Staphylococcal Enterotoxin B Induces an Early and Transient State of Immunosuppression Characterized by Vβ-Unrestricted T Cell Unresponsiveness and Defective Antigen-Presenting Cell Functions[1,2]

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Staphylococcal enterotoxin B (SEB) is a bacterial enterotoxin able to simultaneously bind to class II molecules on APCs and to selected Vβ regions (including Vβ8) of the TCR complex. Administration of SEB to adult BALB/c mice results in clonal activation of T cells bearing Vβ8 receptors, leading to an excessive release of proinflammatory cytokines. This initial immune response is followed by a long-lasting state of Vβ8-specific unresponsiveness, thought to benefit both the host (as it contributes to the down-regulation of the inflammatory response) and the bacterium (through ligand-specific T cell anergy). However, it is not clear how this type of restricted unresponsiveness can effectively impair the generation of an antibacterial response. To gain insight into the mechanism by which Gram-positive bacteria subvert the host immune response, we have investigated the immune competence of SEB-treated mice 48 h following SEB administration. We demonstrate in this report that in vivo, SEB induces a transient but profound state of unresponsiveness affecting both T and Ag-presenting cell functions. Although in vivo activation by SEB appears to be Vβ-restricted under our experimental conditions, SEB-treated mice displayed an early (lasting 48 to 72 h postinjection) and Vβ-unrestricted unresponsive state characterized by the inability to produce IL-2 in response to polyclonal TCR mitogens including third party bacterial superantigens (staphylococcal enterotoxin A and toxic shock syndrome toxin 1, SEA and TSST-1, respectively), Abs to non-SEB reactive Vβ regions (Vβ6), anti-CD3ε Abs, and a lectin (Con A). Spleen cell populations from SEB-treated mice also displayed defective APC functions, possibly related to a selective decrease in splenic dendritic cells numbers. Taken together, these observations indicate that SEB induces an early and transient state of immunodeficiency in vivo, representing a potential mechanism for escaping host immune surveillence. *The Journal of Immunology,* **1997, 158: 2638–2647.**

Bacterial enterotoxins comprise a large group of proteins produced by several bacterial strains, including *Staphylococcus aureus* (SA),[4] that have long been recognized as pathogenic both in humans and animals (1). There is compelling evidence that the pathophysiology associated with SA infections is related to the ability of these enterotoxins to polyclonally activate T cells in vivo, initiating a cascade of events leading to excessive production of proinflammatory cytokines and septic shock (2). The mitogenic properties of these compounds (named superantigens, SAgs) are linked to their ability to interact simultaneously with selected Vβ regions of the clonally distributed TCR and with a conserved domain of the class II molecule (MHC II), in an unprocessed fashion. Because the relative number of Vβ genes is limited in both mouse and humans, a given SAg is capable of interacting with a large fraction (5–30%) of the TCR αβ expressing T cells, causing polyclonal in vivo and in vitro immune stimulation. Remarkably, several different microorganisms (3) produce proteins with superantigenic properties, although these proteins are not necessarily closely related to each other in sequence. Moreover, the same toxins bind to MHC II and to Vβ in mammals as diverse as human (4), nonhuman primates (5), rats (6) and mice (7), in spite of weak sequence homology.

The expression of highly immunogenic proteins by several bacterial strains is surprising, as it is important for parasites to devise strategies allowing them to escape immune surveillance. As previously suggested (3), it is possible that the local inflammatory response induced by SAgs favors bacterial growth through increased blood supply and nutrients. Another well studied property of SAg that might affect host responses in favor of the bacterium is the induction of immune unresponsiveness. Staphylococcal enterotoxin B (SEB) in BALB/c mice reacts with murine T cells expressing members of the Vβ8 gene family (8). Following in vivo administration of SEB, Vβ8-positive T cells produce cytokines and undergo clonal expansion between days 1 and 3, followed by apoptotic death of up to 50% of the responding population (9). The remaining $V\beta8^+$ cells have been shown to be unresponsive to

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[4] Abbreviations used in this paper: SA, *Staphyolcoccus aureus*; SEA, staphylococcal enterotoxin A; SEB, staphyloccocal enterotoxin B; SAg, superantigen; DC, dendritic cells; TSST-1, toxic shock syndrome toxin 1; PBS-BR, PBS containing 0.5% blocking reagent.

