Anti-Vβ8 antibodies induce and maintain staphylococcal enterotoxin B-triggered Vβ8+ T cell anergy

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The mechanism involved in the maintenance of staphylococcal enterotoxin B (SEB)-induced T cell anergy is poorly understood. We demonstrated earlier that B cells play an important role in the maintenance of SEB-induced T cell anergy *in vivo* and *in vitro*. Here, we demonstrate that B cells are not essential in SEB-induced T cell activation, but are important for the maintenance of T cell memory phenotype and anergy *in vivo*. Studying the activated B cell repertoire, we observe that SEB treatment increases serum anti-Vβ8 antibody titer as detected by enzyme-linked immunosorbent assay using soluble Vβ8 chains as antigens, and by staining of a Vβ8-expressing thymoma. These antibodies disappear gradually after immunization with SEB, whereas the capacity of the T cells to respond to SEB *in vitro* is restored. Anti-Vβ8 monoclonal antibody treatment causes Vβ8+ T cell unresponsiveness to SEB *in vitro* (anergy), without affecting CD4Vβ8+ T cell frequency. Together, these results suggest a new mechanism to explain the maintenance of SEB-induced T cell anergy, which is dependent on B cells and on anti-Vβ8 antibody that specifically interacts with Vβ8+ T cells.

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1 Introduction

The superantigen (Sag) model has recently been used to study mechanisms involved in peripheral T cell tolerance. Intact Sag binds to both MHC class II and TCR Vβ chain, in contrast to conventional antigen that must be processed and presented in the context of MHC [1]. Sag establishes tolerance by induction of deletion and anergy in reactive T cells [2–5].

The *in vivo* response to staphylococcal enterotoxin B (SEB) involves several lymphocyte activation steps that culminate in the deletion of or induction of anergy in reactive T cells [2, 6]. After an initial phase following SEB immunization that is characterized by the production and liberation of large amounts of lymphokines [7], the specific activated T cells characterized by the expression of Vβ8 undergo clonal expansion. Two days later, the Vβ8+ T cell frequency is twice that observed in PBS-treated mice [2, 8]. CD4Vβ8+ T cells are thereafter deleted to frequencies below those observed in PBS-treated mice, whereas CD8Vβ8+ T cells are deleted to frequencies similar to those seen in control mice [8, 9]. Early evidence demonstrates that T cell deletion is dependent on the activation and terminal differentiation that culminates in apoptosis [9, 10]. The remaining Vβ8+ T cell population is refractory to SEB, but not to SEA stimulation *in vitro*, indicating a state of anergy [2, 6]. Anergic T cells are characterized by their incapacity to secrete IL-2 and to proliferate after stimulation with antigen *in vitro* [11]. This ability of SEB to trigger deletion and induce anergy in Vβ8+ T cells has also been explored with success in the experimental allergic encephalomyelitis (EAE) model, in which SEB treatment modulates encephalitogenic Vβ8+ T cells and prevents disease development [12–15].

Anergy is a long-lasting, antigen-specific phenomenon. The mechanisms involved in its triggering and maintenance remain to be determined. It is an unstable phenomenon that reverts, depending on the system, in the absence of antigen [16], after infection [17], and with time [18]. Even more interesting, anergy is less apparent

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Abbreviations: SEB: Staphylococcal enterotoxin B **Sag:** Superantigen

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in vivo. T cells, which are unable to grow after restimulation *in vitro*, can proliferate [19], secret interleukins and aid humoral immune responses [20] following a second contact with the antigen *in vivo*. We recently demonstrated that B cells are important in the maintenance of SEB-induced T cell anergy *in vitro*, since B cells from SEB-, but not from PBS- or SEA-treated mice, sustain T cell anergy *in vitro* [21]. The role of B cells in SEB-induced T cell anergy maintenance was confirmed *in vivo* in experiments in which spleen cells from B cell-deficient mice were unable to maintain T cell anergy longer than 13 days. The mechanism through which B cells participate in the maintenance of T cell anergy is not mediated by suppressor factors or anti-SEB antibodies produced by B cells from SEB-primed mice, inasmuch as they are unable to alter the reactivity of unprimed T cells *in vitro* [21].

