

CD4⁺CD25⁺ Regulatory T Cells Specific for a Thymus-Expressed Antigen Prevent the Development of Anaphylaxis to Self¹

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A role for CD4⁺CD25⁺ regulatory T cells (Tregs) in the control of allergic diseases has been postulated. We developed a mouse model in which anaphylaxis is induced in SJL mice by immunization and challenge with the fragment of self myelin proteolipid protein (PLP)_{139–151}, that is not expressed in the thymus, but not with fragment 178–191 of the same protein, that is expressed in the thymus. In this study, we show that resistance to anaphylaxis is associated with naturally occurring CD4⁺CD25⁺ Tregs specific for the self peptide expressed in the thymus. These cells increase Foxp3 expression upon Ag stimulation and suppress peptide-induced proliferation of CD4⁺CD25[−] effector T cells. Depletion of Tregs with anti-CD25 in vivo significantly diminished resistance to anaphylaxis to PLP_{178–191}, suggesting an important role for CD4⁺CD25⁺ Tregs in preventing the development of allergic responses to this thymus-expressed peptide. These data indicate that naturally occurring CD4⁺CD25⁺ Tregs specific for a peptide expressed under physiological conditions in the thymus are able to suppress the development of a systemic allergic reaction to self. *The Journal of Immunology*, 2008, 180: 4433–4440.

Allergic diseases have been steadily increasing over the past two decades, and now affect up to 15% of the population in the Western countries (1). Inappropriate responses to otherwise innocuous environmental allergens, regulated by a set of Th2 cytokines, including IL-4, IL-5, and IL-13, are thought to underlie the development of such disorders (2, 3). However, despite the advances in our understanding of the pathophysiology of allergic diseases, the mechanisms that influence the immune system of an individual to develop a skewed Th2 response leading to allergies against certain Ags (“allergens”) and not others remains unknown.

We previously showed that a single immunization and re-exposure of SJL mice (H-2^s) to myelin proteolipid protein (PLP)³ fragment 139–151 (PLP_{139–151}), a self peptide of the myelin of the CNS, leads to the development of anaphylaxis (4), the most severe manifestation of an allergic reaction (5). This finding recently was confirmed by others (6, 7). Conversely, immunization and re-exposure of the same strain of mice to PLP_{178–191}, a different fragment of PLP, did not induce anaphylaxis (4, 6). PLP_{178–191} and PLP_{139–151} are differentially expressed in the thymus of SJL mice. Only the DM20 isoform of PLP, which lacks residues

116–150, is expressed in the thymus (8). Thus, a lack of expression of PLP_{139–151} in the thymus results in escape from central tolerance of T cells recognizing this peptide and a high frequency in the periphery of these autoreactive cells (9). Because both these peptides are able to effectively prime the immune system of SJL mice to develop the organ-specific autoimmune disease, experimental autoimmune encephalomyelitis (EAE) (4, 6, 10), whereas anaphylactic shock develops only upon re-exposure to PLP_{139–151} (4, 6), such a mouse model offered us the unique opportunity for investigating the role of the thymus in the development of allergic responses to self Ags.

We demonstrate that resistance to anaphylaxis against PLP_{178–191} is associated with naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs) specific for this self peptide expressed in the thymus. CD4⁺CD25⁺ Tregs derived from mice immunized with PLP_{178–191}, but not from mice immunized with PLP_{139–151}, increase the mRNA expression of forkhead/winged helix transcription factor Foxp3 upon Ag-specific stimulation and effectively suppress Ag-induced proliferation of effector CD4⁺CD25[−] T cells. We also provide evidence that in vivo depletion of CD4⁺CD25⁺ Tregs with anti-CD25 mAb significantly reduces the resistance of SJL mice to anaphylaxis against PLP_{178–191}, suggesting that these cells might play an important role in preventing anaphylaxis against self peptides expressed in the thymus.

Materials and Methods

Mice

Female 8- to 12-wk-old SJL mice were purchased from Charles River Laboratories. All procedures involving animals were approved by the ethical committee of the Institute and performed according to the Principles of Laboratory Animal Care (European Communities Council Directive 86/609/EEC).

Peptide synthesis, immunization protocols, and induction of anaphylaxis

PLP_{139–151} (HSLGKWLGHDPKF), PLP_{178–191} (NTWTTCQSIAPFSK), myelin basic protein (MBP)_{89–101} (VHFFKNIVTPRTP), MBP_{84–104} (VHFFKNIVTPRTPPPSQGKGR), and control peptide (rat P0; DGDFAI VKFTKVLDDYTGH) were synthesized using standard 9-Fmoc chemistry

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Received for publication July 9, 2007. Accepted for publication January 25, 2008.

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¹ This investigation was supported by grants from the National Multiple Sclerosis Society, New York, Fondazione Italiana Sclerosi Multipla (2003/R/42), Ministero della Sanità (“ricerca finalizzata”), and Cariplo Foundation (to R.P.).

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³ Abbreviations used in this paper: PLP, myelin proteolipid protein; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; Treg, regulatory T cell; LNC, lymph node cell.

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and purified by HPLC. The purity of each peptide was >95% as assessed by analytical reverse-phase HPLC. Mice were immunized, s.c. in their flanks, with 100 μ g of each PLP or MBP peptide emulsified in incomplete Freund's adjuvant containing 2 mg/ml heat-killed mycobacterium tuberculosis H37Ra (Difco Laboratories), and assessed daily for neurological signs of EAE according to a 5-point scale (4). Six weeks later, mice were challenged i.p. with 100 μ g of the same Ag used for immunization, dissolved in PBS, and were observed for clinical signs of anaphylaxis. For each mouse temperature was recorded with a rectal probe (Physitemp Instruments) at baseline and 5, 10, 20, 30, and 60 min after challenge. Data are depicted as mean changes in body temperature $\pm$ SEM for each time point. Mice were considered as having anaphylaxis when suggestive clinical signs (i.e., reddening of the skin, piloerection, prostration, reduced or lack of response to stimuli) were accompanied by a drop of body temperature of $\geq 1^\circ\text{C}$, and as having a systemic allergic response when the clinical signs were less severe and were accompanied by a drop of body temperature of at least 0.5°C . Based on the clinical signs and level of alertness of the mice, the severity of systemic allergic reactions was evaluated with the following scoring system: 0, no signs of systemic allergic reaction and normal behavior; 1, reddening of the skin with decreased spontaneous activity; 2, reduced response to stimuli and prostration (11). To determine susceptibility to anaphylaxis against thymus-expressed peptide PLP₁₇₈₋₁₉₁ in mice depleted of CD25⁺ cells or IL-10, 400 μ g of anti-CD25 mAb (clone PC61; BioExpress), anti-IL10 mAb (clone JES5; Bioexpress), or rat IgG control Ab (Rat IgG; Sigma-Aldrich) were injected i.p. in mice on day -5 and -3 before immunization (12, 13).

