAG2-deficient B10.S mice revealed that the percentages of DN among CD3hi cells in the thymi of 4E3 tg mice with or without RAG-2 deficiency were comparable (mean 18.5 ± 2.2% and 20.7 ± 2.8%) (Fig. 4A). In the spleens, the percentage of DN TCRβ+ cells was comparable or slightly increased in RAG2-deficient compared with RAG2-intact 4E3 tg mice (mean 7.5 ± 0.5% and 5.7 ± 0.7%) (Fig. 4B). These data indicate that the vast majority of mature DN T cells in thymus and spleen of 4E3 TCR tg B10.S mice were positively selected and expressed the 4E3 αβTCR exclusively.

DN TCRβ+ tg T cells up-regulate CD4 following TCR-mediated stimulation

The increased DN and a proportionate decrease of CD4+ T cells in the thymus of 4E3 tg B10.S mice suggested that CD4 co-receptor might be down-regulated resulting in the conversion of CD4+ into DN CD3hi thymocytes (Fig. 1A). To examine the possible down-modulation of CD4, we analyzed expression of CD4 on DN T cells following T cell activation. Splenocytes from 4E3 tg mice were stimulated with PLP

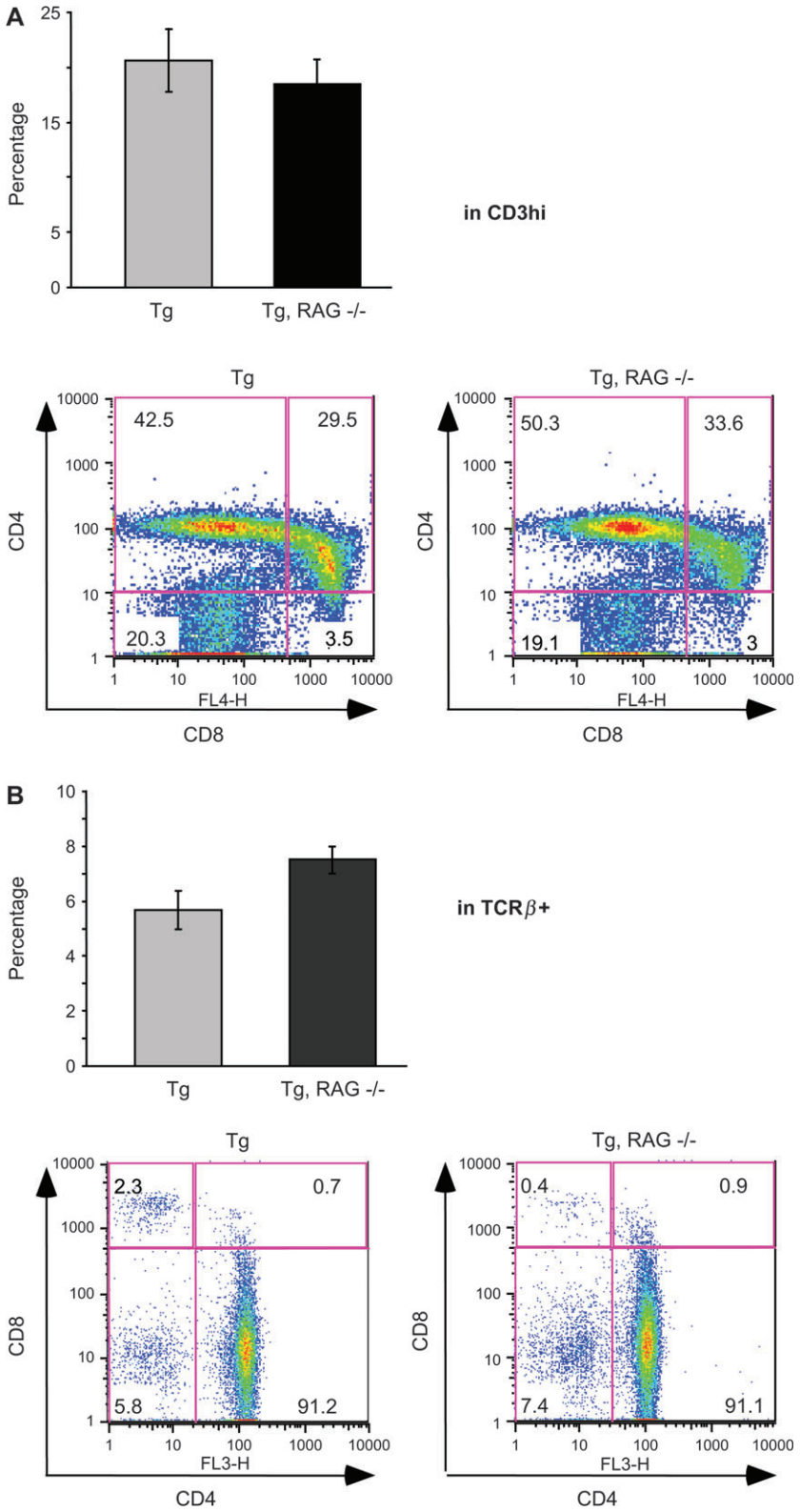

Fig. 4. The percentage of DN T cells is increased in the thymus and spleen of RAG-2-deficient 4E3 TCR tg mice. (A) Surface expression of CD4 and CD8 was examined on CD3+ thymocytes in seven female 4E3 TCR tg mice and in 4E3 TCR tg mice on a RAG-2-deficient background. Columns indicate percentage of DN CD3hi T cells and representative FACS data are shown. (B) Surface expression of CD4 and CD8 was examined on TCRβ+ splenocytes in seven female 4E3 TCR tg mice and in 4E3 TCR tg mice on RAG-2-deficient background. Columns indicate percentage of DN TCRβ+ T cells and representative FACS data are shown.