Here we have characterized the mechanism employed by B cells in maintaining SEB-induced T cell anergy, which correlates with the presence of a "memory" phenotype, and demonstrate that *in vivo* treatment with anti-Vβ8 chain antibody modulates the Sag-induced *in vitro* T cell response. Analysis of sera from SEB-injected mice shows that anti-Vβ8 antibodies are generated specifically in response to SEB immunization and that their presence is related to persistence of anergy. These results suggest that anti-Vβ8 antibodies are important in SEB-induced T cell anergy.

2 Results

2.1 B cell-deficient B6 μmt mice are unable to maintain SEB-induced T cell anergy

Sag-induced T cell anergy is a long-lasting phenomenon that may persist for up to 4 months in BALB/c mice [22]; the mechanism behind this process remains to be understood. Based on earlier experiments, we have shown that B cells participate in the maintenance of SEB-induced T cell anergy *in vitro* and *in vivo* [21]. We have now adopted a more reliable system using B6 wild-type and B6 μmt mice, in which we have compared the effect of SEB *in vivo* and followed the changes in Vβ8 T cell frequency and their capacity to proliferate *in vitro* (Fig. 1). At day 13, spleens from SEB-treated B6 mice have approximately 50 % Vβ8 T cells and the *in vitro* response is only 24 % of that observed in PBS-treated control B6 mice, here considered to have maximum CD4Vβ8$^+$ T cell frequency and *in vitro* proliferative response. In contrast, CD4$^+$ spleen T cells from SEB-treated B6 μmt mice have the same Vβ8$^+$ T cell frequencies (approximately 50 %), and generate a response that accounts for 50 % of the SEB-induced *in vitro* response observed in PBS-treated B6 μmt mice (Fig. 1). This demonstrates that, at day 13 after SEB treatment, virtually all SEB-reactive T cells from B6 μmt mice proliferate following *in vitro* SEB stimulation, indicating rapid SEB-induced T cell anergy loss in the absence of B cells. B6 and B6 μmt spleen cells respond to SEA *in vitro*, whether they are treated with SEB or with PBS (data not shown).

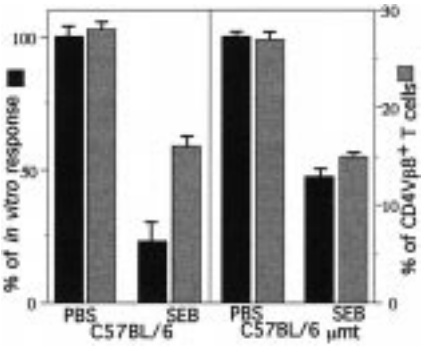

Figure 1. Comparison of SEB-induced *in vitro* T cell responses in B6 versus B6 μmt mice. Spleen CD4Vβ8$^+$ T cell frequency (gray bar) and proliferation following *in vitro* stimulation with SEB (black bar) were determined as described in Sect. 4.7. To calculate the percentage of response, we assume that PBS-treated mice of each strain show 100 % response. % of response = (response of SEB-treated mice/response of PBS-treated mice) × 100. The mean of three animals per group is shown; similar results were obtained in three independent experiments.

2.2 The effect of SEB treatment on B cell-deficient mouse Vβ8$^+$ T cells

As shown earlier [23] and confirmed here (Fig. 2), Sag treatment induces CD4$^+$, but not CD8$^+$ T cell deletion in B6 and B6 μmt mice. CD4 and CD8Vβ8$^+$ T cell kinetics were similar in both B6 and B6 μmt mouse strains (Fig. 2A, B), demonstrating that SEB-induced T cell activation and deletion are not B cell dependent.

Immunization with antigen drives naive T cell differentiation into effector and memory cells. Virgin and memory T cells can be distinguished by the different expression of a number of surface molecules [24–26]. For example, membrane MEL-14 expression is low in primed T and high in virgin T cells [27]; we thus measured MEL-14 levels to differentiate virgin form memory T cells. CD4 and CD8Vβ8$^+$ MEL-14^{low} T cell frequencies were determined in B6 and B6 μmt mice at different times after SEB treatment (Fig. 2C, D). CD4Vβ8$^+$ T cells from B6, but not from B6 μmt mice, retained the memory phenotype for longer

