Chronic exposure to superantigen induces regulatory CD4^+ T cells with IL-10-mediated suppressive activity

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Abstract
The repeated injection of bacterial superantigens (SAg), such as staphylococcus enterotoxin (SE) A or B, has been shown in mice to induce a state of unresponsiveness characterized by the lack of secretion of Th1 lymphokines, such as IL-2 and IFN-γ, following subsequent SAg challenge. We made the observation, in vivo as well as in vitro, that unresponsiveness to SAg could be transferred from SEA- to SEB-reactive T cells (and reversibly from SEB- to SEA-specific T cells) in C57BL/6 mice but not in BALB/c mice. Since C57BL/6 mice, unlike BALB/c mice, possess TCR Vβ3^+ and Vβ11^+ T cells able to react with both SEA and SEB, we hypothesized that SAg-unresponsive Vβ3^+ and Vβ11^+ T cells could mediate linked suppression of other SAg-reactive T cells. To analyze further this possibility, spleen cells from BALB/c mice made unresponsive to SEB were tested for their capacity to suppress the response of normal BALB/c cells to SEB. The production of both IFN-γ and IL-2 following SEB stimulation was greatly impaired in co-cultures containing CD4^+ T cells, but not CD8^+ T cells, isolated from unresponsive animals. In vivo, the production of both IFN-γ and IL-2 responses to SEB was dramatically reduced in animals adoptively transferred with unresponsive spleen cells. This suppression was abrogated in recipients injected with neutralizing anti-IL-10 antibodies. Moreover, in animals made unresponsive to SEB, SAg-reactive CD4^+ T cells were found to express high levels of CTLA-4, a molecule recently described to play an essential role in the suppressive function of regulatory T cells. Taken together these results demonstrate that the repetitive injection of SAg induces the differentiation of regulatory CD4^+ T cells capable of suppressing SAg-reactive naive T cells.

Introduction
Induction and maintenance of peripheral tolerance are important mechanisms required for a balanced immune system. In addition to clonal deletion of T cells (1,2) and functional inactivation among antigen-specific T cells (3–5), active suppression involving T cell-derived soluble factors has been proposed for maintaining peripheral T cell tolerance. Early work by Gershon and Kondo showed that T cells from tolerant mice disabled naive lymphocytes so that they too could not respond to antigenic stimulation (6). This ‘infectious’ immunological tolerance, as termed by the authors, could spread to third-party antigens linked on the same antigen-presenting cell, the process being also described as linked suppression (7). Analysis of immune suppression, however, was hampered for many years by the inability to isolate and characterize suppressor T cells. It was only recently that, by repetitive antigenic stimulation in the presence of IL-10, Groux et al. generated in vitro human and murine CD4^+ T cells with suppressive capability (8). The T cells, termed T regulatory 1 cells (T(1), were able in vivo to suppress the development of colitis in SCID mice transferred with purified pathogenic CD45RB(high)CD4^+ T cells. Further phenotypic characterization of T(1) cells has shown that they express CD25 and their immune-suppressive function in vivo is dependent on signaling via the negative regulator of T cell activation, CTLA-4 (9–12). Finally, T cell-derived factors, such as IL-10 and transforming growth factor (TGF)-β, are considered as soluble mediators of T cell suppression by T(1) cells (13–16).

In vivo exposure to bacterial superantigen (SAg) selectively
induces the rapid proliferation of T cells carrying SAg-specific TCR Vβ fragments (17,18). This is associated with the release of various cytokines such as IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ and tumor necrosis factor (TNF)-α into the circulation (19–22). After their initial expansion, T cells undergo activation-induced cell death, or apoptosis, leading to the clonal deletion of SAg-reactive CD4+ and CD8+ T cells from the periphery (17,18). SAg-reactive T cells which escape death, however, fail to proliferate and to secrete IL-2 in response to subsequent SAg exposure (19,20,23). This phenomenon, often referred to as anergy, is specific for SAg-reactive T cells, since responsiveness to non-cross-reactive SAg remains normal (18). This state of unresponsiveness seems specific for IL-2 production as shown in models of SAg-induced anergy in which IFN-γ and IL-10 production is induced, or increased, after challenge with SAg (24,25). Further exposure to SAg, however, down-regulates the production of IFN-γ. Indeed, following the repeated injection of SAg, IFN-γ disappears from the serum of injected animals, while T1,2 lymphokines, such as IL-10, persist (25,26).