Measurement of serum Ab responses

Blood was collected from the tails of SJL mice 6 wk after immunization and specific IgG, IgG1, IgG2a, IgG2b, and IgG3 Abs were measured by ELISA (4). In brief, 96-wells microtiter plates (Immunol, Thermo Lab-systems) were coated overnight at 4°C with 0.1 ml of PLP₁₃₉₋₁₅₁ and PLP₁₇₈₋₁₉₁ diluted in 0.1 M NaHCO₃ buffer, pH 8, at a concentration of 0.010 mg/ml. The plates were blocked with PBS 10% FCS (blocking buffer) for 2 h. Samples were diluted in blocking buffer at 1/100 for IgG, IgG1, IgG2a, IgG3, 1/5000 for IgG2b, and 1/50 for IgE. Ab binding was tested by the addition of peroxidase-conjugated monoclonal goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, and IgE (Southern Biotechnology Associates), each at 1/5000 dilution in blocking buffer. Enzyme substrate was added and plates were read at 450 nm on a micro plate reader. Data are represented as mean ODs $\pm$ SEM. Total IgE was analyzed by ELISA with capture and detection Abs for IgE according to the manufacturer's protocol (anti-mouse OptEIA ELISA Set; BD Pharmingen).

T cell proliferation assays

Draining lymph node cells (LNCs) were isolated from mice 10 to 14 days p.i. and cultured in vitro with immunization peptide, ConA (2 μ g/ml), control peptide, or medium alone. Cells were cultured in 96-well microtiter plates at a density of 500×10^3 cells/well in 200 μ l of RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5×10^{-2} M), HEPES buffer (0.01 M), and 10% FCS (enriched RPMI 1640). After 72 h of incubation (37°C , 5% CO₂) cultures were pulsed for 18 h with 0.5 μ Ci/well of [³H]thymidine, and proliferation was measured from triplicate cultures on a β -counter (PerkinElmer). Data are shown as mean cpm $\pm$ SEM. To test the inhibitory effects of Treg cells, CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ effector cells were obtained from suspensions of LNCs depleted of B220, CD11b, CD8, CD49, and Ter-119-positive cells by magnetic separation (Miltenyi Biotec). The purity of separated CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells was typically >96% and >98%, respectively. CD4⁺CD25⁻ T cells and CD4⁺CD25⁺ (200×10^3 /well) were stimulated in triplicate cultures, either alone or in combination, in a 96-well plate with anti-CD3 mAb (clone 41452C11, 1 μ g/ml; BD Pharmingen) or PLP peptides (100 μ g/ml) for 48 h in enriched RPMI 1640 in the presence of 100×10^3 γ -irradiated (3000 rad) splenocytes as an APCs source (14). T cell proliferation was assessed by [³H]thymidine incorporation as described above.

Cytokine measurements

Supernatants from LNCs and purified CD4⁺CD25⁺ or CD4⁺CD25⁻ cells cultured in parallel with those cells used in proliferation assays were used for cytokine analysis. IL-10, IFN- γ , IL-4 (anti-mouse optEIA ELISA set; BD Pharmingen), and TGF- β (R&D Systems) were analyzed by ELISA according to the manufacturer's protocols. Supernatants were collected from cultured cells at 48 h for IFN- γ and at 96 h for IL-10, TGF- β , and

IL-4 measurements. Results are shown as mean of triplicates $\pm$ SEM; SEM were within 10% of the mean.

Flow cytometry analysis

Staining reactions were performed at 4°C after incubation of cells with anti-CD16/32 mAb (clone 2.4G2; BD Pharmingen) for 30 min. LNCs were stained with Cy5.5-conjugated anti-CD4 and FITC-conjugated anti-CD25 mAbs (BD Pharmingen). The intracellular staining of Foxp3 was done with the anti-mouse Foxp3-PE staining set (clone FJK-16s; eBioscience) following the manufacturer's instruction (14). The intracellular staining of Foxp3 on magnetically purified CD25⁺ and CD25⁻ populations cultured in vitro was done with anti-mouse Foxp3-FITC staining set (clone FJK-16s; eBioscience), PE-conjugated anti-CD25 and Cy5.5-conjugated anti-CD4 mAbs (BD Pharmingen). Isotype controls rat IgG2a-FITC/PE/Cy5.5 (BD Pharmingen) were matched for fluorochrome. Cells were acquired on a flow cytometer (FACS Vantage; BD Biosciences) and analyzed using CellQuest Pro software (BD Biosciences).

Real-time PCR

Total RNA was isolated from magnetically purified CD4⁺CD25⁺ and CD4⁺CD25⁻ cells, either ex vivo or upon in vitro stimulation with anti-CD3 mAb or specific Ag, using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Invitrogen) and random primers as described by the manufacturers. The expression of Foxp3 and GAPDH was performed using specific primers and probes (Applied Biosystems) (15). A comparative threshold cycle (C_T) was used to determine Foxp3 mRNA expression relative to housekeeping GAPDH. C_T value was normalized for each sample using the formula: $\Delta C_T = C_{T(\text{Foxp3})} - C_{T(\text{GAPDH})}$ and the relative expression of Foxp3 was then calculated using the expression $2^{-\Delta C_T}$.