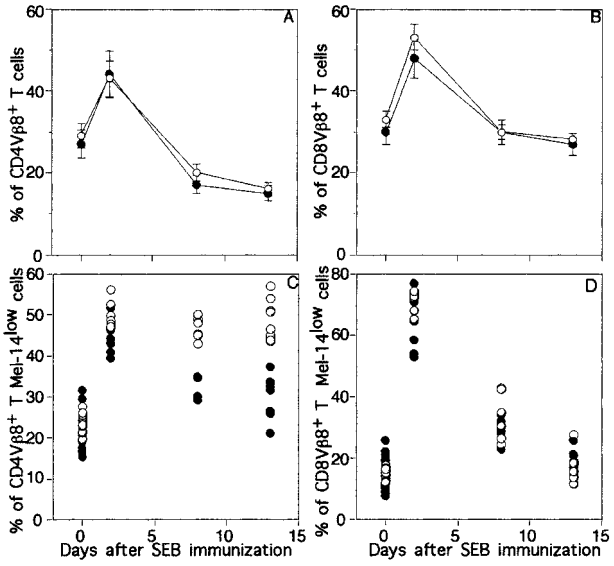

Figure 2. In vivo SEB-reactive T cell response in C57BL/6 and B6 μmt mice. B6 (open circles) and B6 μmt (filled circles) mice were treated with PBS or SEB and the frequency was analyzed by flow cytometry of CD4+ (A) and CD8+ (B) cells expressing Vβ8 chain at different times after treatment. The phenotypes of spleen C57BL/6 and B6 μmt mouse CD4Vβ8+ (C) and CD8Vβ8+ (D) T cells expressing MEL-14low were analyzed on different days after SEB treatment. Double positive T cells were electronically gated and MEL-14 expression analyzed. The mean ± SD of each group are represented in A and B. In C and D, individual animals from three distinct experiments are shown.

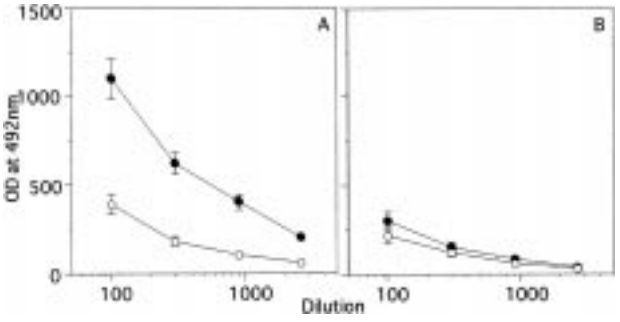

Figure 3. Effect of immunization with SEB on anti-Vβ8 and anti-OVA antibodies. BALB/c mice were treated with PBS (○) or SEB (●), bled 10 days later and the sera analyzed for anti-Vβ8 (A) and anti-OVA antibodies (B). The results represent the mean ± SD of five animals in each group. Similar results were obtained in three independent experiments.

than 13 days (Fig. 2C), suggesting that B cells are involved in maintenance of the CD4+ T cell memory phenotype. Both B6 and B6 μmt mice present the same CD8Vβ8+ T cell memory phenotype following SEB treatment, suggesting that B cells have no relation with the CD8+ T cell memory phenotype. As another internal control, we observed no difference between B6 and B6 μmt mice in the frequency of Vβ6+ T cells expressing memory phenotype (data not shown).

2.3 Immunization with SEB induces anti-Vβ8 antibody production

We next analyzed the modification of the B cell repertoire induced by *in vivo* SEB treatment. We showed earlier that SEB treatment induces an anti-SEB humoral immune response [20], but that anti-SEB antibody does not block *in vitro* SEB-induced T cell proliferation [21]. We have now evaluated the effect of SEB treatment on the humoral response to different TCR, and specifically, to the Vβ8 TCR expressed on SEB-activated T cells. We found that SEB treatment induces a significant increase in serum anti-Vβ8 antibody levels (Fig. 3A).

To exclude the possibility that this is the result of SEB-induced polyclonal B cell activation, we analyzed the effect of SEB immunization on serum anti-OVA antibody expression and found that SEB immunization does not alter its levels (Fig. 3B). This suggests that the increased serum anti-Vβ8 antibody level is a specific phenomenon, mediated by Vβ8+ T cell stimulation after SEB treatment (Fig. 3B).