139–151 peptide presented by irradiated antigen presenting cells APC, and CD4 expression was analyzed by flow cytometry on TCRβ+ T cells after 72 h. In unstimulated control cultures, only CD4+ and CD4− TCRβ+ (DN) subsets were detected. After stimulation with the PLP peptide, four different TCRβ+ subsets could be distinguished based on the expression level of CD4: CD4− (DN), CD4low, CD4+ and CD4hi (Fig. 5A). This suggested that both DN and CD4+TCRβ+ tg cells were able to modulate expression of CD4 after stimulation with the PLP antigen. However, we could not exclude the possibility that the CD4low subset originated from CD4+ T cells by down-regulating the co-receptor. To distinguish between these two possibilities, we sorted CD4+ and DN TCRβ+ cells by flow cytometry from spleens of female 4E3 mice, and then stimulated each subset separately with plate-bound anti-CD3 and soluble anti-CD28 mAbs for 72 h. A subset of CD4− (DN) tg T cells up-regulated the CD4 co-receptor upon activation (2.1 ± 0.6% versus mean 12.4 ± 1.6%, $P < 0.01$). On the other hand, we did not observe any down-regulation of the co-receptor in the CD4+ tg T cell cultures (Fig. 5B). The up-regulation of CD4 co-receptor upon stimulation suggested that DN tg T cells retained the potential to modulate the expression of the co-receptor and matured DN T cells might be generated by down-modulation of CD4 on SP T cells.

DN T cells demonstrate blunted responses to CD4/MHC II-dependent TCR stimulation compared with CD4+ T cells

Our data indicate that tg CD3hi DN thymocytes with a modulated CD4 co-receptor rapidly migrate to the periphery and constitutes a part of the peripheral repertoire in 4E3 tg B10.S mice. Since CD4 has been shown to modulate the activation threshold of responding T cells (23, 31, 32), we hypothesized that CD4 down-regulation on tg T cells would modulate the autoreactive response mediated by the high-affinity 4E3 TCR. To address this issue, DN TCRβ+ and CD4+ splenic T cells were isolated by cell sorting and stimulated with either PLP 139–151 or with plate-bound anti-CD3 and soluble anti-CD28 mAbs. Both subsets proliferated in response to stimulation with the peptide, but the response of DN tg T cells was significantly lower compared with that of CD4+ T cells. In contrast, the two subsets proliferated equally well to anti-CD3/CD28 stimulation. These data demonstrated that only the response of DN tg T cells to peptide antigen were blunted (Fig. 6A). Consistent with these data, reduced PLP 139–151-specific activation was also observed in whole spleen cell cultures, where 33% of DN TCRβ+ cells compared with 70% of CD4+ T cells expressed the early activation marker CD69 in a 3-day culture (data not shown).

We also examined the cytokine production of sorted CD4+ and DN TCRβ+ cells after stimulation with either PLP 139–151 or anti-CD3/CD28. Both DN and CD4+ T cell subsets produced T_h1 cytokines (IL-2, IFN-γ), but failed to produce detectable amounts of IL-4 or IL-10 to either stimulus, indicating that DN T cells in 4E3 tg mice do not produce anti-inflammatory cytokines or represent a regulatory subset described previously (21). Consistent with the decreased proliferative response, DN tg T cells produced less IL-2 and IFN-γ in response to PLP 139–151 compared with CD4+ tg cells, while there was no difference in the production of these cytokines after activation with the polyclonal stimulus of anti-CD3/CD28 (Fig. 6B). Likewise, both T cell subsets produced equal amounts of IFN-γ after stimulation with phorbol myristate acetate/ionomycin (data not shown). These data indicate that DN T cells showed no defect in proliferative or cytokine responses to CD4/MHC II-independent TCR stimulation.

DN CD3hi tg cells induce EAE but are less encephalitogenic than CD4+ tg T cells

To determine the *in vivo* response of 4E3 tg DN T cells to the autoantigen, we examined the encephalitogenicity of these cells upon adoptive transfer. We first tested whether tg SP CD3+ T cells obtained from the thymus can induce EAE. Sorted CD3high, CD4+ T cells obtained from thymus or spleen induced EAE equally well upon adoptive transfer into RAG-2-deficient B10.S mice (data not shown). Next, we tested the encephalitogenic potential of DN CD3hi thymocytes by transferring 5×10^6 DN TCRβ+ thymocytes into RAG-2-deficient B10.S recipients. As a control, we also transferred tg CD4+CD3hi cells into WT B10.S recipients. The majority of recipients receiving CD4 SP thymocytes (70%) developed severe disease (mean maximal score of 2.4 ± 0.3) in 3 weeks after transfer. In contrast, transfer of DN CD3hi thymocytes induced milder EAE (mean maximal score of 1 ± 0) in significantly fewer mice (25%) (Fig. 7 and Table 2). There was no difference in the day of onset of disease between the two groups (Table 2). Adoptive transfer of CD4+ tg T cells induced histological EAE in the CNS of all mice (100%), while DN tg T cells induced histological EAE in only a small portion of recipients (16%) (Table 3). We observed similar numbers of inflammatory foci in the meninges and parenchyma exhibited within each group; however, significantly less inflammatory foci in recipients of DN 4E3 cells compared with recipients of CD4+ tg T cells were detected (Table 3). Based on these observations, we conclude that DN T cells transfer EAE less efficiently into RAG-2-deficient B10.S recipient mice than do CD4+ T cells indicating a blunted antigen-specific response of DN tg T cells *in vivo*.

