

Anti-V β 8 antibodies induce and maintain staphylococcal enterotoxin B-triggered V β 8⁺ 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-V β 8 antibody titer as detected by enzyme-linked immunosorbent assay using soluble V β 8 chains as antigens, and by staining of a V β 8-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-V β 8 monoclonal antibody treatment causes V β 8⁺ T cell unresponsiveness to SEB *in vitro* (anergy), without affecting CD4V β 8⁺ 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-V β 8 antibody that specifically interacts with V β 8⁺ T cells.

Key words: Anergy / T cell receptor / Antibody / Staphylococcal enterotoxin B

Received	18/5/98
Revised	30/10/98
Accepted	30/10/98

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 V β 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 V β 8 undergo clonal expansion. Two days later, the V β 8⁺

T cell frequency is twice that observed in PBS-treated mice [2, 8]. CD4V β 8⁺ T cells are thereafter deleted to frequencies below those observed in PBS-treated mice, whereas CD8V β 8⁺ 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 V β 8⁺ 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 V β 8⁺ T cells has also been explored with success in the experimental allergic encephalomyelitis (EAE) model, in which SEB treatment modulates encephalitogenic V β 8⁺ 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

[1 18420]

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

in vivo. T cells, which are unable to grow after restimulation *in vitro*, can proliferate [19], secrete 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-V β 8 chain antibody modulates the Sag-induced *in vitro* T cell response. Analysis of sera from SEB-injected mice shows that anti-V β 8 antibodies are generated specifically in response to SEB immunization and that their presence is related to persistence of anergy. These results suggest that anti-V β 8 antibodies are important in SEB-induced T cell anergy.

2 Results

2.1 B cell-deficient B6 μ mt 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 μ mt mice, in which we have compared the effect of SEB *in vivo* and followed the changes in V β 8 T cell frequency and their capacity to proliferate *in vitro* (Fig. 1). At day 13, spleens from SEB-treated B6 mice have approximately 50 % V β 8 T cells and the *in vitro* response is only 24 % of that observed in PBS-treated control B6 mice, here considered to have maximum CD4V β 8⁺ T cell frequency and *in vitro* proliferative response. In contrast, CD4⁺ spleen T cells from SEB-treated B6 μ mt mice have the same V β 8⁺ T cell frequencies (approximately 50 %), and generate a response that accounts for 50 % of the SEB-induced *in vitro* response observed in PBS-treated

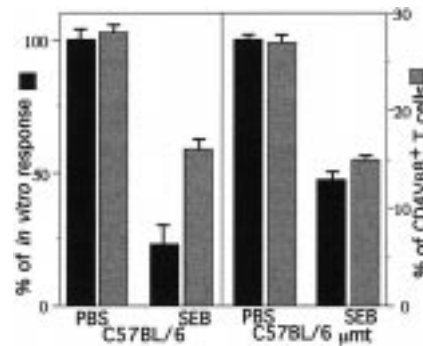


Figure 1. Comparison of SEB-induced *in vitro* T cell responses in B6 versus B6 μ mt mice. Spleen CD4V β 8⁺ T cell frequency (gray bar) and proliferation following *in vitro* stimulation with SEB (black bar) were determined as described in Sect. 4.7. To calculate the percentage of response, we assume that PBS-treated mice of each strain show 100 % response. % of response = (response of SEB-treated mice/response of PBS-treated mice) \times 100. The mean of three animals per group is shown; similar results were obtained in three independent experiments.

B6 μ mt mice (Fig. 1). This demonstrates that, at day 13 after SEB treatment, virtually all SEB-reactive T cells from B6 μ mt mice proliferate following *in vitro* SEB stimulation, indicating rapid SEB-induced T cell anergy loss in the absence of B cells. B6 and B6 μ mt spleen cells respond to SEA *in vitro*, whether they are treated with SEB or with PBS (data not shown).