Anti-Vβ8 antibodies were evaluated in two different systems, an ELISA using recombinant Vβ8 peptide-coated plates as decribed in Sect. 4.6, as well as by their capacity to stain YAC-1 cells, a thymoma that expresses TCR Vβ8. In both systems, it is clearly shown that SEB immunization triggers anti-Vβ8 antibody production. YAC-1 cells are thus stained by the anti-Vβ8 F23.1 mAb (Fig. 4D), as well as by serum from SEB-treated mice (Fig. 4C), but not by normal serum (Fig. 4B) or secondary antibodies (Fig. 4A).

The anergic response to SEB is specific, since SEB treatment induces T cell anergy to SEB, but not to SEA, which stimulates T cells expressing a different TCR Vβ family [21]. To determine whether anti-TCR Vβ8 antibodies are specific for activated T cells, we estimated the effect of SEA treatment *in vivo* on the serum anti-Vβ8 antibody titer. Mice were treated with SEA, SEB or PBS and the sera obtained 10 days later were analyzed for anti-Vβ8 antibodies. SEB treatment, but not SEA or PBS, induced an increase in serum anti-Vβ8 antibody levels (Fig. 5). Immunization with SEA, which stimulates Vβ3+ and Vβ11+ T cells, did not increase serum anti-Vβ8 levels.

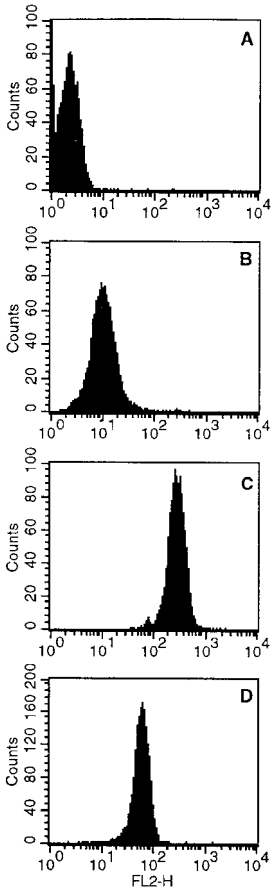

Figure 4. Serum from SEB-treated mice stains Vβ8-expressing T cell clones. YAC-1 cells (10^6) were incubated for 15 min with serum from PBS- (B) or SEB-treated (C) mice or F23.1 mAb (D), washed and incubated with a PE-conjugated secondary anti-mouse antibody. YAC-1 cells incubated with anti-Ig alone were used as control (A). Cells were then analyzed by flow cytometry.

Since SEB-induced T cell anergy is reversible *in vivo*, we evaluated the temporal effect on SEB-induced anti-Vβ8 antibody. BALB/c mice were treated with PBS or SEB and, at different times after treatment, mice were bled to study serum anti-Vβ8 antibody levels. Spleen cells from mice in each group were analyzed for *in vitro* proliferation to SEB at each time point (Fig. 6). After immunization with SEB, the increment in anti-Vβ8 antibody titer parallels the increase in anergy of the SEB-reactive T cells. By 120 days post-treatment, SEB-treated mouse spleen T cells proliferate after *in vitro* SEB stimulation, indicating loss of anergy (Fig. 6B). At that time, sera from SEB- and PBS-treated mice show similar anti-Vβ8 antibody concentrations (Fig. 6A). In these studies, we also analyzed the CD4Vβ8$^+$ T cell frequency and found that SEB-treated mice have the same frequency as PBS-treated mice (data not shown), suggesting that the reduction in

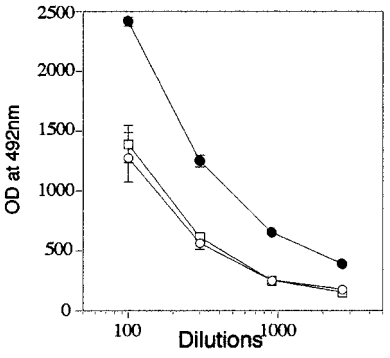

Figure 5. Anti-Vβ8 antibody production is an SEB-specific phenomenon. BALB/c mice were treated with PBS (○), SEB (●) or SEA (□), bled 10 days later and the sera analyzed for anti-Vβ8 antibodies. The results represent the mean ± SD of five animals in each group. Similar results were obtained in three independent experiments.

anti-Vβ8 antibodies may be related to the loss of SEB-induced T cell anergy. Finally, *in vitro* SEB-induced T cell proliferation and serum anti-Vβ8 antibody levels do not change in PBS-treated mice.