Thymic expression of PLP does not drive the generation of tg DN CD3hi T cells

We have shown that a high frequency of DN CD3hi T cells is selected in 4E3 TCR tg mice on the resistant B10.S background. These T cells respond poorly to PLP 139–151 and consequently are weakly encephalitogenic. A possible explanation for these observations is that thymocytes bearing the high-affinity 4E3 tg TCR down-modulate CD4 upon encounter of self-antigen in the thymus in order to avoid negative selection. To address whether the expression of PLP in the thymus is responsible for the generation of DN cells in 4E3 tg B10.S mice, we crossed 4E3 TCR tg mice with PLP-deficient mice (28) on the B10.S background and examined T cell development in the thymus. As shown in Fig. 8(B), we found that the frequency of DN cells present in 4E3 tg PLP-deficient mice was not significantly different compared with 4E3 tg WT littermate controls. Thus, the expression of PLP in the thymus does not appear to play a role in the thymic down-modulation of CD4 on 4E3 tg T cells on the B10.S background.

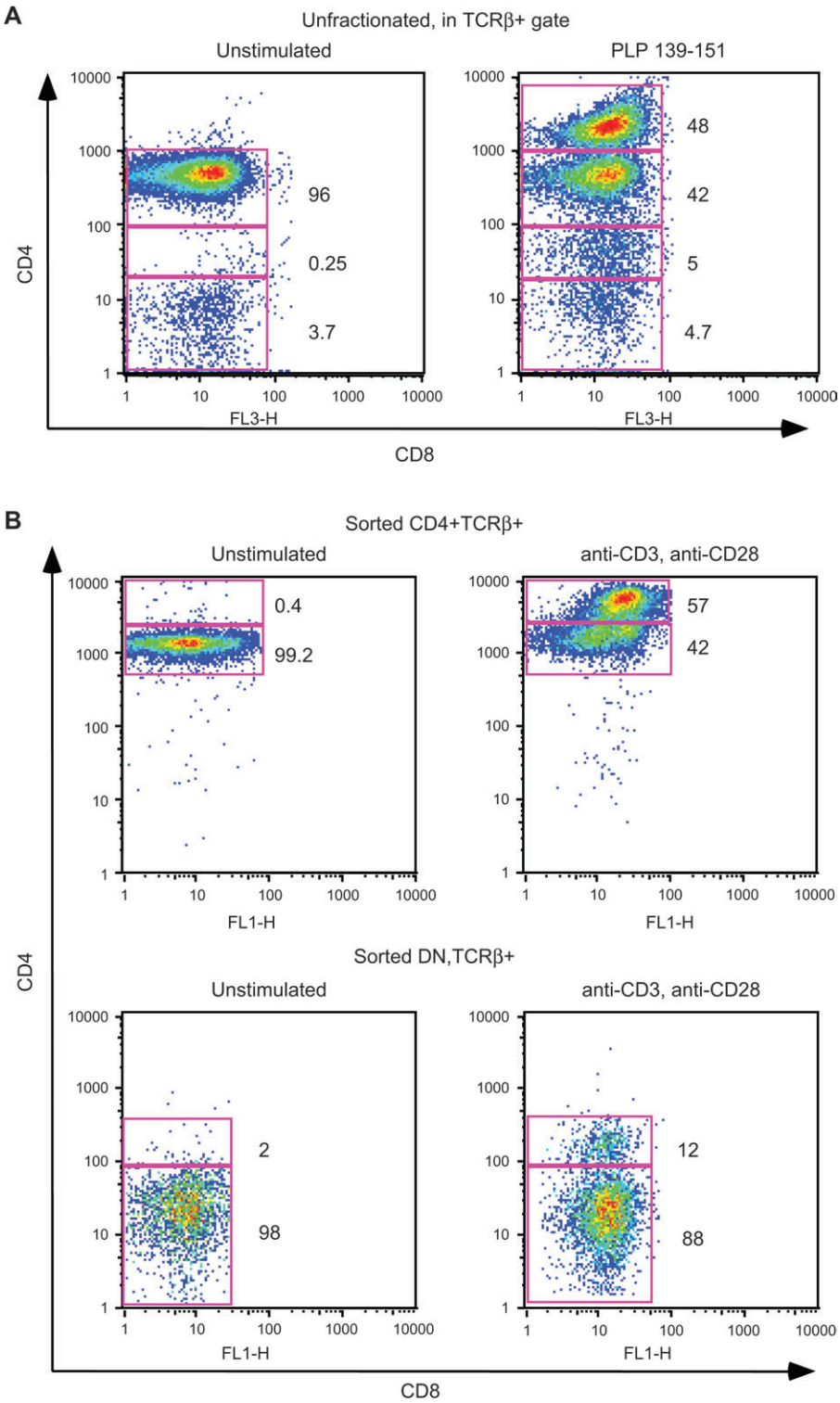

Fig. 5. 4E3 TCR tg T cells up-regulate the CD4 co-receptor upon stimulation *in vitro*. (A) Splenocytes (4×10^5) of tg 4E3 mice were stimulated with 50 μg ml^{-1} PLP 139–151 *in vitro* in the presence of irradiated antigen presenting cells. The expression