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further TCR stimulation by SEB or anti-TCR complex Abs, while retaining responsiveness to pharmacologic agents bypassing the early steps of receptor signaling (10). SEB induces therefore a Vβ subset-specific functional unresponsiveness (termed anergy) that appears to be due to a membrane proximal defect in the TCR signaling pathway. It is however not clear how this type of restricted unresponsiveness can be beneficial to the pathogen, as the functional deletion (by apoptosis/anergy) of a restricted number of Vβ-bearing T cells is not likely to affect the host response to most pathogen-born Ags. Indeed, there is no compelling evidence indicating that mice expressing a limited set of Vβ receptors (due to Mls-like neonatal negative selection or genetic deletion) are immunocompromised (11).

A more relevant finding is provided by early studies suggesting that SEB induces in vivo a state of profound immunosuppression affecting both humoral and cellular responses to complex Ags such as SRBC or allo-MHC (12). In the present study, we have performed a detailed kinetic analysis to evaluate the immunocompetence status of splenic lymphocytes isolated from SEB-treated mice. Our data demonstrate that injection of single SEB dose induced a short-term but profound state of immunosuppression characterized by the inability of peripheral T cells to respond to all T cell mitogens tested, followed by a long lasting SEB-specific hyporeactivity. SAg-treated mice also displayed an APC defect, probably related to a reduction in splenic dendritic cell (DC) numbers. Thus, in addition to the well described Vβ-specific unresponsiveness, SEB appears to induce an early and transient generalized immunosuppressive status, affecting the function of both T and APCs.

Materials and Methods

Animals and reagents

Female BALB/c (H-2^d), 6 to 8 wk old, were purchased from Charles River Wiga (Sulzfeld, Germany), and maintained in our own pathogen-free facility. Staphylococcal enterotoxin A (SEA), SEB, and toxic shock syndrome toxin 1 (TSST-1) (highly purified form) were purchased from Toxin Technology, Inc., (Sarasota, FL) and solubilized in pyrogen-free NaCl 0.9%. Con A, calcium ionophore A23187, and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium ionophore was dissolved in DMSO at the concentration of 1 mg/ml. PMA was dissolved in anhydrous ethanol at a concentration of 10 μg/ml. These reagents had no effect on IL-2 determination (not shown).

In vivo treatment

BALB/c mice were injected intravenously into the lateral tail vein with SAg solubilized in pyrogen-free NaCl 0.9%. Control animals were injected with the same volume of diluent. Serum IL-2 content was determined by ELISA, using the rat anti-mIL-2 mAb S4B6 (13) as capture reagent and a rabbit anti-mIL-2 serum (produced in our laboratory) as revealing reagent.

In vitro responses

The complete medium used in all experiments was RPMI 1640 (Seromed Biochem KG, Berlin, Germany) supplemented with 2% HY ultroser (a serum-free media purchased from Life Technologies, Merelbeke, Belgium), penicillin, streptomycin, nonessential amino acids, sodium pyruvate, 2-ME, and L-glutamine (Flow ICN Biomedicals, Bucks, U.K.). An adequate number of spleen cells (see *Results* for cell numbers) was stimulated with graded doses of γ-irradiated (3000 rad) allogeneic spleen cells, lectins, mAbs to CD3ε (hamster mAb 145–2C11; Ref. 14), or SAgs in a total volume of 0.2 ml in 96-well U-bottom plates. Spleen cell suspensions were depleted of adherent cells by passage over Sephadex G10 (Pharmacia Bioprocess, Uppsala, Sweden) columns as described (15). Splenic T cells were purified following incubation of spleen cell suspensions with mouse Ig-specific, biotin-coupled, rabbit Ig (produced in our laboratory). SIg^+ cells were negatively removed using a Magnetic Cell Sorter (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer recommendations. The resulting population contained <1% Ig^+ cells and comprised between 75 and 80% $CD3^+$ cells. APC were obtained following complement-mediated lysis of anti-Thy-1.2 (clone HO-13-4A, available through the American Type Culture Collection (ATCC), Bethesda, MD) labeled spleen cells and comprised <1% $CD3^+$ cells. Cultures were maintained at 37°C in a humidified incubator (7% CO_2). Supernatants were collected after 24 or 48 h culture, frozen, and assayed for IL-2 content by a bioassay using a subclone of the CTL.L cell line insensitive to murine IL-4 (16). Data represent the mean of triplicate determinations. SD never exceeded 15% and were omitted for simplicity.