Statistical analysis

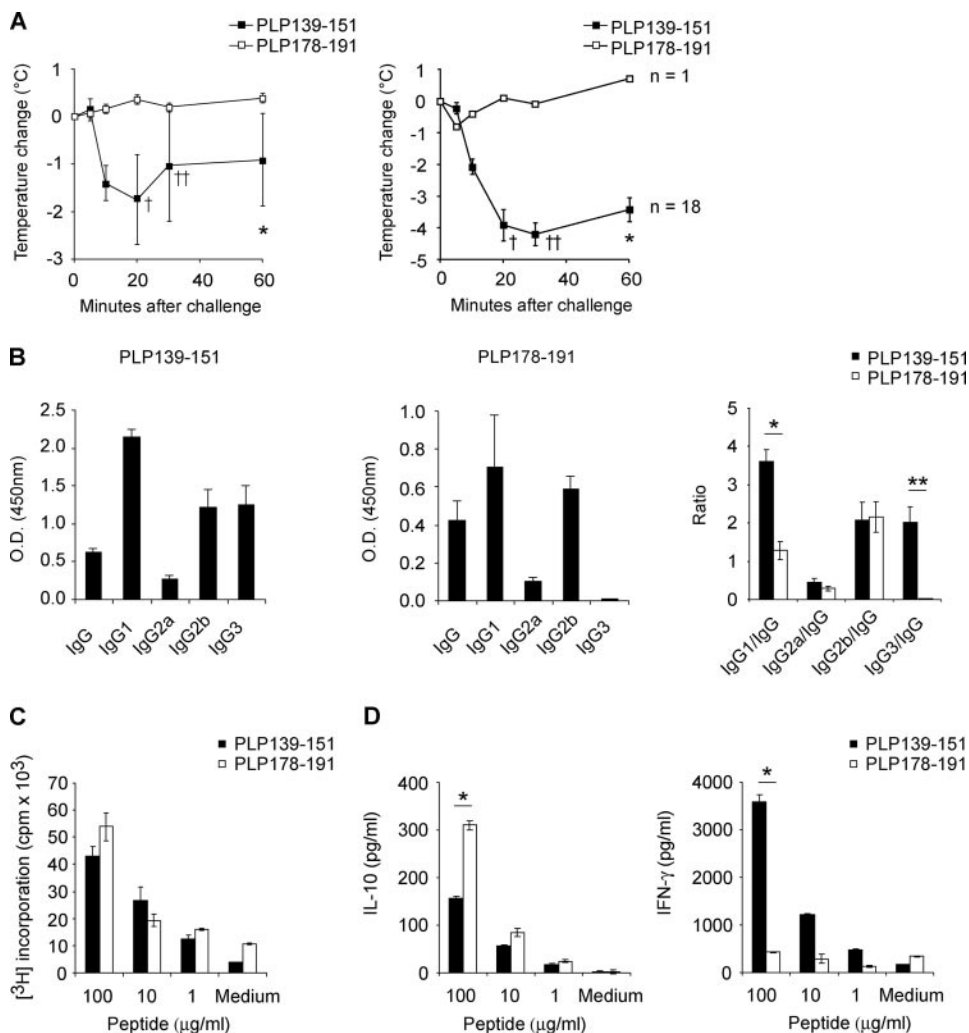
Differences among groups in the time course of body temperature were examined by ANOVA. Differences among groups in the number of mice exhibiting systemic allergic reactions were analyzed by the Fisher's exact test. Student's t test, 2 tailed, was used to compare results between two groups. In all tests, $p < 0.05$ was considered statistically significant.

Results

Titers of Ag-specific IgG1 and IgG3 Ab are lower in mice immunized with PLP₁₇₈₋₁₉₁ than in mice immunized with PLP₁₃₉₋₁₅₁

Anaphylaxis in mice can be mediated both by the Ag+IgE/Fc ϵ RI and the IgG-immune complex/Fc γ RIII pathways (16, 17). Anaphylaxis to PLP₁₃₉₋₁₅₁ has been associated with Ag-specific IgG1 Abs (4). We first investigated whether a defect of B cell priming could be responsible for the resistance to anaphylaxis against PLP₁₇₈₋₁₉₁. As expected (4, 6), only 1 of 10 mice immunized and challenged with PLP₁₇₈₋₁₉₁ developed evidence of a mild systemic allergic reaction, whereas 18 of 22 mice immunized and challenged with PLP₁₃₉₋₁₅₁ developed severe anaphylaxis, with 125 dead ($p < 0.0005$) (Fig. 1A). Immunization of SJL mice with either PLP₁₇₈₋₁₉₁ or PLP₁₃₉₋₁₅₁ induced large amounts of Ag-specific IgG Abs (Fig. 1B), confirming that these peptides serve as efficient B cell epitopes (6). However, analysis of IgG isotypes revealed that Ag-specific IgG1 and IgG3 Ab concentrations were significantly lower in sera of mice immunized with PLP₁₇₈₋₁₉₁ than in those of mice immunized with PLP₁₃₉₋₁₅₁ (mean IgG1/IgG ratio was 1.28 ± 0.23 in PLP₁₇₈₋₁₉₁ vs 3.61 ± 0.30 in PLP₁₃₉₋₁₅₁ immunized mice, $p < 0.005$ by Student's t test; IgG3/IgG ratio was 0.00001 ± 0.001 in PLP₁₇₈₋₁₉₁ vs 2.01 ± 0.39 in PLP₁₃₉₋₁₅₁ immunized mice, $p < 0.0001$ by Student's t test). Notably, IgG3 Abs, the only murine T cell-independent IgG subclass recognizing primarily polysaccharides and appearing early in the immune response (18), were virtually absent in sera of mice immunized with PLP₁₇₈₋₁₉₁. As previously reported (4, 6), we could not detect Ag-specific IgE in the sera of either