Similar data were obtained in B6 mice. B6 mouse T cells lose SEB-induced anergy 60 days after SEB treatment,

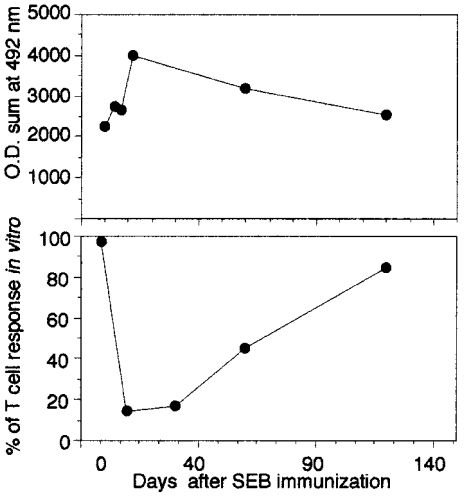

Figure 6. Temporal effect on T cell anergy and anti-Vβ8 antibodies. BALB/c mice were treated with PBS or SEB and, at different times, three mice from each group were bled and spleen cells assayed for *in vitro* proliferative response to SEB. Serum was analyzed for anti-Vβ8 antibodies; the result is represented as the mean of individual values (top). Spleen cells (10^5) were cultured in the presence of SEB (bottom). After 3 days, the proliferative response was measured as described in Sect. 4.7. Percentage of response was determined as in Fig. 1.

when serum anti-Vβ8 antibody levels in SEB-treated mice are similar to those in PBS-treated mouse sera (data not shown). The results thus indicate an inverse correlation between serum anti-TCR antibody level and specific anergy in the Vβ8CD4⁺ T cell population.

2.4 Reconstituted nude mice do not produce anti-Vβ8 antibodies in response to SEB immunization, nor do their Vβ8 T cells become anergic

We recently showed that SEB treatment is unable to induce Vβ8⁺ T cell deletion or anergy in T cell-reconstituted nude (r-nude) mice [28]. To determine the effect of SEB treatment on circulating anti-Vβ8 antibody in a situation in which anergy does not occur, nude mice were reconstituted with T cells and treated with SEB 1 month later. We observed that nude mice have lower natural anti-Vβ8 antibody levels than age-matched normal BALB/c mice, but they are normalized by 1 month after T cell reconstitution (data not shown). Thereafter, normal, nude or r-nude mice were treated with PBS or SEB and their sera analyzed 10 days later for anti-Vβ8 antibody. The anti-Vβ8 serum antibody level in r-nude mice is not altered in response to SEB (Fig. 7), despite massive T cell activation and lymphokine secretion [28], suggesting that anti-Vβ8 antibodies may be important for maintenance of T cell anergy *in vivo*.

2.5 *In vivo* anti-Vβ8 antibody injection modulates the *in vitro* T cell response to SEB

Early evidence showed that anti-Vβ8 antibodies modulate Vβ8⁺ T cell reactivity *in vivo* and *in vitro* [29, 30]; the results presented above suggest an inverse correlation between serum anti-Vβ8 antibody levels and the state of CD4Vβ8⁺ T cell reactivity. Most previous studies selected anti-TCR antibodies on the basis of high anti-TCR affinity. We have now produced hybridomas secreting anti-Vβ8 antibodies and tested the effect of *in vivo* administration of one of these in the SEB response. To study the role of anti-TCR antibody in the establishment and maintenance of anergy, we evaluated the effect of *in vivo* anti-Vβ8 IgM antibody (C1-23) treatment on SEB-reactive T cell responsiveness *in vitro*. Mice received three injections (one each day) of C1-23 (10 μg) or an unrelated IgM antibody (10 μg) every other day. The day after the last injection we analyzed the frequency of CD4Vβ8⁺ T cells and their ability to proliferate upon SEB stimulation *in vitro*. C1-23 treatment did not alter CD4Vβ8⁺ T cell frequency (Table 1) and, when stimulated with SEB *in vitro*, spleen cells from C1-23-treated mice proliferated less than cells from mice treated with an unrelated IgM antibody. Two days after first injection the frequency of Vβ8⁺ cells expressing memory phenotypes was higher in the group treated with C1-23 than that treated with control IgM. This indicates that the increase in serum anti-Vβ8 antibody levels by passive C1-23 administration renders Vβ8⁺ T cells either more resistant to SEB stimulation *in vitro*, or anergic. The combination of these results strongly suggests that anti-Vβ8 antibodies are involved in the maintenance of SEB-induced T cell anergy.