Purification of low density spleen cells

Spleens were digested with collagenase (CLSIII; Worthington Biochemical Corp., Freehold, NJ) and separated into low and high density fractions on a BSA gradient (Bovuminar Cohn fraction V powder; Armour Pharmaceutical Co., Tarrytown, NY), according to a procedure described in Reference 18.

Cytofluorometric analysis

Cells were analyzed by flow cytometry with a FACScan cytometer (Becton Dickinson, Mountain View, CA). The cells were preincubated with 2.4G2 (a rat anti-mouse Fc receptor mAb) for 10 min before staining to prevent Ab binding to FcR and then further labeled with the following Abs to murine determinants: 14.4.4-S (murine IgG2a anti-$I\text{-}E^{k,d}$, available through the ATCC) and N418 (hamster mAb, anti-CD11c; see Ref. 18). These Abs were purified from ascitic fluids and coupled to FITC or biotin in our laboratory according to standard procedures. Cells were gated according to size and scatter to eliminate dead cells and debris from analysis.

Immunohistochemistry

Tissues samples were frozen in isopentane and cryostat sections of 12 μm were prepared and stored at −80°C. Samples were fixed in neat acetone for 10 min, air-dried, and incubated in PBS containing 0.5% blocking reagent (PBS-BR; Boehringer Mannheim, Brussels, Belgium) for 30 min. Sections were incubated for 20 min with culture supernatant of the mAb rat 2.4G2 anti-mouse Fc receptors to prevent nonspecific staining. Slides were washed in PBS, incubated for 1 h at room temperature with biotinylated mAbs (10 μg/ml in PBS-BR) and washed in PBS. Slides were then incubated in a 1:100 dilution of avidin-biotin-peroxidase complex (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) in PBS-BR for 30 min and washed in PBS. The peroxidase activity was revealed with a solution of diaminobenzidine tetrahydrochloride (DAB tablets, SigmaFAST). The sections were stained for 10 min at room temperature, counterstained with hematoxylin and mounted with Poly-mount (Polysciences, Inc., Warrington, PA).

Results

In vivo administration of SEB induces a transient state of spleen cell unresponsiveness to heterologous T cell mitogens

Recently, a kinetic analysis of the in vitro reactivity of splenic T cells from thymectomized BALB/c mice that had been injected with SEB 10 days to 4 mo before, has demonstrated that Vβ8 anergy lasts for a long period of time (>2 mo) and is reversible (19). However, no attempts have been made to date to fully characterize the immunocompetence of SEB-treated mice during the early phases of the response preceeding the establishment of the Vβ-selective unresponsiveness. For this purpose, adult BALB/c mice were injected with 50 μg SEB and the in vitro response of spleen cells to SEA, SEB, anti-CD3ε Abs, and Con A was assayed 2, 4, and 15 days later. As shown in Figure 1, spleen cells from mice injected 2 days earlier with SEB were not only unable to produce IL-2 in response to SEB, but also to a third party SAg, SEA (known to interact predominantly with Vβ1 in BALB/c mice; see Ref. 20), and to non-Vβ-restricted T cell mitogens such as anti-CD3ε Abs or Con A. This generalized unresponsive state lasted for at least 4 days following treatment. When SEB-treated spleen cells were examined 15 days later, they had partially recovered the ability to produce IL-2 when stimulated by SEA and anti-CD3ε Abs, while displaying a marked reduction in the SEB response. Indeed, and in agreement with previous studies (21), stimulation of cells from SEB-treated mice required a 50-fold higher SEB concentration than stimulation of primary T cells. When graded doses of enterotoxin were injected in vivo, induction