In the present study, we made the observation that spleen cells from mice made unresponsive to SAg were able to suppress the primary response of SAg-specific T cells. The suppression was dependent on the presence of CD4+ T cells in the suppressor cell population and on the secretion of IL-10. The suppressive activity affected both IL-2 and IFN-γ responses. These results show that, in addition to clonal deletion and T cell anergy, active suppression is also part of the mechanisms that maintain tolerance to bacterial SAg.

**Methods**

**Mice**

BALB/c and C57BL/6 mice of both sexes were obtained from IFFA-Credo Belgium (Brussels, Belgium). They were maintained in the animal facilities of the Faculty of Medicine of the Université Libre de Bruxelles. All mice used in our experiments were 8–12 weeks old.

**Flow cytometry**

Spleen cells were stained with various combinations of fluorochrome- and biotin-conjugated antibodies were all from PharMingen (Erembodegem-Aalst, Belgium). Anti-TCR Vβ6 (RR4-7) and Vβ8 (F23.1) mAb were labeled with FITC. Anti-CD4 mAb (GK1.5) were biotinylated and anti-CTLA-4 mAb (JES5-2A5, IgG1, 500 µg/ml of SEA or SEB (Toxin Technologies, Saratoga, FL) or 20 µg of SEA (Toxin Technologies) in 200 µl of physiological saline. Mice were bled by retro-orbital puncture 4 h after SAg injection. Serum levels of IFN-γ, IL-2 and IL-10 were determined by ELISA (see below).

**Rechallenge in vivo after tolerization with SAg**

BALB/c or C57BL/6 mice were made unresponsive by three i.p. injections (one injection every 3 days) of 20 µg of SEB. The animals were then compared to un.injected control mice for their capacity to produce IFN-γ and IL-10 following challenge with SEA or SEB (25 µg i.p.). Four hours after SAg challenge, animals were bled and serum levels of lymphokines were determined by ELISA (see below). Neutralization of IL-10 in vivo was carried out by one peritoneal injection of anti-IL-10 mAb (JES5-2A5, IgG1, 500 µg per injection) 3 h before rechallenge with SEB.

**Transfer of tolerized spleen cells**

BALB/c mice were made unresponsive with three injections of 20 µg of SEB at 3-day intervals. Twenty-four hours after the last injection, spleens were removed and homogenized to single-cell suspension. The cells were injected i.p. in naive BALB/c mice (100–150×106 cells/mouse in 500 µl). The mice were then immunized with 25 µg of SEB 24 h after the transfer of tolerized spleen cells. In some of the experiments, neutralizing anti-IL-10 mAb was given i.p. to mice receiving adoptively transferred cells 3 h before the injection of SEB. Control groups consisted of mice with no cell transfer or receiving naive spleen cells. Mice were bled 4 h after the last injection of superantigen and serum samples were assayed for serum levels of cytokines (IL-2 and IFN-γ) by ELISA.

**Production of cytokines in vitro**

Spleens of mice were homogenized to single-cell suspensions. The cells were then stimulated with 10 µg/ml of SEA or SEB in 48-well culture plates (Life Technologies, Merialbeke, Belgium) in 1 ml of RPMI 1640 (Biowhittaker, Petit-Rechain, Belgium) supplemented with 10% FCS, 1% sodium pyruvate, 1% l-glutamine, 1% non-essential amino acids, penicillin, streptomycin and 5×10−3 M 2-mercaptoethanol. The cells were incubated at 37°C in 5% CO2 atmosphere. Levels of IL-2 were determined in supernatants of 24 h cultures, while levels of IFN-γ, IL-4, IL-5 and IL-10 were assessed after 72 h of culture.