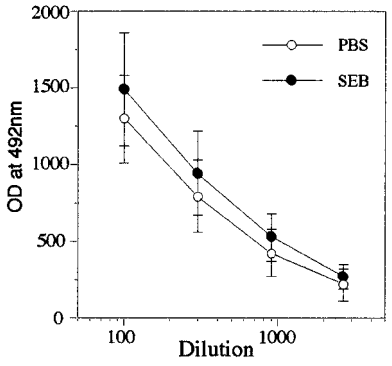

Figure 7. The effect of SEB immunization on serum anti-Vβ8 antibody concentration in reconstituted nude mice. One month after T cell reconstitution, nude mice were treated with PBS (○) and SEB (●), bled 10 days later and sera anaylzed for anti-Vβ8 antibodies. The results represent the mean ± SD of five animals in each group. Similar results were obtained in two independent experiments.

Table 1. *In vivo* natural antibody treatment induces T cell anergy

Treatment *in vivo*	CD4Vβ8⁺ cells (%)[a]	Vβ8⁺ Mel-14^{low} (%)[b]	*In vitro* stimuli[c] SEB	SEA
Irrelevant	21.2 ± 1.2	26.92 ± 1.3	138 577 ± 10 355	135 734 ± 8 563
C1-23	22.3 ± 1.1	34.8 ± 2.7	50 124 ± 15 421	136 783 ± 5 764

a) The results are the mean of four animals/group ± SEM.
b) Mice received an injection of 10 μg antibodies, and the frequency of Vβ8⁺ expressing MEL-14^{low} was determined 48 h later.
c) Spleen cells (10^5) were cultured in triplicate and stimulated *in vitro* with 10 μg SEB or SEA. After 72 h, the proliferative response was measured as described in Sect. 4.7. The results are the mean of four animals/group ± SEM.

3 Discussion

It was demonstrated earlier that *in vivo* SEB-induced T cell deletion [23] and anergy [31] are not B cell dependent, since spleen T cells from B6 μmt mice treated 2 days earlier with SEB are refractory to SEB stimulation *in vitro* [31]. The data presented here suggest, however, that B cells may have a critical role in the maintenance of T cell anergy. T cell anergy is a long-lasting phenomenon [23]; we thus evaluated SEB-induced T cell anergy in B6 μmt mice 13 days after Sag treatment. In this case, nearly all remaining SEB-primed Vβ8$^+$ T cells from B6 μmt, but not from B6 mice, proliferated in response to *in vitro* stimulation with SEB (Fig. 1). This indicates that, in the absence of B cells, T cell anergy is short-lived. The absence of anergy is not a result of deficient T cell activation in B6 μmt mice, inasmuch as their Vβ8$^+$ T cells proliferate, acquire memory phenotype and trigger programmed cell death as well as those from B6 mice do (Fig. 2), although B6 μmt mouse CD4Vβ8$^+$ T cells lose their memory phenotype faster than these same T cells from B6 mice.

Memory phenotype has been correlated to an incapacity of cells to proliferate after *in vitro* antigenic stimulation [18]. Loss of CD4Vβ8$^+$ T cell anergy in B6 μmt mice must thus be accompanied by a change from the memory to the virgin phenotype. This indicates that the anergic state is an active phenomenon, dependent on a B-T cell interaction that appears not to participate in the CD8Vβ8$^+$ T cell, since CD8$^+$ T cells from both B6 and B6 μmt mice lose their memory phenotype soon after SEB treatment (Fig. 2D).