2.2 The effect of SEB treatment on B cell-deficient mouse V β 8⁺ 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 μ mt mice. CD4 and CD8V β 8⁺ T cell kinetics were similar in both B6 and B6 μ mt 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 from memory T cells. CD4 and CD8V β 8⁺ MEL-14^{low} T cell frequencies were determined in B6 and B6 μ mt mice at different times after SEB treatment (Fig. 2C, D). CD4V β 8⁺ T cells from B6, but not from B6 μ mt mice, retained the memory phenotype for longer

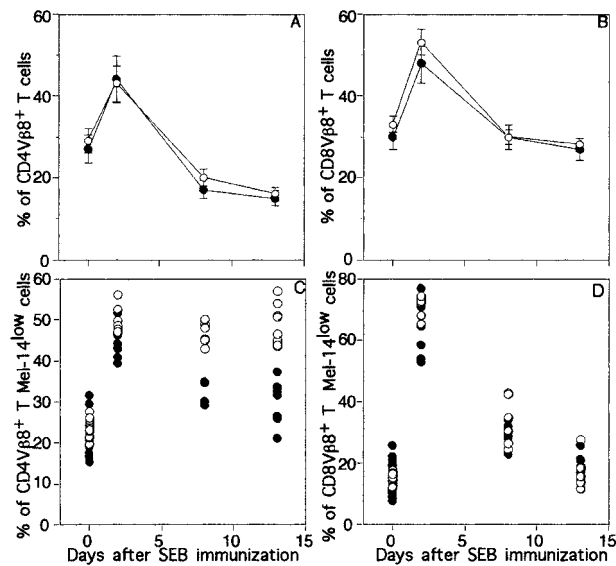


Figure 2. *In vivo* SEB-reactive T cell response in C57BL/6 and B6 μ mt mice. B6 (open circles) and B6 μ mt (filled circles) mice were treated with PBS or SEB and the frequency was analyzed by flow cytometry of CD4⁺ (A) and CD8⁺ (B) cells expressing V β 8 chain at different times after treatment. The phenotypes of spleen C57BL/6 and B6 μ mt mouse CD4V β 8⁺ (C) and CD8V β 8⁺ (D) T cells expressing MEL-14^{low} were analyzed on different days after SEB treatment. Double positive T cells were electronically gated and MEL-14 expression analyzed. The mean \pm SD of each group are represented in A and B. In C and D, individual animals from three distinct experiments are shown.

than 13 days (Fig. 2C), suggesting that B cells are involved in maintenance of the CD4⁺ T cell memory phenotype. Both B6 and B6 μ mt mice present the same CD8V β 8⁺ 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 μ mt mice in the frequency of V β 6⁺ T cells expressing memory phenotype (data not shown).

2.3 Immunization with SEB induces anti-V β 8 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 V β 8 TCR expressed on SEB-activated T cells. We

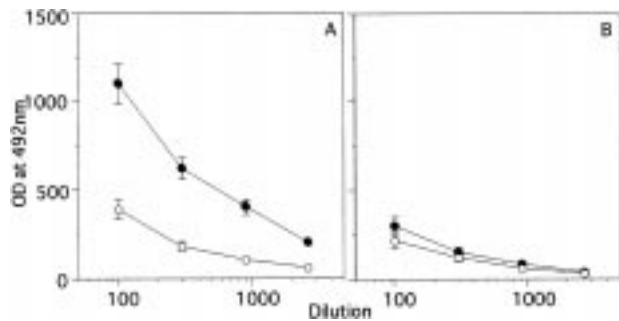


Figure 3. Effect of immunization with SEB on anti-V β 8 and anti-OVA antibodies. BALB/c mice were treated with PBS (○) or SEB (●), bled 10 days later and the sera analyzed for anti-V β 8 (A) and anti-OVA antibodies (B). The results represent the mean \pm SD of five animals in each group. Similar results were obtained in three independent experiments.

found that SEB treatment induces a significant increase in serum anti-V β 8 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-V β 8 antibody level is a specific phenomenon, mediated by V β 8⁺ T cell stimulation after SEB treatment (Fig. 3B).