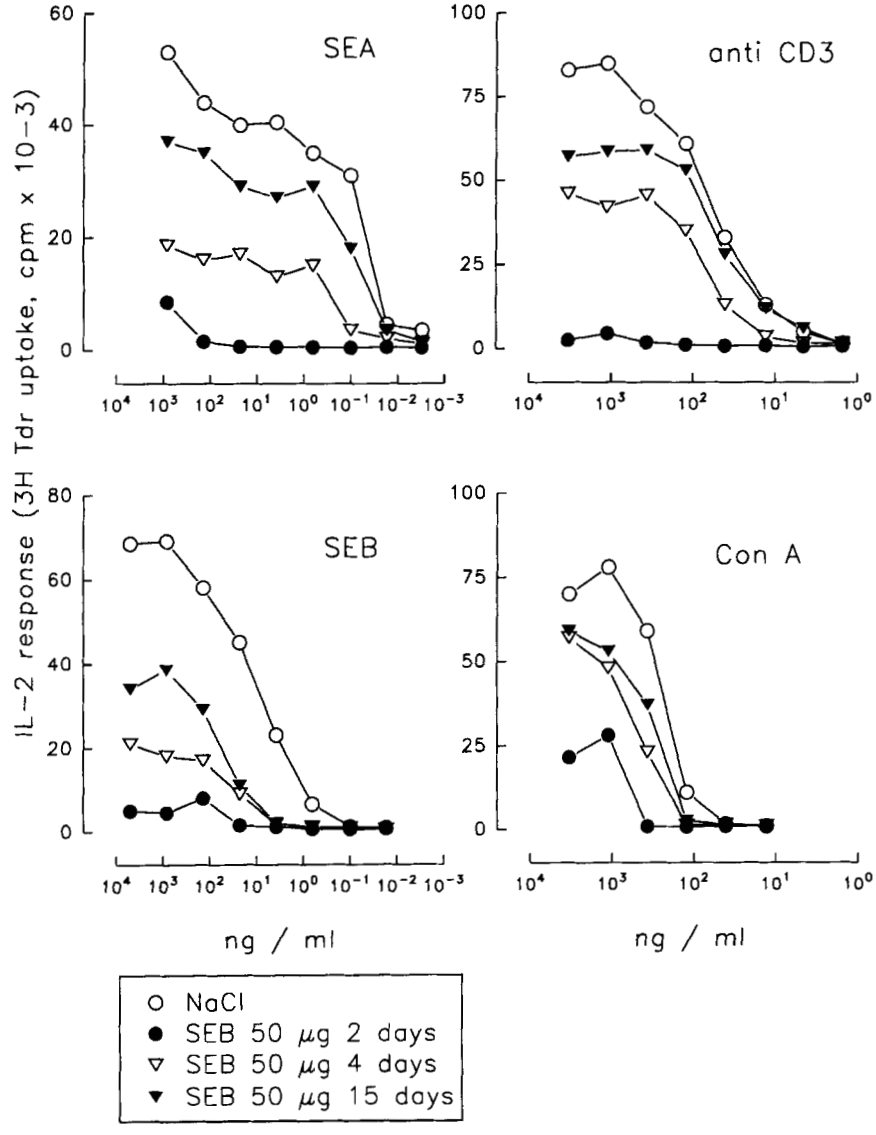

FIGURE 1. Effects of SEB administration on mitogen-induced in vitro IL-2 response. BALB/c mice were injected with 50 μg SEB i.v. and their immunocompetence tested in vitro 2, 4, and 15 days later. Unfractionated splenic cells (5×10^5/well) were cultured in microtiter plates with graded doses of SEA, SEB, anti-CD3ε, or Con A, and IL-2 production assayed as described in *Materials and Methods*. Results are expressed as cpm of [^{3}H]thymidine incorporation (mean of triplicate wells) by the IL-2-dependent cell line CTL.L. These results are representative of more than 20 independent experiments.

of immunosuppression (i.e., inhibition of IL-2 production in response to both SEA, SEB, Con A, and anti-CD3) was observed in mice injected with as low as 0.1 μg SEB, a dose that does not induce detectable systemic cytokine production nor lethality in D-galactosamine sensitized mice (our unpublished observations).

To verify whether SEB induced a similar unresponsive state in vivo, SEB-primed mice were challenged with SEB or third party SAgs (SEA and TSST-1; see Refs. 20 and 22, respectively) known to interact with nonoverlapping sets of Vβ families, and the systemic release of IL-2 was assayed in the serum as previously described. Figure 2 demonstrates that in addition to a marked SEB-specific unresponsiveness, SEB-primed mice displayed a reduced in vivo response to both SEA and TSST-1 in agreement with the in vitro data presented above.