of CD4 was examined on unstimulated (left panel) and stimulated (right panel) TCRβ+ cells by flow cytometry after 72 h. Representative data of three separate experiments are shown. (B) CD4+TCRβ+ (upper panel) and DN TCRβ+ subsets (lower panel) were isolated by FACS sorting from spleens of 4E3 tg mice. T cells (3×10^4) were stimulated with 5 μg plate-bound anti-CD3 and 2 μg ml^{-1} soluble anti-CD28 and the expression of CD4 was examined by flow cytometry after 72 h. Representative data of three separate experiments are shown.

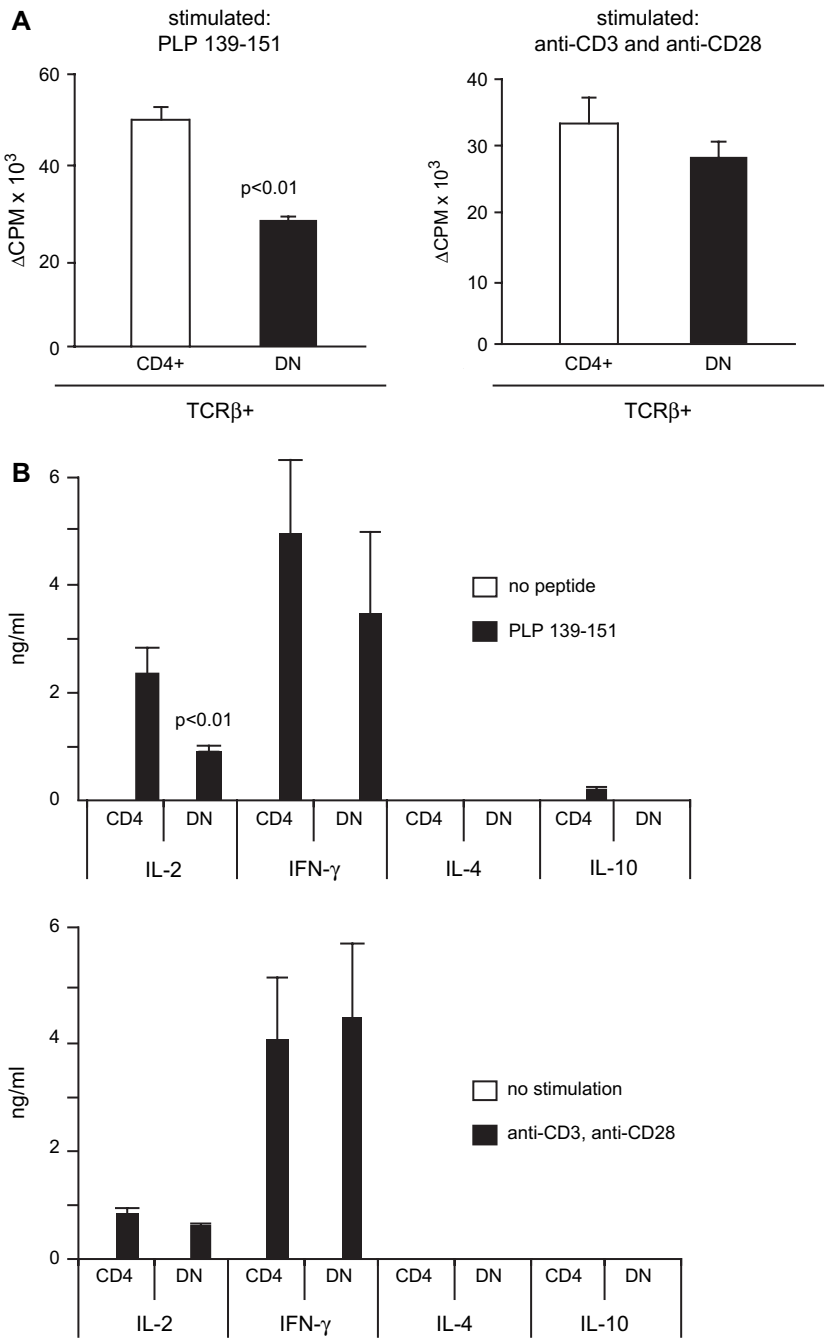

Fig. 6. Proliferative response and cytokine production of sorted CD4+TCRβ+ and DN TCRβ+ subsets of 4E3 TCR tg mice. (A) CD4+TCRβ+ and DN TCRβ+ subsets were sorted by FACS from spleens of 4E3 tg mice. T cells (3×10^4) were stimulated with 50 μg ml^{-1} PLP 139–151 in the presence of 5×10^5 irradiated antigen presenting cells (left panel) or with 5 μg plate-bound anti-CD3 and 2 μg ml^{-1}soluble anti-CD28 (right panel). Proliferative responses were measured by incorporation of [^{3}H]thymidine after 72 h. Data of three separate experiments are shown. (B) The production of IL-2, IFN-γ, IL-4 and IL-10 was examined by ELISA in the above cultures after 48-h stimulation with PLP 139–151 (upper panel) or anti-CD3 and anti-CD28 (lower panel). Data of three separate experiments are shown.