**In vitro assay for suppression**

Spleen cells (5×105/well) from BALB/c mice made unresponsive to SEB by three injections of the SAg were mixed with normal BALB/c spleen cells (5×105/well) in 48-well culture plates and stimulated for 3 days by SEA or SEB (5 µg/ml). Their suppressive effect on lymphokine secretion was assayed by comparing levels of IL-2, IFN-γ and IL-10 from these mixed cultures with those present in cultures of normal spleen cells alone stimulated by SEB. For the depletion of CD4+ or CD8+ T cells, spleen cells were incubated with rat anti-mouse CD4 (GK1.5) and CD8α (H35-17.2) respectively for 30 min at room temperature, and then with rabbit complement (Cedarlane, Hornby, Canada) at dilution 1/10 for 45 min at 37°C. The efficiency of depletion was assessed by flow cytometry with FITC-conjugated anti-CD4 (RM4.4) and anti-CD8α (53-6.7)
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Fig. 1. Repeated injection of SEA and SEB induces unresponsiveness in C57BL/6 and BALB/c mice respectively. SEA and SEB (20 µg/injection) were administered twice a week to C57BL/6 and BALB/c mice (five per group) respectively. Blood samples were taken 3 h after injections 1, 2, 4, 6 and 8, and serum levels of IFN-γ and IL-10 were determined by ELISA. Results are expressed as the mean ± SD of individual determinations. Asterisks indicate values for samples below the limit of detection. ND, not done; at these time points, acute hypovolemia did not allow collection of sufficient serum for determination of both IFN-γ and IL-10.

mAb specific for epitopes different than those recognized by GK1.5 and H35-17.2 mAb. After depletion, cell populations always contained <0.3% CD4⁺ cells or <0.4% CD8⁺ cells.

For purification of CD4⁺ T cells, spleen cells were filtered on nylon wool column. Then they were further purified by complement-mediated lysis using a cocktail of mAb to mouse CD8 (H35-17.2), Ia (M5/114) and B220 (RA3-6B2) molecules. The purity of the population was assessed by immunostaining with FITC-conjugated anti-CD4, anti-CD8, anti-Ia (25-9-17) mAb specific for epitope different than those recognized by H35-17.2 and M5/114 mAb. After purification, the percentage of CD4⁺ cells varied between 85 and 92%, depending on the experiment, whereas Ia⁺ and CD8⁺ cells represented <0.8 and <0.2% respectively.

Determination of cytokine by ELISA

Serum samples and culture supernatants were assayed for their content in lymphokines by solid-phase ELISA. IFN-γ and IL-2 were determined using ELISA kits from R & D Systems (Abingdon, UK). Kits for IL-4 and IL-10 were obtained from Biosource (Fleurus, Belgium) and for IL-5 from PharMingen (Erembodegem-Aalst, Belgium) respectively. Assays were carried out according to manufacturer’s instructions. Sensitivity limits of the assays were >30 pg/ml for IFN-γ, >15 pg/ml for IL-2 and IL-4, >60 pg/ml for IL-5 and >30 pg/ml for IL-10.

Results

Repeated injection of bacterial SAg induces a state of unresponsiveness in C57BL/6 mice that is transferable to naive cells

Recent studies suggest that staphylococcal SAg may be involved in the pathogenesis of arthritis and other autoimmune disorders (27). In a model of chronic exposure to SEB in BALB/c mice, we have shown a feature of hyperglobulinemia in a context where Th2-type cytokine production correlated always contained 0.3% CD4⁺ cells or 0.4% CD8⁺ cells.

For puriﬁcation of CD4⁺ T cells, spleen cells were ﬁltered (26). Deposits of IgG were observed by immunoﬂuorescence on nylon wool column. Then they were further puriﬁed by the mesangium of glomeruli while examination by light microscopy of kidney did not reveal significant lesions. The current study was initially undertaken to extend this observation and to evaluate whether chronic exposure to bacterial SAg could lead to the development of autoimmune disease. Thus, BALB/c and C57BL/6 mice were stimulated by repeated injection of SEB and SEA respectively. SEA was chosen to stimulate I-E⁺ C57BL/6 mice because it has a higher afﬁnity for I-E than SEB (28). Every second injection, mice were bled and the serum levels of IFN-γ and IL-10 were determined by ELISA. The production of IFN-γ was not detected as soon as after the third injection in both BALB/c and C57BL/6 mice (Fig. 1). The presence of IL-10 in the sera of injected mice persisted even after the eighth injection when the experiment was terminated (Fig. 1). These results conﬁrmed and extended the observations made by Florquin et al. in BALB/c mice and by Sundstedt et al. in Vβ3 TCR transgenic C57BL/6 mice where repeated injection of SAg induced an IL-10-dominated response (25,26).