In vivo administration of SEB induces Vβ-unrestricted, T cell unresponsiveness

As previously described (23), administration of SEB induced a Vβ8-specific blastogenesis and IL-2R expression at day 2 post-treatment, while Vβ6-expressing cells (a subset of peripheral T cells unresponsive to SEB; see Ref. 8) remained unaffected (data not shown). In spite of this Vβ8-specific in vivo early effects, purified T cells from SEB-primed animals, were equally unresponsive to plastic-coated anti-Vβ6 and anti-Vβ8 Abs, as shown in Figure 3. These observations suggest that induction of the early in vitro unresponsiveness does not necessarily unfold from in vivo clonal activation.

To clearly establish that SEB induced a T cell-specific unresponsive state, the response of purified T cells from animals primed 2 days earlier with SEB was tested in vitro using T cell-depleted spleen cells from naive, untreated animals as APCs. Figure 4 demonstrates that purified T cells from SEB-primed animals failed to respond to all mitogens tested (SEA, SEB, and anti-CD3ε Abs) when stimulated in the presence of naive APCs, while retaining the ability to respond to pharmacologic agents bypassing receptor signaling. Note that Vβ-unrestricted unresponsiveness is not maintained through active suppression, as shown by cell mixing experiments (note cell number in the legend of Fig. 4). These data clearly demonstrate that failure to respond to T cell mitogens was not simply a consequence of defective Ag presentation.

SEB administration induces an APC defect

The above-mentioned results demonstrated that SEB induces in vivo a generalized T cell defect, but did not preclude the possibility that APC functions were also affected by this enterotoxin. To directly evaluate the APC functions of SEB-treated animals, mice

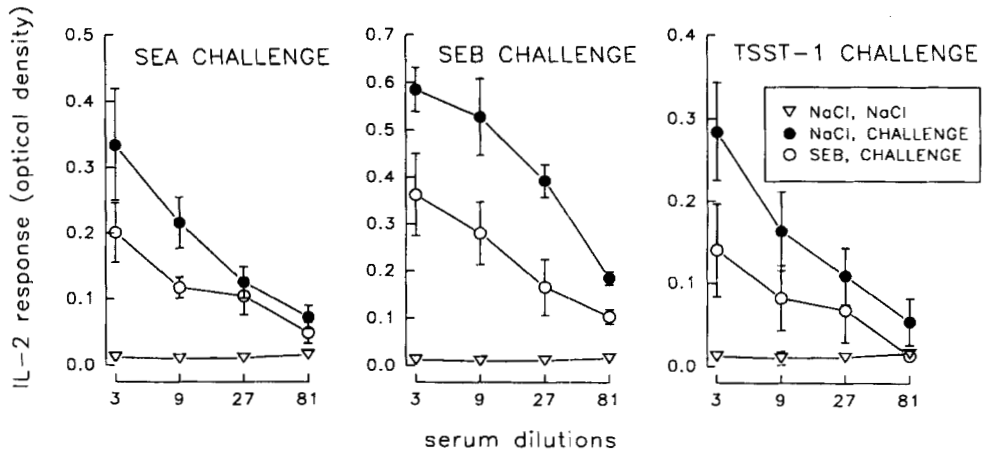

FIGURE 2. SEB induced Vβ-unrestricted in vivo unresponsiveness. Animals (six per group) were pretreated with pyrogen-free NaCl or 50 μg SEB i.v. 2 days before challenge with 50 μg of SEA, SEB, or TSST-1. Mice were bled 2 h later and the serum IL-2 activity tested by ELISA. Results are expressed as the mean absorbance (492 nm) ± SD of individual determinations. These results are representative of three independent experiments.