To elucidate the mechanism by which B cells maintain Vβ8$^+$ T cell anergy, we studied SEB-induced B cell repertoire modifications. We showed earlier that immunization with SEB induces a humoral immune response, with anti-SEB antibody production [20]. The possibility that this antibody may play a role in the maintenance of T cell unresponsiveness by blocking Sag presentation appears to be untrue, based on two pieces of evidence. First, anergic T cells express the memory phenotype, suggesting that they are activated. If anti-SEB antibodies block antigenic interaction with TCR, anergic Vβ8$^+$ T cells should not have “memory” phenotype. Second, anti-SEB antibodies either produced *in vitro* by primed B cells or added in culture did not impair SEB-induced T cell responses in PBS-treated mice [21].

Previous studies indicate that anti-Vβ8 antibodies can induce Vβ8$^+$ T cell anergy. Indeed, it has been shown in *in vitro* systems that anti-Vβ8 mAb added in culture modulates SEB-induced T cell proliferation and IL-2 production through a mechanism that induces T cell anergy [30]. We show here that *in vivo* SEB treatment induces a significant increase in serum anti-Vβ8 antibody (Fig. 3A) and that this phenomenon is specific, as we detected no changes in serum anti-OVA antibody (Fig. 3B), nor after treatment with SEA, a Sag that stimulates T cells expressing receptor Vβ8 and Vβ11 (Fig. 5). These results suggest that anti-Vβ8 antibodies are produced in response to specific T cell activation and not as consequence of SEB-induced polyclonal B cell activation.

In agreement with the role of anti-Vβ8 TCR antibodies in maintaining T cell anergy, the injection of an IgM anti-Vβ8 antibody (C1-23) into normal mice induces T cell anergy as measured by the T cell unresponsiveness to SEB, but not to SEA *in vitro* (Table 1).This treatment does not induce Vβ8$^+$ T cell deletion (Table 1) or modulation of TCR expression (data not shown), suggesting that C1-23 treatment induces modifications in Vβ8$^+$ T cell function, culminating in CD4Vβ8$^+$ T cell anergy.

We demonstrate an inverse correlation between the anti-Vβ8 antibodies and the ability of Vβ8$^+$ T cells to generate an *in vitro* response to SEB. Finally, the inability of r-nude mice to acquire SEB-induced tolerance [28] correlates with their inhability to produce anti-Vβ8 in response to SEB treatment (Fig. 7). This may be explained by B cell exhaustion, a result of polyclonal activation during T cell reconstitution (data not shown).

It reamins unclear whether, or how, anti-Vβ8 antibodies maintain SEB-induced T cell anergy, although two distinct mechanisms have been proposed. First, the anti-Vβ8 antibodies may impair antigen presentation by inducing TCR down-regulation or by blocking TCR, consequently avoiding T cell stimulation and proliferation. This alternative is incompatible with the memory phenotype expressed by anergic T cells. A second possibility is that anti-Vβ8 antibodies affect the signaling transduction pathway associated to TCR-SEB interactions, culminating in the establishment and maintenance of T cell anergy. This suggests that anergy is a different cell activation stage, in which the activation threshold is higher than in normal cells [32]. Anergic T cells, rather than being paralyzed, are thus differentiated into a functional state. Of note in this context is that the anergic T cells express the memory phenotype [18] and present high levels of protein tyrosine kinase phosphorylation in tyrosine (personal observation). Anti-Vβ8 antibody may thus play an important role in the maintenance of an elevated activation threshold through continuous stimulation of Vβ8$^+$ T cells. Finally, the mechanism of anti-Vβ8 antibody production requires activated T cells (Figs. 3 and 5). SEB-stimulated Vβ8$^+$ T cells may provide help more efficiently to those B cells that, in spite of presenting SEB, display specificity for that TCR. Any modification in the B

cell repertoire, such as a reduction in serum anti-TCR levels (Fig. 5) or polyclonal B cell activation [17] may break this circuit, causing the reversion to Sag-induced T cell anergy. This proposition has important implications for the way the immune system operates. The maintenance of T cell anergy is a property of the immune system centered on cellular interactions and not determined by antigen persistence, as earlier proposed [16].

Over the last decade, studies have demonstrated that circulating antibodies can select B [33] and T cell repertoires [34, 35]. We now add our results to this issue, and further suggest that anti-Vβ8 antibody may drive T cells to the anergic cell pool. In conclusion, our results demonstrate that SEB-induced T cell anergy is a property of the immune system and that anti-Vβ8 antibody plays an important role in the maintenance of Vβ8$^+$ T cell reactivity. All together, this suggests that B cells and immunoglobulin may participate specifically in the modulation of a potential pathogenic T cell, as already evidenced by the fact that B cell-deficient mice are unable to recover from EAE [36].