FIGURE 1. Resistance to anaphylaxis against thymus-expressed PLP_{178–191} is associated with differences in peptide specific IgG Ab isotype responses and T cell cytokine production. **A**, Changes in body temperature after Ag injection in SJL mice immunized with PLP_{139–151} ($n = 22$) or PLP_{178–191} ($n = 10$) and challenged i.p. with these peptides 6 wk after immunization. Data are shown as mean $\pm$ SEM for all mice in each group, including those that gave no detectable systemic allergic response (left panel) or only for those mice that exhibited any evidence of a systemic allergic response (right panel) (see *Materials and Methods*). †, 12 and ††, 3 mice dead from anaphylactic shock at that time point. *, $p < 0.005$. **B**, IgG Ab isotype response against PLP_{139–151} or PLP_{178–191} in sera collected from immunized mice 6 wk p.i. (10 mice/group) (left and middle panels) and specific IgG isotype/total IgG ratios (mean ratio of individual mice) (right panel). *, $p < 0.05$; **, $p < 0.005$. **C**, T cell proliferation and (D) IL-10 and IFN- γ production of LNCs from PLP_{139–151}- or PLP_{178–191}-immunized mice (3 mice/group). Data are representative of those obtained in the three independent experiments performed. *, $p < 0.05$.



immunized group with our direct ELISA method, and concentrations of total IgE were below the detection limit (1.6 ng/ml) (data not shown).

LNCs from PLP_{178–191}-immunized mice produce more IL-10 and less IFN- γ compared with those from mice immunized with PLP_{139–151}

Ab isotype switching is under the control of Th1 and Th2 cytokines, with IL-4 promoting the production of IgG1 and IgE and the suppression of IgG2a and IgG3, and IFN- γ promoting the production of IgG2a Abs (19–21). Because we observed a difference in the production of different isotypes of peptide-specific IgG Abs in mice either susceptible or resistant to anaphylaxis, we investigated whether the differential Ab responses to PLP_{139–151} or PLP_{178–191} might be associated with the different cytokine profiles of T cells activated by PLP_{139–151} or PLP_{178–191} peptides.

As shown in Fig. 1C, T cells from mice immunized with either peptide proliferated robustly to Ag stimulation, and no significant differences were observed. However, T cells stimulated with PLP_{178–191} produced significantly higher amounts of IL-10 and lower amounts of IFN- γ than did those stimulated with PLP_{139–151} (Fig. 1D). IL-4 was below detection limit (7.8 pg/ml) upon stimulation with either peptide (data not shown). These observations suggest that there are functional differences between T cells activated by PLP_{178–191} vs PLP_{139–151}, not

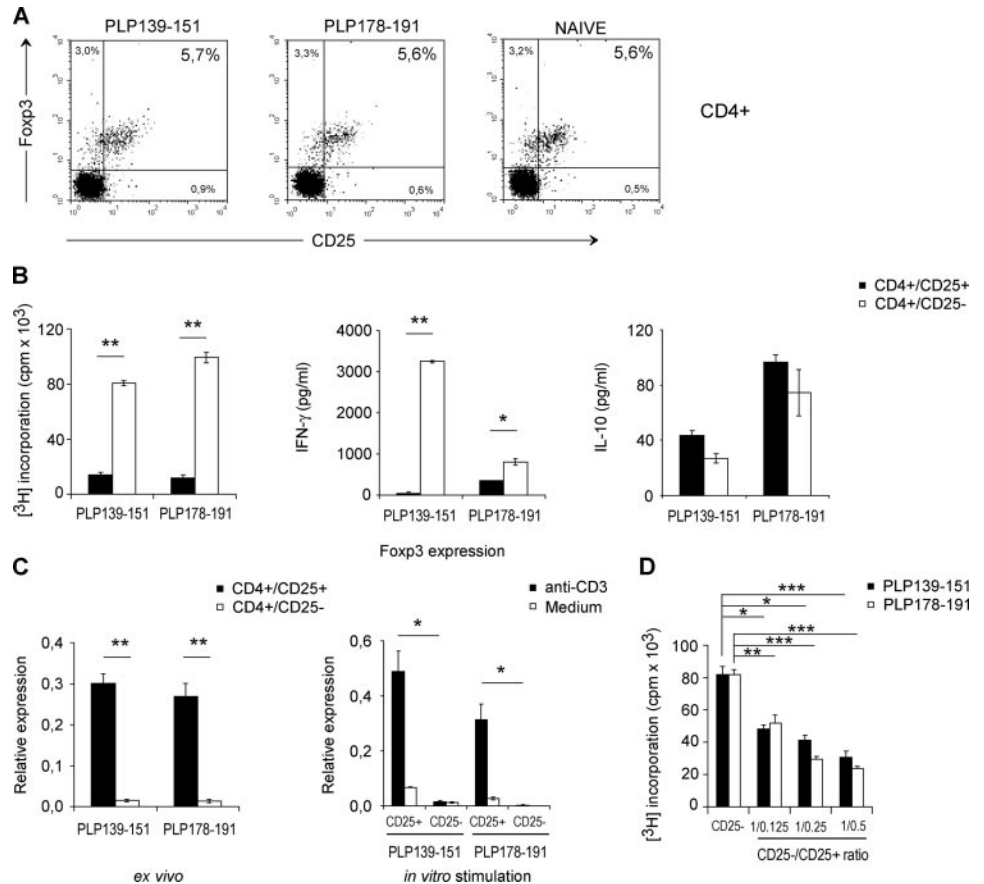
merely differences in the magnitude of T cell activation by the two peptides.

CD4⁺CD25⁺ T cells are present with similar frequency in PLP_{178–191}- or PLP_{139–151}-immunized mice and effectively suppress anti-CD3-induced proliferation of naive CD4⁺CD25[−] T cells

IL-10 is an important suppressor cytokine, and a role for IL-10 in preventing/suppressing the potentially detrimental immune responses that result in autoimmunity or allergy has been reported (22–27). IL-10 can be produced by different subsets of Tregs, that can be functionally distinguished by their capacity to suppress T cell proliferation and effector function (reviewed in Ref. 28). Naturally occurring CD4⁺CD25⁺ Tregs constitutively express the forkhead/winged helix transcription factor Foxp3 and are generated in the thymus by high-affinity interaction of the TCR with MHC II-bound self peptides (29–31).