After eight injections, the animals were sacriﬁced and organs were analyzed for the presence of histological lesions. Morphological analysis of the kidney revealed neither glomerular lesions nor Ig deposits. Heart, liver, lung and skin from these mice were normal and did not show signs of autoimmune lesions. Spleen cells were isolated and stimulated in culture with SEA or SEB. To determine their cytokine proﬁle, culture supernatants were analyzed by ELISA for the production of IFN-γ, IL-2, IL-4, IL-5 and IL-10 (Fig. 2). Upon stimulation with...
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Fig. 2. Spleen cells from C57BL/6 mice being given repeated injection of SEA are unresponsive to SEA or SEB challenge for IL-2 and IFN-γ responses. Spleen cells from normal C57BL/6 (n = 5) (a) and BALB/c (n = 5) (b) mice (open columns), and from C57BL/6 (n = 5) and BALB/c (n = 5) mice which received repeated injection of SEA and SEB (filled columns) respectively were stimulated in vitro by SEA or SEB. Culture supernatants were then assayed by ELISA for the presence of IFN-γ, IL-2, IL-4, IL-5 and IL-10. Results are expressed as the mean ± SD of individual determinations. Asterisks indicate values for samples below the limit of detection.

SEA or SEB, IL-4, IL-5 and IL-10 were produced by spleen cells isolated either from normal control mice or from mice receiving repeated injection of SAg (Fig. 2a and b). Looking at the production of IFN-γ and IL-2 in these systems, we found that, as expected from above in vivo results, spleen cells from C57BL/6 and BALB/c mice exposed to the repeated stimulation of SEA and SEB respectively did not respond to SAg challenge (Fig. 2a and b). Remarkably, spleen cells from C57BL/6 mice exposed to the repeated stimulation of SEA did not produce IL-2 nor IFN-γ in response to control SEB, as if in C57BL/6 mice unresponsiveness to SEA could be transferred to SEB-reactive T cells. On the contrary, such transfer of unresponsiveness was not observed in BALB/c mice, and IL-2 and IFN-γ were both produced upon stimulation of SEB-unresponsive BALB/c cells by SEA (Fig. 2b).

Because some caution should be exercised in interpreting the transfer of SAg-induced unresponsiveness in C57BL/6 mice for IL-2 and IFN-γ responses based only on in vitro assays, we moved to an in vivo model. In these experiments we compared C57BL/6 and BALB/c mice for their capacity to transfer SEB-induced unresponsiveness to SEA-reactive T cells. C57BL/6 and BALB/c mice were made unresponsive to SEB by three i.p. injections and their response to SEA challenge was then determined by analyzing their serum levels of IFN-γ. As shown in Fig. 3(a), the injection of SEA did not induce the secretion of IFN-γ in C57BL/6 mice rendered unresponsive to SEB. This lack of IFN-γ production could not be linked to the absence of SEA-reactive T cells as IL-10 was produced at levels similar to those observed in control animals after SEA injection (Fig. 3b). On the contrary, in BALB/c mice repeatedly injected with SEB, this transfer of unresponsiveness was not observed since both IFN-γ and IL-10 were detected following the injection of SEA (Fig. 3a and b).

Repeated exposure to SAg induces regulatory CD4+ T cells capable of suppressing the response of normal T cells to SAg stimulation

The possibility existed that the absence of response to control SAg in unresponsive C57BL/6 mice might not be the result of a genuine transfer of unresponsiveness but could reflect an intrinsic property of this inbred strain of mouse. To exclude that possibility, we designed experiments demonstrating that transfer of unresponsiveness could also operate in BALB/c mice. Spleen cells isolated from BALB/c mice made unresponsive to SEB were tested in vitro for their suppressive activity on normal BALB/c spleen cells. Cultures containing normal spleen cells or unresponsive spleen cells alone or a mixture of normal and unresponsive cells were stimulated with SEB or SEA. As seen in Fig. 4, normal cells alone responded to both SEA and SEB, and IL-2, IFN-γ and IL-10 could be detected in the culture supernatants. As expected from our previous results, in BALB/c mice, stimulation by SEB of spleen cells from mice exposed to repeated injection of SEB did not produce IL-2 nor IFN-γ in the culture, whereas control SEA stimulation induced secretion of both IL-2 and IFN-γ by these cells. Mixing responding and non-responding spleen cells dramatically reduced the production of IL-2 and IFN-γ by normal cells after stimulation by SEB but not by SEA (Fig. 4). This result demonstrated that chronic exposure to bacterial SAg induced in the spleen of injected mice the
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Fig. 4. Spleen cells from BALB/c mice made unresponsive to SEB by repeated injection of SAg suppress the responses to SEB stimulation developed by normal BALB/c spleen cells. Cultures containing spleen cells from normal BALB/c (5×10^6 cells/well), spleen cells from mice having received three injections of SEB (5×10^6 cells/well) or a mixture of these two types of cells (5×10^6 normal cells + 5×10^6 cells from 3×SEB-injected mice) were stimulated for 72 h with SEA (filled columns) or SEB (open columns). The levels of IL-2, IFN-γ and IL-10 present in the culture supernatants at the end of the culture were determined by ELISA. Cells from 3×SEB-injected mice were either whole spleen cells (WS), CD4^+ T cell-depleted spleen cells (CD4^− SC) or CD8^+ T cell-depleted spleen cells (CD8^− SC). The experiment was repeated on three independent occasions. Results are expressed as the mean ± SD of triplicates.