Discussion

In this study, we show that TCR tg mice expressing a high-affinity TCR for the self myelin antigen PLP 139–151, when bred on the resistant B10.S background, demonstrate a reduction in fulminant spontaneous EAE along with the appearance of a high proportion of DN tg T cells both in the thymus and periphery. We show that the magnitude of DN tg T cell responses to self-antigen PLP 139–151 *in vitro* and *in vivo* are blunted in the absence of CD4, although the responses to anti-CD3 are comparable to CD4+ tg T cells *in vitro*. In addition, DN T cells can partly up-regulate the CD4 co-receptor upon activation, suggesting that CD4 was not lost due to developmental block but is actively down-regulated.

Table 2. Characterization of EAE induced by adoptive transfer of CD4+CD3hi or DN CD3hi thymocytes

Donor	Incidence	Incidence (%)	Mean onset (days)	Mean maximum score	Mortality (%)	Recovery (%)
CD4	7/10	60*	20.6 ± 0.9[a]	2.4 ± 0.3*	14*	0
DN[l]	3/12	25	20 ± 1.1	1 ± 0	0	66*

EAE was induced by adoptive transfer of sorted CD4+CD3hi or DN CD3hi thymocytes into RAG-deficient B10.S mice and scored as described in Methods. [a]SEM. *$P \leq 0.05$.

Table 3. Histologic EAE induced by adoptive transfer of CD4+CD3hi or DN CD3hi thymocytes

Donor	Incidence	Incidence %	Number of inflammatory foci (mean ± SEM)		
			Meninges	Parenchyma	Total
CD4	10/10*	100*	63.1 ± 7.7*	65.5 ± 12.7*	129.6 ± 17.8*
DN	2/12	16	29.9 ± 19.7	20.1 ± 12.8	50.8 ± 30.7

EAE was induced by adoptive transfer of sorted CD4+CD3hi or DN CD3hi thymocytes into RAG-deficient B10.S mice. The number of inflammatory foci was determined as described in Methods. *$P \leq 0.05$.

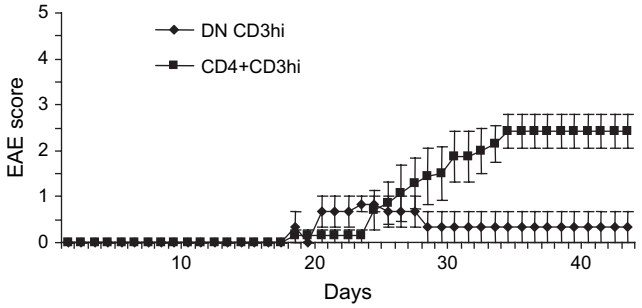

Fig. 7. Induction of EAE with 4E3 TCR tg T cell subsets. Thymi of 16 female 4E3 TCR tg mice were pooled, and CD4+CD3hi and DN CD3hi subsets were sorted by flow cytometry. T cells (5×10^6) of each fraction were injected intravenously into RAG-deficient B10.S mice, respectively. Mice were scored for clinical signs of EAE daily as described in Methods. Data represent average daily scores of mice (DN CD3hi: 3/12, CD4+CD3hi: 7/10) pooled from two independent experiments (see Table 2).

These results suggest that the tg DN T cells originate from CD4+ T cells by down-regulating the co-receptor and that this modulation of co-receptor limits activation of T cells expressing a high-affinity TCR for self-antigen, thereby preventing the development of spontaneous disease. However, this thymic modulation of the CD4 co-receptor was independent from the expression of PLP 139–150.

Approximately 70% of DN TCRβ+ cells were recent thymic emigrants in 4E3 tg B10.S mice. Several lines of evidence indicated that the majority of these cells were generated in the thymus, not extrathymically and were positively selected. First, the high percentage of DN tg T cells in the thymus and spleen were proportional, and a decrease in the percentage of DN T cells with aging in the thymus resulted in decrease in the percentage of DN cells in the periphery (data not shown). In addition, a proportional decrease in the percentage of CD4+ along with an increase of tg DN thymocytes may suggest conversion of SP CD4 T cells into DN thymocytes. Second, the high expression of CD3 on DN thymocytes and the partial expression of CD69 indicate that the DN cells were positively selected in the thymus. Third, generation of DN T cells from DP thymocytes by active down-regulation of CD4 and CD8 co-receptors (11, 14, 33–36) was unlikely in 4E3 mice, since peripheral DN T cells could up-regulate only CD4 but not CD8 following TCR-mediated stimulation, and DN thymocytes were CD3hi while DP cells expressed only low levels of CD3. Although tg DN thymocytes may also maturate without passing the DP stage and up-regulate any co-receptors (37), it is unlikely that 4E3 DN T cells preserved only the potential of expressing CD4 but not CD8 upon proper stimulation in the periphery. Fourth, modulation of CD4 also excluded the abortive expression of CD4 as a result of tg dysgenesis. Gross clonal deletion of tg CD4+ thymocytes was also unlikely, since there was no decrease in the number of T cells or in the percentage of DP cells between tg mice and WT littermates. Finally, DN thymocytes were capable to mediate EAE upon adoptive transfer.