We first explored the possibility that immunization with PLP_{178–191} or PLP_{139–151} peptides, which are differentially expressed in the thymus, influenced differences in the expansion of CD4⁺CD25⁺ Tregs. As shown in Fig. 2A, there were no differences in the frequency of CD4⁺CD25⁺Foxp3⁺ cells in the lymph nodes of mice immunized with either PLP_{139–151} or PLP_{178–191} vs (5.7 and 5.6% of CD4⁺ T cells, respectively), and this frequency was similar to that observed in the LNCs of naive mice (5.6% of CD4⁺ T cells).

FIGURE 2. Lack of differences in CD4⁺CD25⁺ Tregs frequency, phenotype, and suppressive functions in mice immunized with PLP_{139–151} or PLP_{178–191}. LNCs were obtained from mice 10–14 days after immunization with PLP_{139–151} or PLP_{178–191}, or from naive mice. **A**, Percentage of CD25⁺/Foxp3⁺ gated CD4⁺ T cells ex vivo from immunized and naive mice. **B**, Proliferative response, IFN- γ and IL-10 production from magnetically purified CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from LNCs of immunized mice and stimulated in vitro with anti-CD3. **, $p < 0.005$; *, $p < 0.05$. **C**, Foxp3 mRNA expression relative to GAPDH mRNA expression of purified CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells derived from immunized mice ex vivo and after in vitro stimulation with anti-CD3 (in triplicate wells $\pm$ SEM). **, $p < 0.005$; *, $p < 0.01$. **D**, Proliferative responses of CD4⁺CD25⁻ effector T cells derived from naive mice stimulated in vitro with anti-CD3 either alone or in combination with increasing amounts of CD4⁺CD25⁺ T cells derived from immunized mice (functional assay). Data are representative of those obtained in two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.



We then searched for possible differences in the suppressive function of CD4⁺CD25⁺ Tregs derived from mice immunized with PLP_{139–151} or PLP_{178–191}. As described for CD4⁺CD25⁺ Tregs analyzed in other settings (29–31), magnetically sorted CD4⁺CD25⁺ T cells derived from mice immunized with either peptide were anergic, and produced IL-10, as did the CD4⁺CD25⁻ T cells, but significantly lower levels of IFN- γ , when compared with the amounts of this cytokine produced by effector T cells upon polyclonal activation with anti-CD3 (Fig. 2B). Furthermore, CD4⁺CD25⁺ sorted T cells from either group of immunized mice expressed similar levels of Foxp3 ex vivo, which increased upon anti-CD3 stimulation in vitro (Fig. 2C). In vitro stimulation of these cells with anti-CD3 Ab also resulted in increase of frequency of CD4⁺CD25⁺ T cell expressing Foxp3⁺ (Fig. 4, upper and middle panels). Finally, CD4⁺CD25⁺ T cells from

either group of immunized mice effectively suppressed anti-CD3-induced proliferation of effector CD4⁺CD25⁻ T cells from naive mice (Fig. 2D). These observations indicate that the differences in the susceptibility of mice to develop anaphylaxis against PLP_{139–151} vs PLP_{178–191} cannot be explained by global differences in the frequency of Tregs, or by differences in the potential for suppressor function of Tregs (as assessed by anti-CD3 stimulation), in mice immunized with either of these peptides.

CD4⁺CD25⁺ Tregs suppress Ag-induced proliferation of effector CD4⁺CD25⁻ T cells in mice immunized with PLP_{178–191}, but not in those immunized with PLP_{139–151}

As naturally occurring Tregs cells are generated in the thymus (29–31), and PLP_{139–151} and PLP_{178–191} are differentially

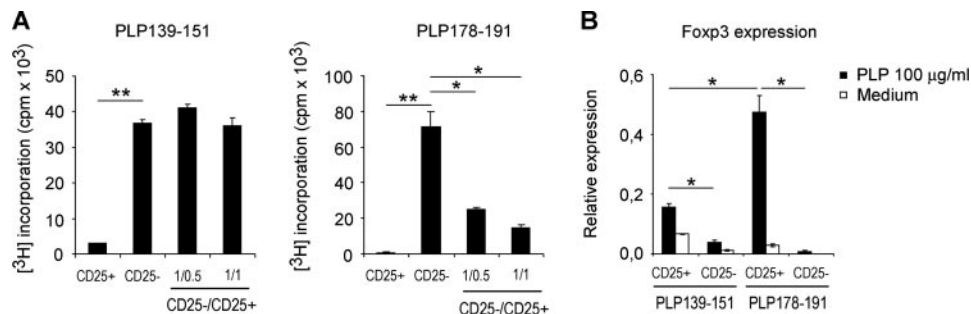


FIGURE 3. CD4⁺CD25⁺ T cells derived from mice immunized with PLP_{178–191}, that is expressed in the thymus, suppress Ag-specific activation of effector T cells. LNCs were obtained from mice 10–14 days after immunization with PLP_{139–151} or PLP_{178–191} ($n = 5$ mice/group), and CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells were magnetically purified. **A**, Proliferative responses of CD4⁺CD25⁻ effector T cells and CD4⁺CD25⁺ Tregs either alone or in combination stimulated in vitro with the specific Ag. **, $p < 0.005$; *, $p < 0.05$. Data are representative of those obtained in three independent experiments. **B**, Foxp3 mRNA expression relative to GAPDH mRNA expression of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells upon Ag-specific in vitro stimulation (in triplicate wells $\pm$ SEM). Data are representative of those obtained in four independent experiments. *, $p < 0.005$.