Fig. 5. CD4^+ T cells purified from BALB/c mice made unresponsive to SEB by repeated injection of SAg suppress the responses to SEB stimulation developed by normal BALB/c spleen cells. Cultures containing spleen cells from normal BALB/c (5×10^6 cells/well) or a mixture of normal cells and CD4^+ cells purified from mice having received three injections of SEB (5×10^6 normal cells + 5×10^6 CD4^+ cells from 3×SEB-injected mice) were stimulated for 72 h with SEB. The levels of IL-2, IFN-γ and IL-10 present in the culture supernatants at the end of the culture were determined by ELISA. The experiment was repeated in two independent occasions. Results are expressed as the mean ± SD of triplicates.

devlopment of SAg-reactive cells capable of suppressing SAg-stimulated primary responses.

To determine the phenotype of the suppressor cell population, complement-mediated depletion of CD4^+ and CD8^+ T cells was carried out on spleen cells isolated from mice repeatedly injected with SEB. The depletion of CD4^+ T cells from spleen cells unresponsive to SEB abolished the production of IL-2 by this cell population upon stimulation by control SEA (Fig. 4). This observation was consistent with previous studies indicating that CD4^+ T cells are the main source of IL-2 in primary response to SAg (20). As depicted in Fig. 4, depletion of CD4^+ T cells, but not of CD8^+ T cells, abrogated the suppressive activity of unresponsive spleen cells. Upon stimulation with SEB, high levels of IL-2 and IFN-γ, comparable to those produced in cultures of resting cells alone, were detected in cultures containing the mixture of normal cells and CD4^+ T cell-depleted suppressive cells. To demonstrate that CD4^+ T cells from unresponsive animals were indeed sufficient to promote suppression, CD4^+ T cells purified from unresponsive animals were co-cultured with naive normal spleen cells. Stimulation by SEB did not induce IL-2 nor IFN-γ in these cultures, demonstrating that unrespons-
ive CD4⁺ T cells were alone capable of suppressing the primary response of normal T cells to SAg stimulation (Fig. 5).

Recent reports have shown that regulatory CD4⁺ T cells constitutively express the negative regulator of T cell activation CTLA-4 and that their immune-suppressive function is dependent on signaling via this molecule (10–12). We have analyzed by flow cytometry the expression of CTLA-4 on SAg-induced suppressor T cells, i.e. SEB-reactive CD4⁺ Vβ8 TCR⁺ T cells isolated from BALB/c mice submitted to repetitive injection of SEB. As shown in Fig. 6, a single injection of SEB to normal BALB/c mice specifically up-regulated the expression of CTLA-4 in both CD4⁺ and CD4⁻ Vβ8 TCR⁺ splenic T cells. This effect was not seen in control CD4⁺ Vβ8 TCR⁺ T cells, known not to react with SEB (data not shown). After three injections of SEB, however, CTLA-4 was only found at high levels on a subpopulation of CD4⁺ Vβ8 TCR⁺ T cells (18.3%), whereas it was not seen on CD4⁻ Vβ8 TCR⁺ T cells (0.9%) nor on control CD4⁺ Vβ6 TCR⁺ T cells (1.2%) (Fig. 6 and data not shown).