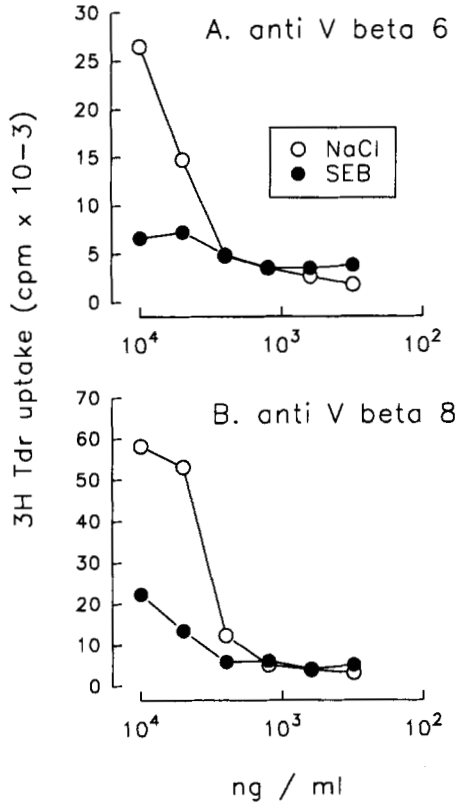

FIGURE 3. Induction of T cell unresponsiveness in SEB nonreactive Vβ6$^+$. Mice were treated with pyrogen-free NaCl or 50 μg SEB i.v. Pooled splenic T cells from three mice were stimulated (5×10^5/well) by graded concentration of anti-Vβ6 (*A*) and anti-Vβ8 (*B*) plastic-coated mAb. Culture supernatants were harvested following 24 h of culture and assayed for IL-2 content by bioassay. Results are expressed as cpm of [^{3}H]thymidine incorporation by the IL-2 dependent cell line CTLL. These results are representative of three independent experiments.

were injected with 50 μg SEB, and their spleen cells used as stimulators in an in vitro MLR. As shown in Figure 5, spleen cells from animals injected 2 days earlier with SEB failed to stimulate an alloreactive in vitro response. Again, this accessory cell defect appeared to be transient, since cells had recovered their immunostimulatory properties 2 wk following SAg administration (Fig. 5*A*). Cell-mixing experiments failed to demonstrate any active suppression of the Ag presenting functions of control spleen cells by spleen cells recovered from SEB-treated animals (Fig. 5*A*). A reduction in accessory cell function (three-fold reduction on a per cell basis) was also observed when purified low density spleen cells from SEB-treated were used as APC (Fig. 5*B*).

Down-regulation of DC numbers following in vivo SEB response

Because DC represent the most potent APC subset within the splenic population (24), we wished to evaluate whether SEB treatment affected splenic DC numbers and phenotype. Surprisingly, spleen cells recovered from animals injected with SEB displayed a marked reduction in the relative number of DC (identified as MHC class II$^+$, CD11c$^+$ cells in Fig. 6). Immunofluorescence studies on DC-enriched, low density spleen cells similarly indicated that SEB-injected mice displayed a reduction in DC relative numbers (corresponding to a loss of ~60% of cells compared with untreated mice). The numbers of DC returned to near control levels 15 days after SAg administration (Fig. 6). Table I demonstrates that the absolute number of splenic DC is also affected by SEB treatment. The data obtained by flow cytometry of splenic low density cells indicate that in spite of an increase in spleen cellularity secondary to SEB injection, the absolute and relative number of CD11c^{high} splenic cells were significantly decreased upon SAg-treatment. To further demonstrate that SEB induced in vivo a reduction in DC numbers rather than down-modulation of a given marker, two-color fluorographic analyses were performed to score the cellular composition of DC-enriched, low density cells, recovered from control and SEB-treated mice. The data detailed in Table II show that SEB induced in vivo a significant loss in the DC population identified as CD11c^{high}, I-E$^+$; CD11c^{high}, CD11b^{low}; CD11c^{high}, CD24$^+$, or I-E$^+$, CD45R$^-$ cells, from 75 to 80% reduction when compared with control cells. Note that no significant reduction in splenic B cells (not shown) and cells expressing a typical macrophage phenotype (see Table II, group A; CD11b^{high}CD11c^{low} cells) was observed 48 h following SEB administration. Finally, DC, identified as CD11c$^+$ cells, were visualized in cryosections from control and SEB-treated spleens. As expected in untreated animals, DC were mostly detected in the inner periarteriolar sheath