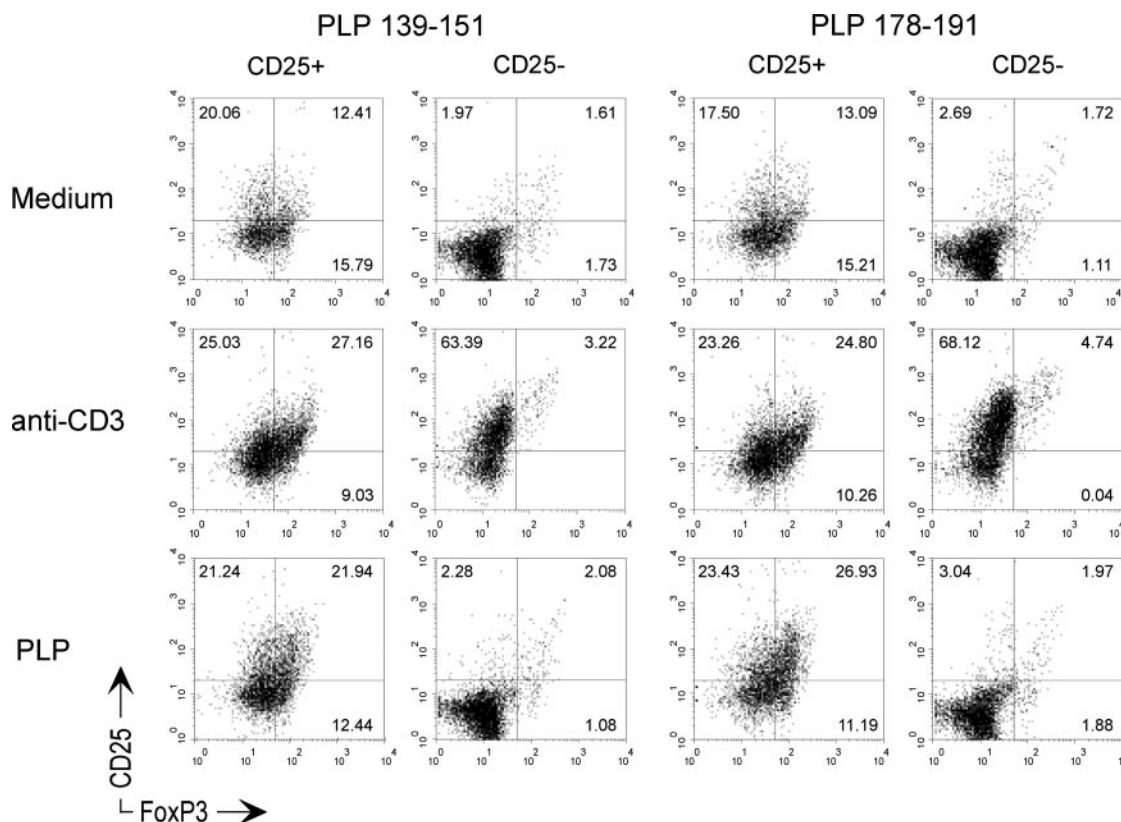


FIGURE 4. Frequency of $CD4^+CD25^+$ cells expressing Foxp3 upon in vitro stimulation of Tregs. LNCs were obtained from mice 10–14 days after immunization with PLP_{139–151} (left panels) or PLP_{178–191} (right panels) ($n = 5$ mice/group), and magnetically purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were stimulated for 48 h in vitro with medium, anti-CD3 mAb (1 μ g/ml) or the specific Ag (100 μ g/ml) as in Fig. 3B. FACS plots show the frequency of $CD25^+Foxp3^+$ T cells gated on $CD4^+$.

expressed in the thymus (9), we next investigated whether there were differences in Ag-specific Tregs in mice immunized with the different peptides. To express their suppressor function, $CD4^+CD25^+$ Tregs require TCR activation (29). We therefore evaluated the ability of $CD4^+CD25^+$ T cells derived from mice immunized with either one or the other PLP peptide to suppress Ag-specific proliferation of effector $CD4^+CD25^-$ T cells derived from the immunized mice.

We found that $CD4^+CD25^+$ Tregs derived from mice immunized with either PLP_{178–191} or PLP_{139–151} exhibited little or no proliferation in response to Ag stimulation (Fig. 3A). However, $CD4^+CD25^+$ Tregs derived from mice immunized with PLP_{178–191}, that is expressed in the thymus, significantly suppressed Ag-specific proliferation of effector cells, whereas those derived from mice immunized with PLP_{139–151}, that is not expressed in the thymus, failed to do so (Fig. 3A). Moreover, expression of Foxp3, which is required for $CD4^+CD25^+$ Tregs to express their regulatory function (32–35), was increased more significantly upon Ag stimulation in Tregs derived from mice immunized with PLP_{178–191} than in those derived from mice immunized with PLP_{139–151} (Fig. 3B), and intracellular Foxp3 expression analysis of these cells by FACS showed an increase of $CD4^+CD25^+$ T cells expressing Foxp3, which was concordant with results observed at the mRNA level (Fig. 4, upper and lower panels). Taken together, these observations suggest that SJL mice contain $CD4^+CD25^+$ Tregs specific for the PLP peptide expressed in the thymus (PLP_{178–191}), and that such cells can suppress the Ag specific activation of primed effector cells by PLP_{178–191}.

In vivo CD25⁺ T cells depletion with anti-CD25 mAb reduces resistance to anaphylaxis to PLP_{178–191}

We next tried to assess whether naturally occurring Tregs, including the population of Ag-specific cells, play a role in conferring resistance to the development of anaphylactic reactivity to thymus-expressed PLP_{178–191}. In vivo treatment with anti-CD25 mAb PC61 at the time of immunization with PLP_{178–191} resulted in depletion of Tregs ($CD25^+$ cells were 5.9% of $CD4^+$ cells in naive mice and 0.5% one day after the second injection of PC61; 10 days after peptide-immunization they were 2.5% in PC61 treated mice and 4.4% in rat IgG Ab-treated mice) (36, 37). This depletion correlated with suppression of the resistance to anaphylaxis to this peptide (Fig. 5A and Table I). Peptide-specific IgG1 Ab concentrations were significantly increased in sera of mice treated with anti-CD25 mAb than in those treated with control Ab or left untreated (Fig. 5B), and LNCs from these treated mice produced higher amounts of IFN- γ upon Ag stimulation (Fig. 5C). These results suggested that these $CD25^+$ T cells play an important role in resistance to anaphylaxis, which might be associated with their ability to suppress T cell and IgG1 Ab responses.