The magnitude of immune responses to PLP 139–151 was blunted in the absence of CD4, although the responses to anti-CD3 were comparable. There was no difference in the expression level of TCR between CD4+ and DN tg T cells, suggesting that the reduced response was not the result of TCR modulation. Since 4E3 tg DN T cells responded to PLP 139–151 and produced significant amounts of IFN-γ and IL-2, DN tg TCRβ+ cells are not anergic and likely lack those regulatory properties that had been previously ascribed to anergic and DN T cells (21, 22, 38). 4E3 DN T cells behaved like CD4+ tg T cells, except that they had a reduced capacity to respond to self-antigen. In addition to the reduced response *in vitro*, the adoptive transfer of DN CD3hi T cells induced less severe disease than the transfer of CD4 T cells, highlighting the significance of CD4 modulation *in vivo*. This modulated expression of co-receptor could increase the activation threshold of tg T cells, thereby decreasing the incidence of spontaneous EAE in 4E3 B10.S mice. Nevertheless, DN 4E3 T cells were encephalitogenic upon adoptive transfer. Our data also imply that the high affinity of the 4E3 TCR could be a crucial factor in the generation of

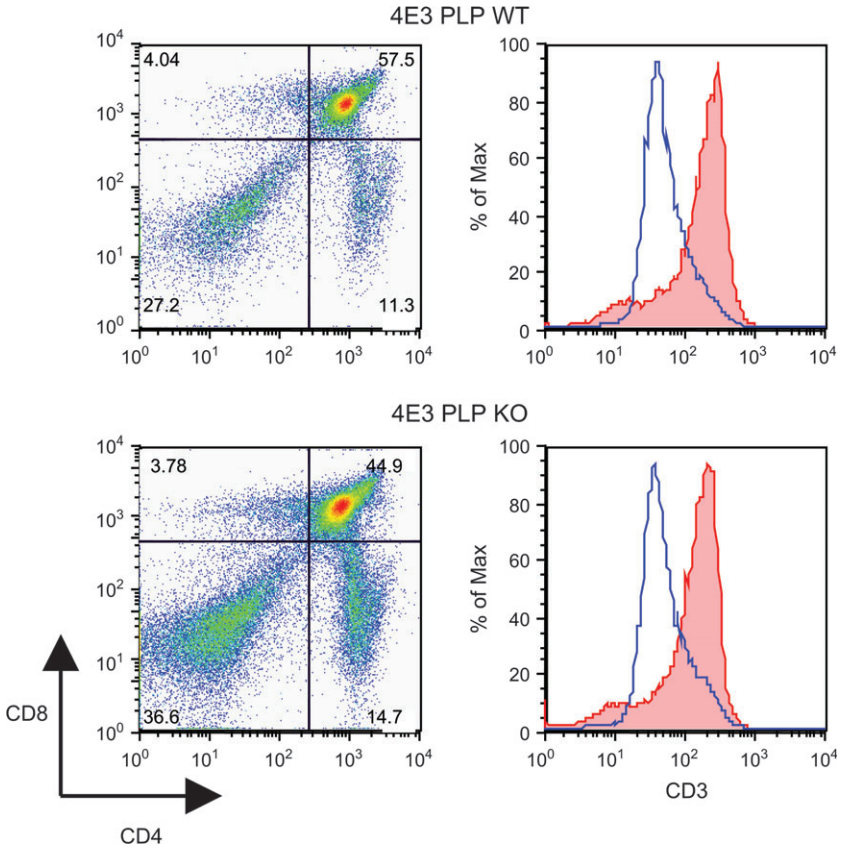

Fig. 8. DN CD3hi cells in the thymus of 4E3 tg PLP-deficient mice. Left panels: CD4 and CD8 expression profile of thymocytes from 4E3 tg WT and 4E3 tg PLP-deficient littermates. Right panels: expression of CD3 on DN cells (closed histogram) relative to DP cells (open histogram). A total of four mice were analyzed. Representative data are shown.

DN T cells. Another PLP 139–151-specific TCR 5B6 has a lower affinity for the antigen/MHC II than the 4E3 TCR (10) and 5B6 TCR on the B10.S background have no increase in the percentage of DN T cells compared with WT littermates. Altered activation thresholds have been previously shown in studies of tg expression of antigen together with high-affinity TCR, and after repeated stimulation with antigen *in vitro* (11, 21–23, 31, 32, 37–40). It should also be noted that the original 4E3 T cell clone had a non-functional CD4 co-receptor, indicating that such modulation of CD4 might also have been responsible for T cells bearing this high-affinity TCR exiting the thymus without deletion (27). Similar increases in DN tg T cells have been previously described in other TCR tg mice and in lpr/gld mice (21, 22, 35, 38, 41, 42) and in human lymphoproliferative disorders and systemic lupus erythematosus (SLE) (43, 44). In these cases, modulation of the CD4 co-receptor as a result of exposure to self-ligands should be also considered as a mechanism for peripheral tolerance, particularly in lpr/gld mice and SLE in which autoreactive T cells with high-affinity TCR may not be deleted due to their defect in apoptosis.