Anti-V β 8 antibodies were evaluated in two different systems, an ELISA using recombinant V β 8 peptide-coated plates as described in Sect. 4.6, as well as by their capacity to stain YAC-1 cells, a thymoma that expresses TCR V β 8. In both systems, it is clearly shown that SEB immunization triggers anti-V β 8 antibody production. YAC-1 cells are thus stained by the anti-V β 8 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 V β family [21]. To determine whether anti-TCR V β 8 antibodies are specific for activated T cells, we estimated the effect of SEA treatment *in vivo* on the serum anti-V β 8 antibody titer. Mice were treated with SEA, SEB or PBS and the sera obtained 10 days later were analyzed for anti-V β 8 antibodies. SEB treatment, but not SEA or PBS, induced an increase in serum anti-V β 8 antibody levels (Fig. 5). Immunization with SEA, which stimulates V β 3⁺ and V β 11⁺ T cells, did not increase serum anti-V β 8 levels.

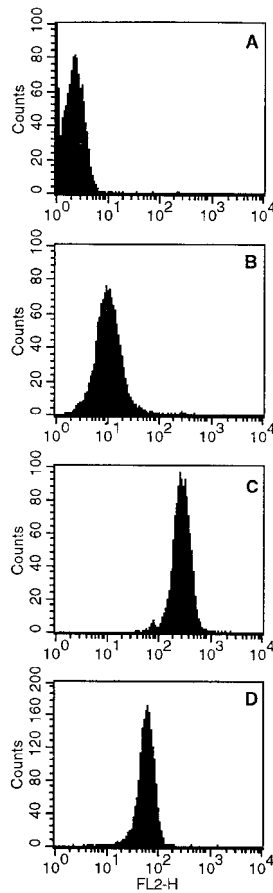


Figure 4. Serum from SEB-treated mice stains V β 8-expressing T cell clones. YAC-1 cells (10^6) were incubated for 15 min with serum from PBS- (B) or SEB-treated (C) mice or F23.1 mAb (D), washed and incubated with a PE-conjugated secondary anti-mouse antibody. YAC-1 cells incubated with anti-Ig alone were used as control (A). Cells were then analyzed by flow cytometry.

Since SEB-induced T cell energy is reversible *in vivo*, we evaluated the temporal effect on SEB-induced anti-V β 8 antibody. BALB/c mice were treated with PBS or SEB and, at different times after treatment, mice were bled to study serum anti-V β 8 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-V β 8 antibody titer parallels the increase in energy 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 energy (Fig. 6B). At that time, sera from SEB- and PBS-treated mice show similar anti-V β 8 antibody concentrations (Fig. 6A). In these studies, we also analyzed the CD4V β 8⁺ 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

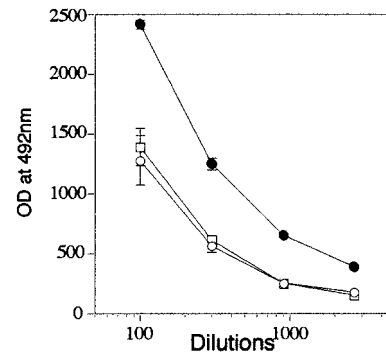


Figure 5. Anti-V β 8 antibody production is an SEB-specific phenomenon. BALB/c mice were treated with PBS (○), SEB (●) or SEA (□), bled 10 days later and the sera analyzed for anti-V β 8 antibodies. The results represent the mean \pm SD of five animals in each group. Similar results were obtained in three independent experiments.

anti-V β 8 antibodies may be related to the loss of SEB-induced T cell energy. Finally, *in vitro* SEB-induced T cell proliferation and serum anti-V β 8 antibody levels do not change in PBS-treated mice.

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

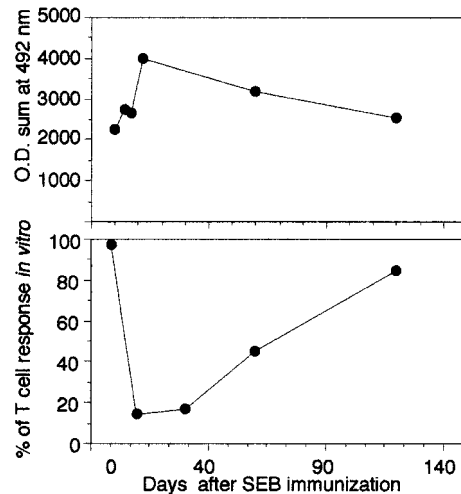


Figure 6. Temporal effect on T cell energy and anti-V β 8 antibodies. BALB/c mice were treated with PBS or SEB and, at different times, three mice from each group were bled and spleen cells assayed for *in vitro* proliferative response to SEB. Serum was analyzed for anti-V β 8 antibodies; the result 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-V β 8 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 V β 8CD4⁺ T cell population.