$CD4^+CD25^+$ T cells are known to mediate their suppressor function via cell-to-cell contact (29). It has been shown that in vitro blockade of IL-10, that can be produced in large amounts by $CD4^+CD25^+$ T cells, does not abrogate their ability to express suppressor function, as it does instead for inducible Tr1 regulatory cells (38–40). To clarify whether the ability of these cells to confer resistance to anaphylaxis is mediated by IL-10, which they

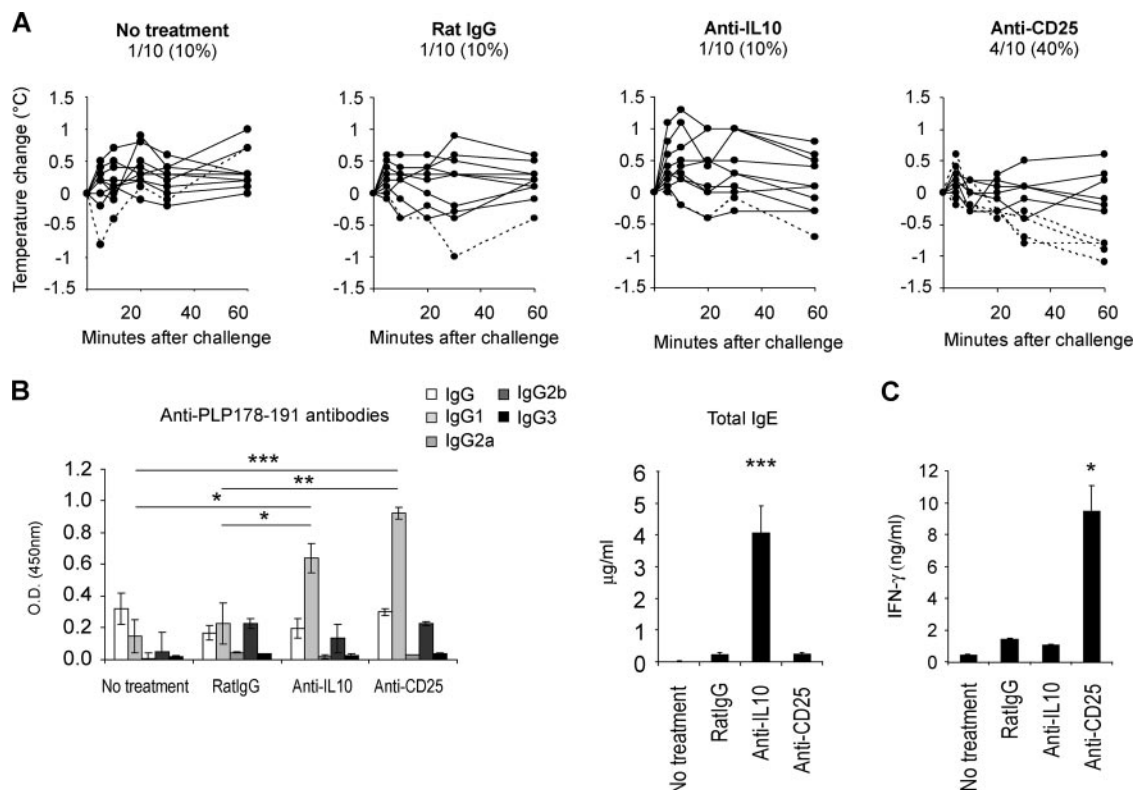


FIGURE 5. Anti-CD25 Ab treatment reduces resistance to anaphylaxis against thymus-expressed PLP₁₇₈₋₁₉₁. **A**, Mice treated with anti-CD25, anti-IL10, control rat IgG Abs, or left untreated before immunization with PLP₁₇₈₋₁₉₁ ($n = 10$ mice/group) were challenged i.p. with the peptide 6 wk later. For each treatment group, changes in body temperature of individual mice at each time point are represented. Mice were considered as having a systemic allergic reaction (dotted lines and text in bold) when suggestive clinical signs were accompanied by a drop of body temperature of $>0.5^{\circ}\text{C}$ (see *Materials and Methods*). Data are representative of those obtained in two independent experiments. **B**, IgG Ab isotype response against PLP₁₇₈₋₁₉₁ (left panel) and total IgE Ab response (right panel) in sera collected from immunized mice 6 wk p.i. ($n = 5-7$ mice/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. **C**, IFN- γ was measured on LNCs obtained from mice treated with anti-CD25, anti-IL10, control rat IgG Abs, or left untreated 10 days after immunization with PLP₁₇₈₋₁₉₁ and stimulated in vitro with the Ag (100 $\mu\text{g}/\text{ml}$) ($n = 5$ mice/group). *, $p < 0.05$.

produce (as shown in Fig. 2B), we attempted to induce anaphylaxis to PLP₁₇₈₋₁₉₁ in mice in which IL-10 was neutralized in vivo. As shown in Fig. 5A and Table I, blockade of IL-10 with the JES5 Ab failed to abrogate resistance to anaphylaxis against PLP₁₇₈₋₁₉₁. Notably, peptide-specific IgG1 Ab concentrations were increased also in sera of mice treated with anti-IL10 monoclonal compared with those of mice treated with control Ab or left untreated, and total IgE Abs, which were undetectable in naive or either PLP₁₃₉₋₁₅₁ or PLP₁₇₈₋₁₉₁ SJL immunized

mice, were elevated (Fig. 5B). However, also in the sera of these treated mice we failed to detect Ag-specific IgE (data not shown). This observation suggests that IL-10, despite its ability to influence peptide-specific IgG1 and total IgE Ab responses in this model, is not required to induce CD4⁺CD25⁺ Treg cell-mediated resistance to anaphylaxis against this thymus-expressed peptide. Instead, such cells might exert their suppressor function through a cell-to-cell contact mechanism at the site/s where the immunological response against this peptide develops.