4 Materials and methods

4.1 Animals

For these experiments, we used 1 to 2-month-old athymic BALB/c (nude), euthymic BALB/c, C57BL/6 (B6) and C57BL/6 background B cell-deficient (B6 μmt) mice of different ages and both sexes, purchased from IFFA-Credo (Lyon, France) and B&K Universal Ltd. (London, GB) and bred in our facilities.

4.2 Anti-Vβ8 mAb

Spleen cells from normal 1-month-old mice were fused with 635 B cell myeloma cells and cultured in selective medium. Clones were screened for anti-Vβ8 antibody production in an ELISA as below, and an IgM anti-Vβ8 clone (C1-23) was obtained. Mice received an injection of C1-23 (10 μg) or an irrelevant IgM antibody (10 μg) every other day for a week. The frequency of CD4Vβ8$^+$ T cells and their capacity to respond to SEB *in vitro* was determined 2 days after the last injection.

4.3 Antibodies and flow cytometry

Peroxidase-labeled goat anti-mouse Ig (H+L chain) (Southern Biotechnologies, Birmingham, AL) were used for ELISA assays. FITC-conjugated anti-CD4, anti-CD8 mAb anti-rat Ig (Boehringer Mannheim, Mannheim Germany), biotin-conjugated F23.1 (anti-Vβ8.1, 8.2, 8.3) and PE-conjugated streptavidin (Southern Biotechnology) were used for staining. MEL-14 supernatant was obtained in our laboratory. Spleen cells were stained with various combinations of antibodies described above and analyzed on an Epics XL flow cytometer (Coulter Electronics, Miami, FL).

4.4 Antigens and immunization

Staphylococcus aureus enterotoxins A and B (Toxin Technology, Sarasota, FL) were used as antigen. Immunization was performed by i.p. injection of SEB (50 μg) or SEA (50 μg) in 0.2 ml of PBS; control mice received 0.2 ml PBS alone. OVA grade III (Sigma Chemical, St. Louis, MO) was used for ELISA assays.

4.5 Vβ8-chain preparation and purification

A plasmid modified by the insertion of Vβ8.2 chain genes containing a C-terminal His_6 inserted to allow affinity purification on Ni^{2+}-NTA-agarose columns, designed for expression and secretion of the immunoglobulin domain, was produced [37]. Vβ8.2 was produced and purified as described [37]. After purification, the Vβ8 concentration was determined using the Micro-BCA Protein Assay (Pierce, Rockford, IL).

4.6 ELISA

The presence of antigen-specific antibody in serum was assessed by standard ELISA procedures. In short, polystyrene plates were coated overnight at 4 °C with 5 μg/ml of synthetic Vβ8.2 chain or OVA grade III in 0.1 M carbonate-bicarbonate buffer, pH 9.5. Serum was incubated for 1 h at 37 °C. Anti-Vβ8 antibody was visualized using peroxidase-conjugated anti-mouse Ig (H+L) (Southern Biotechnology). The reaction was performed using buffer containing 0.1 M citric acid and 0.2 mM Na_2HPO_4 with H_2O_2 and orthophenylenediamine and terminated 15–30 min later with 3 M H_2SO_4. Absorbance was measured at 492 nm.

4.7 Proliferative response to SEB and SEA

Splenocytes (10^5) were cultured in triplicate in 96-well microtiter plates in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 10 % FCS, 50 mM 2-ME, 1 mM Hepes and 2 mM glutamine. Cells were cultured in the presence of various concentrations of SEB or 1 μg/ml SEA. After 72 h, cultures were pulsed for 16 h with [^{3}H]thymidine (1 μCi/ml, Amersham Intl., Aylesbury, GB), cells harvested and radioactivity measured using a liquid scintillation β-counter.

4.8 T cell reconstitution

Spleen cells (10^6) were transferred to nude mice by an i.v. injection in the tail. A group of animals was bled 10 days

after T cell reconstitution and serum analyzed for the presence of anti-Vβ8 antibodies. One month after T cell reconstitution, mice were treated with PBS or SEB and bled 10 days later for determination of anti-Vβ8 antibodies in serum.

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