2.4 Reconstituted nude mice do not produce anti-V β 8 antibodies in response to SEB immunization, nor do their V β 8 T cells become anergic

We recently showed that SEB treatment is unable to induce V β 8⁺ T cell deletion or anergy in T cell-reconstituted nude (r-nude) mice [28]. To determine the effect of SEB treatment on circulating anti-V β 8 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-V β 8 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-V β 8 antibody. The anti-V β 8 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-V β 8 antibodies may be important for maintenance of T cell anergy *in vivo*.

2.5 *In vivo* anti-V β 8 antibody injection modulates the *in vitro* T cell response to SEB

Early evidence showed that anti-V β 8 antibodies modulate V β 8⁺ T cell reactivity *in vivo* and *in vitro* [29, 30]; the results presented above suggest an inverse correlation between serum anti-V β 8 antibody levels and the state of CD4V β 8⁺ 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-V β 8 antibodies and tested the effect of *in vivo* administration of one of these in the SEB response. To study the

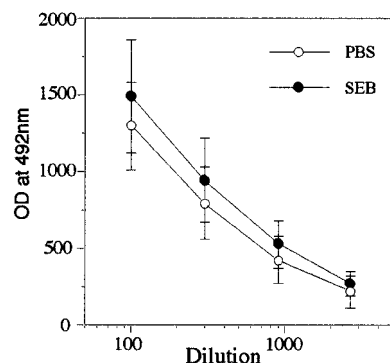


Figure 7. The effect of SEB immunization on serum anti-V β 8 antibody concentration in reconstituted nude mice. One month after T cell reconstitution, nude mice were treated with PBS (○) and SEB (●), bled 10 days later and sera analyzed for anti-V β 8 antibodies. The results represent the mean \pm SD of five animals in each group. Similar results were obtained in two independent experiments.

role of anti-TCR antibody in the establishment and maintenance of anergy, we evaluated the effect of *in vivo* anti-V β 8 IgM antibody (C1-23) treatment on SEB-reactive T cell responsiveness *in vitro*. Mice received three injections (one each day) of C1-23 (10 μ g) or an unrelated IgM antibody (10 μ g) every other day. The day after the last injection we analyzed the frequency of CD4V β 8⁺ T cells and their ability to proliferate upon SEB stimulation *in vitro*. C1-23 treatment did not alter CD4V β 8⁺ 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 V β 8⁺ 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-V β 8 antibody levels by passive C1-23 administration renders V β 8⁺ T cells either more resistant to SEB stimulation *in vitro*, or anergic. The combination of these results strongly suggests that anti-V β 8 antibodies are involved in the maintenance of SEB-induced T cell anergy.

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

Treatment <i>in vivo</i>	CD4V β 8 ⁺ cells (%) ^{a)}	V β 8 ⁺ Mel-14 ^{low} (%) ^{b)}	<i>In vitro</i> stimuli ^{c)}	
			SEB	SEA
Irrelevant	21.2 \pm 1.2	26.92 \pm 1.3	138 577 \pm 10 355	135 734 \pm 8 563
C1-23	22.3 \pm 1.1	34.8 \pm 2.7	50 124 \pm 15 421	136 783 \pm 5 764

a) The results are the mean of four animals/group \pm SEM.

b) Mice received an injection of 10 μ g antibodies, and the frequency of V β 8⁺ expressing MEL-14^{low} was determined 48 h later.

c) Spleen cells (10⁵) were cultured in triplicate and stimulated *in vitro* with 10 μ g 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 \pm 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 μ mt 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 μ mt mice 13 days after Sag treatment. In this case, nearly all remaining SEB-primed $V\beta 8^+$ T cells from B6 μ mt, 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 μ mt mice, inasmuch as their $V\beta 8^+$ T cells proliferate, acquire memory phenotype and trigger programmed cell death as well as those from B6 mice do (Fig. 2), although B6 μ mt mouse CD4 $V\beta 8^+$ 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 CD4 $V\beta 8^+$ T cell anergy in B6 μ mt 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 CD8 $V\beta 8^+$ T cell, since CD8 $^+$ T cells from both B6 and B6 μ mt mice lose their memory phenotype soon after SEB treatment (Fig. 2D).