Table I. Systemic allergic reaction scores and body temperature changes upon challenge with PLP₁₇₈₋₁₉₁ in mice treated with anti-CD25, anti-IL10, control rat IgG, or left untreated

Treatment	No. of Mice with Allergic Reactions	Clinical Systemic Allergic Reaction Score ^a			Peak of Body Temperature Drop ^b
		0	1	2	
None	1/10	9	1	0	-0.8
Rat IgG	1/20	19	0	1	-1.0
Anti-IL10	1/10	9	1	0	-0.7
Anti-CD25	9/20 ^c	11	6	3	-1.0 $\pm$ 0.08

^a Data represent the number of mice in each treatment group reaching the specified systemic allergic reaction score during the observation period of 1 h after peptide challenge.

^b Data are mean $\pm$ SEM only for mice that exhibited any evidence of a systemic allergic response.

^c $p < 0.01$ vs rat IgG treated mice.

Discussion

Taken together, our data support a model in which naturally occurring Tregs specific for a peptide that is expressed under physiological conditions in the thymus both can suppress Ag-specific stimulation and can play an important role in preventing the development of anaphylactic responses to such self peptides. Indeed, in vivo depletion of these cells with an anti-CD25 mAb partially restored susceptibility to anaphylaxis against this thymus-expressed peptide. Several findings obtained in animal models provide evidence for the generally accepted concept of a thymic origin of naturally occurring CD4⁺CD25⁺ Tregs (reviewed in Ref. 29). However, the specificity for thymic Ags of naturally occurring Tregs is still an open field of investigation. Indeed, because Tregs require TCR activation to exert their suppressive function, polyclonal activation with anti-CD3 Abs

Table II. Anaphylactic response to self-peptides in SJL mice^a

Priming Peptide	Thymic Expression	i.p. Challenge	No. of Mice with Anaphylaxis (%)	No. of Mice Dead From Anaphylaxis (%)
PLP _{139–151}	—	PLP _{139–151}	18 of 22 (81.8)	15 of 22 (68.2)
		PLP _{178–191}	0 of 13 (0)	0 of 13 (0)
PLP _{178–191}	+	PLP _{178–191}	1 of 20 (0.5)	0 of 20 (0)
MBP _{89–101}	+	MBP _{89–101}	2 of 21 (9.5)	1 of 21 (4.8)
MBP _{84–104}	+	MBP _{84–104}	1 of 7 (14.3)	0 of 7 (0)

^a Mice were immunized with 100 μ g of each peptide emulsified in complete Freund's adjuvant and challenged 6 wk later with the indicated peptides (100 μ g in PBS i.p.).

is often used for in vitro assays of suppressive function of naturally occurring Tregs. In models of autoimmunity, Kuchroo and colleagues (12) have shown that unmanipulated B10.S mice, which express PLP_{139–151} in their thymus and are resistant to the induction of EAE, contain CD4⁺CD25⁺ T cells that are specific for this encephalitogenic Ag and that can suppress activation of effector T cells in an Ag-specific manner. However, it has recently been shown that such Tregs fail to suppress T-effector cells isolated during EAE from the inflamed CNS (41, 42).

In the field of allergy, in which foreign (i.e., non-self) Ags most typically are the targets of allergic responses, work on Tregs has focused mostly on “inducible” Tregs, such as Tr1 (reviewed in Ref. 28). Indeed in humans, it has been reported that successful allergen immunotherapy is associated with the generation of IL-10-producing Tregs (43, 44). However, the spontaneous development in *Foxp3* mutant mice of allergic airway inflammation, hyperIgE and eosinophilia, in addition to several autoimmune syndromes, which are reversed by transfer in these mice of CD4⁺CD25⁺ Tregs from wild type mice, provide evidence for an important role of naturally occurring CD4⁺CD25⁺ Treg in the prevention of allergic diseases (34, 45, 46). In atopic subjects, a reduced numbers or defective suppressive function of CD4⁺CD25⁺ Tregs has been demonstrated (47, 48). More recent evidence, obtained using mice genetically predisposed or resistant to allergen-induced airway hyperresponsiveness, has shown a protective role for naturally occurring CD4⁺CD25⁺ Tregs in the development of allergic asthma (49). Furthermore, in a rat asthma model, reversal of airway hyperresponsiveness by chronic exposure to aerosolized OVA has been shown to be associated with the induction of airway mucosal CD4⁺CD25⁺ Tregs (50).

Our results provide evidence of an important role for naturally occurring Tregs specific for a thymus-expressed peptide in conferring resistance to the development of systemic anaphylaxis, the most dramatic expression of allergic responses. Thus, these findings corroborate the hypothesis of a role for naturally occurring Ag-specific Tregs in inhibiting the primary immune response. The mechanism/s by which these cells protect against anaphylaxis is/are not known, but cell-to-cell contact that suppresses activation of effector T cells, with indirect modulation of B cell responses, and/or a direct effect of Tregs on B cells (51), could be hypothesized. The modulation of T cell and Ab responses that we observed in mice treated with anti-CD25 Ab, a treatment that depleted CD25⁺ T cells irrespectively of their Ag specificities, supports this hypothesis. Our studies have focused on a single peptide expressed in the thymus. However, the resistance to anaphylaxis that we have observed to other myelin peptides that are expressed in the thymus of SJL mice (8, 52), the strain that we used throughout our study (Table II), raises the possibility that naturally occurring Tregs also may

have a role in preventing anaphylaxis against other self peptides that are expressed in the thymus.

Acknowledgments

We thank Renato Longhi for providing the peptides used in this study, Cinthia Farina for help with flow cytometry analysis, Chiara Falcone for help with the statistical analysis, Massimo Costanza for fruitful discussion, Giulia Fragola for technical help, and Stephen J. Galli for advice and review of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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