The suppressive activity of spleen cells isolated from mice made unresponsive to SAg can be adoptively transferred to naive recipients and is mediated by IL-10

Spleen cells isolated from BALB/c mice made unresponsive to SEB were adoptively transferred to unprimed syngeneic recipients. Twenty-four hours later, the recipients were stimulated by a single injection of SEB and serum levels of IFN-γ and IL-2 were determined by ELISA. As seen in Fig. 7, the adoptive transfer of SEB-unresponsive spleen cells suppressed the secretion of IFN-γ and IL-2 following SEB priming of the recipients. This was not observed in control animals not receiving cell transfer or injected with normal spleen cells.

Because in our experiments IL-10 production persisted after repeated injection of SAg (see above), we tested whether SAg-mediated suppression was dependent on IL-10 activity. Recipients of adoptive cell transfer were treated with neutralizing anti-mouse IL-10 antibodies. As seen in Fig. 7, this treatment abolished the suppression of IL-2 and IFN-γ responses observed after adoptive transfer of SEB-unresponsive spleen cells and both lymphokines were detected in the serum of SEB-stimulated recipients at levels similar to those observed in control mice.

Unresponsiveness induced by repeated injection of SAg is not IL-10 dependent

In view of the above results, we wished to determine whether the state of unresponsiveness induced by repetitive injection of SAg could be the direct result of IL-10-mediated suppression. BALB/c mice were made unresponsive by repeated injection of SEB and their response to further SEB challenge in the presence of neutralizing anti-IL-10 antibodies was then analyzed (Fig. 8). In normal control mice, the neutralization of IL-10 increased the levels of IFN-γ following stimulation by SEB. In unresponsive animals, however, the inhibition of IL-10 activity did not restore the secretion of both IL-2 and IFN-γ.

Conclusion

The data presented in this paper demonstrate that (i) in addition to clonal deletion and functional anergy, active T cell suppression by SAg-reactive CD4⁺ T cells develops after bacterial SAg exposure, (ii) this suppression can be adoptively transferred to naive recipients and (iii) is dependent of IL-10 secretion. The important implication of these findings is the negative effect that the development of SAg-specific suppressor T cells might have on the generation of T cell responses to bacterial antigens. Presumably, during infection, the same APC can simultaneously present bacterial epitopes and SAg to T cells. Therefore, the possibility exists that individuals chronically exposed to bacterial infection develop SAg-driven linked suppression capable of disabling T cells specific to bacteria antigens and whose TCR does not express Vβ fragments bound by SAg.

Precisely how T cells do transfer their state of unresponsiveness to naive T cells is not clear. However, the contribution of regulatory cytokines, such as IL-10 and TGF-β, to this process has been demonstrated in several studies (13–16). In our present report, IL-10 was shown to be required for the
induced CD4
conclusions can be drawn from these observations. (i) SAg-
result from failing to produce IL-10 (Fig. 3). Thus, important model of adoptive transfer of unresponsiveness, anergic
present in the spleen of SEB-treated BALB/c mice did not
of suppression of SEA-reactive T cells by regulatory cell tion of naive antigen-speci
not suf
(ii) There appears to be one or more unknown components other than IL-10 required for suppression. Importantly, our observation that IL-10 is secreted in cultures depleted of regulatory CD4+ T cells (Fig. 4) also shows that regulatory CD4+ T cells are not the only
source of IL-10. This observation is, however, in direct contrast to the report previously made by Sundstedt et al. who observed that, after repeated injection of SEA in C57BL/6 mice, CD4+ T cells were the only source of IL-10 (25). One possible explanation for this discrepancy is the different strain of mouse used in our study. Indeed, in BALB/c mice, CD8+ T cells have been shown by several investigators to be an important source of IL-10 following repeated SEB stimulation (29–31).

We also observed that, in mice made unresponsive by repeated exposure to SAg, neutralization of IL-10 did not restore the secretion of IL-2 and IFN-γ in mice made unresponsive by repeated injection of SAg. BALB/c mice injected 3 times with SEB and uninjected controls were challenged with SEB in the presence (open column) or absence (filled column) of anti-IL-10 antibodies. Three hours after SEB challenge, mice were bled and serum levels of IL-2 and IFN-γ were determined by ELISA. Data presented are representative of two independent experiments including three individual mice. They are expressed as the mean ± SD of individual determinations.