Since mice 4E3 tg mice died on the SJL/J background very early due to fulminant disease, the lines could not be maintained, so we were not able to directly compare 4E3 tg SJL/J and B10.S mice. However, our previous data indicated no such increase in the number of 4E3 tg DN T cells in SJL/J as observed in B10.S mice, suggesting that the high number of tg DN T cells might be indeed related to the resistant background (27). Since we have previously shown that differential thymic expression of thymic PLP may influence the frequency of PLP-specific T cells and susceptibility to autoimmunity (4), we considered the possibility that higher expression of PLP in the thymus of B10.S mice along with a high-affinity tg TCR may induce modulation of CD4. However, backcrossing the 4E3 B10.S tg mice onto a PLP-deficient background did not decrease the percentage of thymic DN T cells, thus factors other than thymic expression of autoantigen may influence the modulation of CD4. While thymic expression of PLP antigen does not seem to influence the co-receptor modulation, the TCR signal might be important. Two different lines bearing the 4E3 TCR exhibited high number of DN T cells, while the percentage of DN T cells was not significantly increased in the case of another tg PLP-specific TCR, 5B6, which has a lower affinity for PLP 139–151. Thus, while cross-reactivity of the 4E3 PLP-specific TCR with other thymic antigens can be one possible mechanism responsible to the modulated expression of CD4, strength or duration of TCR signals and transcriptional regulation of co-receptors similar to differentiation of DP into SP T cells may also play role (45). In any case, down-modulation of CD4 on tg T cells resulted in a reduced response to the autoantigen in the periphery *in vitro* and *in vivo*.

In summary, our data indicate that highly autoreactive T cells can down-regulate the CD4 co-receptor in the thymus on a resistant genetic background when there is a large autoreactive repertoire. This modulation of CD4, irrespective of the expression of PLP 139–150 in the thymus, can result in tolerance to self-antigen in the periphery, thereby preventing or reducing the frequency of spontaneous autoimmune disease.

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Abbreviations

BrdU	5-bromo-2-deoxyuridine
CNS	central nervous system
DN	double negative
DP	double positive
EAE	experimental autoimmune encephalomyelitis
PLP	proteolipid protein
SLE	systemic lupus erythematosus
SP	single positive
tg	transgenic
WT	wild type

References

1 Janeway, C. A. Jr 1994. Thymic selection: two pathways to life and two to death. *Immunity* 1:3.
2 Ramsdell, F., Lantz, T. and Fowlkes, B. J. 1989. A nondeletional mechanism of thymic self tolerance. *Science* 246:1038.
3 Schonrich, G., Momburg, F., Hammerling, G. J. and Arnold, B. 1992. Anergy induced by thymic medullary epithelium. *Eur. J. Immunol.* 22:1687.
4 Anderson, A. C., Nicholson, L. B., Legge, K. L., Turchin, V., Zaghouani, H. and Kuchroo, V. K. 2000. High frequency of autoreactivemyelin proteolipid protein-specific T cells in the periphery of naive mice: mechanisms of selection of the self-reactive repertoire. *J. Exp. Med.* 191:761.
5 Klein, L., Klugmann, M., Nave, K. A., Tuohy, V. K. and Kyevski, B. 2000. Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat. Med.* 6:56.
6 Liston, A., Lesage, S., Wilson, J., Peltonen, L. and Goodnow, C. C. 2003. Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* 4:350.
7 Goldrath, A. W. and Bevan, M. J. 1999. Selecting and maintaining a diverse T-cell repertoire. *Nature* 402:255.
8 Ohashi, P. S., Oehen, S., Buerki, K. *et al.* 1991. Ablation of ''tolerance'' and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65:305.
9 Oldstone, M. B., Nerenberg, M., Southern, P., Price, J. and Lewicki, H. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell* 65:319.
10 Waldner, H., Collins, M. and Kuchroo, V. K. 2004. Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. *J. Clin. Invest.* 113:990.
11 Schonrich, G., Kalinke, U., Momburg, F. *et al.* 1991. Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. *Cell* 65:293.
12 Jenkins, M. K. and Schwartz, R. H. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. Exp. Med.* 165:302.
13 Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science* 248:1349.
14 Rocha, B., Tanchot, C. and von Boehmer, H. 1993. Clonal anergy blocks *in vivo* growth of mature T cells and can be reversed in the absence of antigen. *J. Exp. Med.* 177:1517.
15 Forster, I., Hirose, R., Arbeit, J. M., Clausen, B. E. and Hanahan, D. 1995. Limited capacity for tolerization of CD4+ T cells specific for a pancreatic beta cell neoantigen. *Immunity* 2:573.
16 Munder, M., Bettelli, E., Monney, L., Slavik, J. M., Nicholson, L. B. and Kuchroo, V. K. 2002. Reduced self-reactivity of an autoreactive T cells after activation with cross-reactive non-self ligand. *J. Exp. Med.* 196:1151.
17 Illes, Z., Waldner, H., Reddy, J., Nicholson, L. B., Bettelli, E. and Kuchroo, V. K. 2005. T cell tolerance induced by cross-reactive TCR ligands can be broken by superagonist resulting in anti-inflammatory T cell cytokine production. *J. Immunol.* 175:1491.
18 Jones, L. A., Chin, L. T., Longo, D. L. and Kruisbeek, A. M. 1990. Peripheral clonal elimination of functional T cells. *Science* 250:1726.
19 Zhang, L. I., Martin, D. R., Fung-Leung, W. P., Teh, H. S. and Miller, R. G. 1992. Peripheral deletion of mature CD8+ antigen-specific T cells after *in vivo* exposure to male antigen. *J. Immunol.* 148:3740.
20 Jameson, S. C., Hogquist, K. A. and Bevan, M. J. 1994. Specificity and flexibility in thymic selection. *Nature* 369:750.
21 Wang, R., Wang-Zhu, Y. and Grey, H. 2001. Interactions between double positive thymocytes and high affinity ligands presented by cortical epithelial cells generate double negative thymocytes with T cell regulatory activity. *Proc. Natl Acad. Sci. USA*. 99:2181.
22 Viret, C. and Janeway, C. A. Jr 2003. Self-specific MHC class II-restricted CD4−CD8− T cells that escape deletion and lack regulatory activity. *J. Immunol.* 170:201.
23 Nicholson, L. B., Anderson, A. C. and Kuchroo, V. K. 2000. Tuning T cell activation threshold and effector function with cross-reactive peptide ligands. *Int. Immunol.* 12:205.
24 Tuohy, V. K., Lu, Z., Sobel, R. A., Laursen, R. A. and Lees, M. B. 1989. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J. Immunol.* 142:1523.
25 McRae, B. L., Kennedy, M. K., Tan, L. J., Dal Canto, M. C. and Miller, S. D. 1992. Induction of active and adoptive chronic-relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. *J. Neuroimmunol.* 38:229.
26 Greer, J. M., Sobel, R. A., Sette, A., Southwood, S., Lees, M. B. and Kuchroo, V. K. 1996. Immunogenic and encephalitogenic epitope clusters of myelin proteolipid protein. *J. Immunol.* 156:371.
27 Waldner, H., Whitters, M. J., Sobel, R. A., Collins, M. and Kuchroo, V. K. 2000. Fulminant spontaneous autoimmunity of the central nervous system in mice transgenic for the myelin proteolipid protein-specific T cell receptor. *Proc. Natl Acad. Sci. USA* 97:3412.
28 Klugmann, M., Schwab, M. H., Puhlhofer, A. *et al.* 1997. Assembly of CNS myelin in the absence of myelin proteolipid protein. *Neuron* 18:59.
29 Tough, D. F. and Sprent, J. 1994. Turnover of naive and memory-phenotype T cells. *J. Exp. Med.* 179:1127.
30 von Boehmer, H. 1994. Positive selection of lymphocytes. *Cell* 76:219.
31 Hampl, J., Chien, Y. H. and Davis, M. M. 1997. CD4 augments the response of a T cell agonist but not antagonist ligands. *Immunity* 7:379.
32 Madrenas, J., Chau, L. A., Smith, J., Bluestone, J. A. and Germain, R. N. 1997. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *J. Exp. Med.* 185:219.