To elucidate the mechanism by which B cells maintain $V\beta 8^+$ 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 $V\beta 8^+$ 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- $V\beta 8$ antibodies can induce $V\beta 8^+$ T cell anergy. Indeed, it has been shown in *in vitro* systems that anti- $V\beta 8$ 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- $V\beta 8$ 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 $V\beta 8$ and $V\beta 11$ (Fig. 5). These results suggest that anti- $V\beta 8$ 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- $V\beta 8$ TCR antibodies in maintaining T cell anergy, the injection of an IgM anti- $V\beta 8$ 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 $V\beta 8^+$ T cell deletion (Table 1) or modulation of TCR expression (data not shown), suggesting that C1-23 treatment induces modifications in $V\beta 8^+$ T cell function, culminating in CD4 $V\beta 8^+$ T cell anergy.

We demonstrate an inverse correlation between the anti- $V\beta 8$ antibodies and the ability of $V\beta 8^+$ 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 inability to produce anti- $V\beta 8$ 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 remains unclear whether, or how, anti- $V\beta 8$ antibodies maintain SEB-induced T cell anergy, although two distinct mechanisms have been proposed. First, the anti- $V\beta 8$ 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- $V\beta 8$ 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- $V\beta 8$ antibody may thus play an important role in the maintenance of an elevated activation threshold through continuous stimulation of $V\beta 8^+$ T cells. Finally, the mechanism of anti- $V\beta 8$ antibody production requires activated T cells (Figs. 3 and 5). SEB-stimulated $V\beta 8^+$ 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-V β 8 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-V β 8 antibody plays an important role in the maintenance of V β 8⁺ 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 μ mt) 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-V β 8 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-V β 8 antibody production in an ELISA as below, and an IgM anti-V β 8 clone (C1-23) was obtained. Mice received an injection of C1-23 (10 μ g) or an irrelevant IgM antibody (10 μ g) every other day for a week. The frequency of CD4V β 8⁺ 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-V β 8.1, 8.2, 8.3) and PE-conjugated streptavidin (Southern Biotechnology) were used for stain-

ing. 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 μ g) or SEA (50 μ g) 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 V β 8-chain preparation and purification

A plasmid modified by the insertion of V β 8.2 chain genes containing a C-terminal His₆ inserted to allow affinity purification on Ni²⁺-NTA-agarose columns, designed for expression and secretion of the immunoglobulin domain, was produced [37]. V β 8.2 was produced and purified as described [37]. After purification, the V β 8 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 μ g/ml of synthetic V β 8.2 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-V β 8 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 Na₂HPO₄ with H₂O₂ and orthophenylenediamine and terminated 15–30 min later with 3 M H₂SO₄. Absorbance was measured at 492 nm.

4.7 Proliferative response to SEB and SEA

Splenocytes (10⁵) 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 μ g/ml SEA. After 72 h, cultures were pulsed for 16 h with [³H]thymidine (1 μ Ci/ml, Amersham Intl., Aylesbury, GB), cells harvested and radioactivity measured using a liquid scintillation β -counter.

4.8 T cell reconstitution

Spleen cells (10⁶) 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-V β 8 antibodies. One month after T cell reconstitution, mice were treated with PBS or SEB and bled 10 days later for determination of anti-V β 8 antibodies in serum.

Acknowledgments: We thank Dr. José Mengel for critical reading of the manuscript and C. Mark for editorial assistance. Luiz Stark Aroeira was supported by a fellowship from the Conselho Nacional de Pesquisa (CNPq), Brasil. The Department of Immunology and Oncology was founded and its supported by the Spanish Research Council (CSIC) and Pharmacia & Upjohn.

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