![Fig. 7](image)

**Fig. 7.** Adoptive transfer of spleen cells from BALB/c mice receiving repeated injection of SEB to naive BALB/c recipients suppresses IL-2 and IFN-γ responses that follow injection of SEB. (Left panels) Spleen cells from normal BALB/c mice or BALB/c mice injected 3 times with SEB were isolated. The cells were then injected to normal BALB/c recipients (n = 5 per group). A control group included mice not receiving cell transfer. After SEB challenge, serum levels of IL-2 (a) and IFN-γ (b) were determined by ELISA. (Right panels) Spleen cells from BALB/c mice injected 3 times with SEB were adoptively transferred to normal BALB/c recipients or to BALB/c recipients treated with anti-mouse IL-10 neutralizing antibodies. After challenge with SEB, recipient serum levels of IL-2 (a) and IFN-γ (b) were determined by ELISA. Data presented are representative of two independent experiments including four individual mice in each experimental group. They are expressed as the mean ± SD of individual determinations.

![Fig. 8](image)

**Fig. 8.** Neutralization of IL-10 activity does not restore secretion of IL-2 and IFN-γ in mice made unresponsive by repeated injection of SAg. BALB/c mice injected 3 times with SEB and uninjected controls were challenged with SEB in the presence (open column) or absence (filled column) of anti-IL-10 antibodies. Three hours after SEB challenge, mice were bled and serum levels of IL-2 and IFN-γ were determined by ELISA. Data presented are representative of two independent experiments including three individual mice. They are expressed as the mean ± SD of individual determinations.
subpopulation of unresponsive SEB-reactive T cells suggests that T \text sub cells and anergic T cells are different. Whether CTLA-4 is necessary for SAg-mediated suppression is currently under investigation.

Our study was based on the initial observation that C57BL/6 mice made unresponsive to SEA could neither synthesized IL-2 nor IFN-\( \gamma \) upon stimulation with SEB, as if SEA non-responsive cells had been able to suppress SEB-reactive T cells. Such suppression, however, was not observed in BALB/c mice. In the mouse, both SEA and SEB can stimulate T cells expressing the \( V_3^\beta \) TCR fragment, and possibly \( V_{11}^\beta \) TCR fragments (34–36). In addition, SEA binds to mouse \( V_3^\beta, V_{10}^\beta \) and \( V_{12}^\beta \) fragments, while SEB binds \( V_3^\beta, V_{8.1}^\beta, V_{8.2}^\beta \) and \( V_{8.3}^\beta \) fragments (34–36). Therefore, it is possible that, in C57BL/6 mice made unresponsive to SEA, \( V_3^\beta \) and \( V_{11}^\beta \) T cells are capable of suppressing SEB-reactive \( V_3^\beta, V_{8.1}^\beta, V_{8.2}^\beta \) and \( V_{8.3}^\beta \) T cells upon stimulation by SEB. In BALB/c mice, however, \( V_3^\beta \) and \( V_{11}^\beta \) T cells have been deleted early in life from the periphery due to thymic expression of endogenous retroviral superantigen, Mtv-6, -8 and -9, respectively (37,38). Thus, transfer of unresponsiveness from SEB-specific T cells could not efficiently operate following SEA challenge because T cell repertoire stimulated by SEA and SEB in BALB/c mice do not overlap.

In our model of SAg-mediated transfer of unresponsiveness, tolerant and naive T cells are both stimulated by SAg–MHC class II molecule complexes present on the same APC. This raises the possibility that tolerant T cells could regulate the stimulatory function of APC, making them improper for stimulating responses involving the production of IL-2 and IFN-\( \gamma \). TGF-\( \beta \) has been shown to induce IL-10 secretion by APC, raising the possibility that lymphokines produced by tolerant T cells might alter the stimulating capabilities of APC (39). The observation by Maldonado-Lopez et al. that antigen-pulsed CD8\( ^{\text{+}} \)-dendritic cells are potent stimulators of IL-10 production by T cells but do not support T\( _{1,1} \) responses demonstrates the existence of APC with specific stimulatory capacities (40). Whether this is the result of interaction with T\( _{1} \) cells remains to be determined.  

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Abbreviations

\textit{APC} antigen-presenting cell  
\textit{SAg} superantigen  
\textit{SE} staphylococcal enterotoxin  
\textit{TGF} transforming growth factor  
\textit{TNF} tumor necrosis factor  
\( T_{1,1} \) T regulatory 1

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