33 Gapin, L., Matsuda, J. L., Surh, C. D. and Kronenberg, M. 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat. Immunol.* 10:971.
34 Teh, H. S., Kishi, H., Scott, B. and von Boehmer, H. 1989. Deletion of autospecific T cells in T cell receptor (TCR) transgenic mice spares cells with normal TCR levels and low levels of CD8 molecules. *J. Exp. Med.* 169:795.
35 Caveno, J., Zhang, Y., Motyka, B., Teh, S. J. and Teh, H. S. 1999. Functional similarity and differences between selection-independent CD4−CD8− αβT cells and positively selected CD8 T cells expressing the same TCR and the induction of anergy in CD4−CD8− αβT cells in antigen-expressing mice. *J. Immunol.* 163:1222.
36 Landolfi, M. M.T., Van Houten, N., Russell, J. Q., Scollay, R., Parnes, J. R. and Budd, R. C. 1993. CD2−CD4−CD8−lymph node T lymphocytes in MLR lpr/lpr mice are derived from a CD2+CD4+CD8+ thymic precursor. *J. Immunol.* 151:1086.
37 Liu, C.-P., Kappler, J. W. and Marrack, P. 1996. Thymocytes can become mature T cells without passing through the CD4+CD8+, double-positive stage. *J. Exp. Med.* 184:1619.
38 Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A. and Nagata, S. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356:314.
39 Shen, X. and Konig, R. 1998. Regulation of T cell immunity and tolerance *in vivo* by CD4. *Int. Immunol.* 10:247.
40 Zhang, L., Fung-Leung, W. and Miller, R. G. 1995. Down-regulation of CD8 on mature antigen-reactive T cells as a mechanism of peripheral tolerance. *J. Immunol.* 155:3464.
41 Harrington, C. J., Paez, A., Hunkapiller, T. *et al.* 1998. Differential tolerance is induced in T cells recognizing distinct epitopes of myelin basic protein. *Immunity* 8:571.
42 Targoni, O. S. and Lehmann, P. V. 1998. . Endogeneous myelin basic protein inactivates the high avidity T cell repertoire. *J. Exp. Med.* 187:2055.
43 Shivakumar, S., Tsokos, G. C. and Datta, S. K. 1989. T cell receptor alpha/beta expressing double-negative (CD4−/CD8−) and CD4+ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. *J. Immunol.* 143:103.
44 Straus, S. E., Sneller, M., Leonardo, M. J., Puck, J. M. and Strober, W. 1999. An inherited disorder of lymphocyte apoptosis: the autoimmune lymphoproliferative syndrome. *Ann. Intern. Med.* 130:591.
45 Sarafova, S. V., Erman, B., Yu, Q. *et al.* 2005. Modulation of coreceptor transcription during positive selection dictates lineage fate independently of TCR/coreceptor specificity. *Immunity* 23:75.