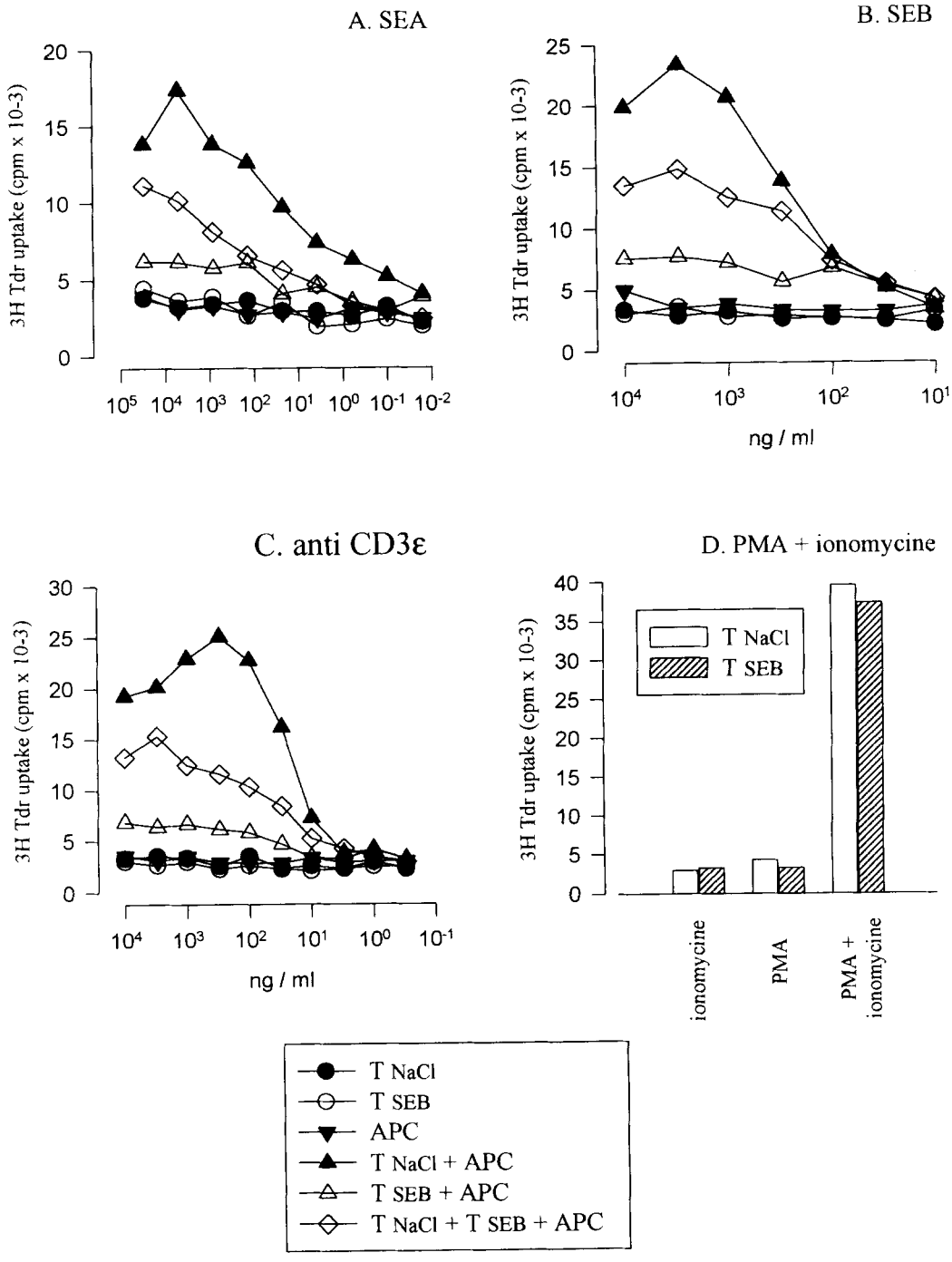

FIGURE 4. Failure of naive APC to restore SEB-induced T cell unresponsiveness. T cells (3×10^5/wells) from pyrogen-free NaCl or 50 μg SEB-treated BALB/c mice were stimulated as indicated in the insert for 24 h in presence of naive APC (5×10^5) and graded doses of SEA (*A*), SEB (*B*), anti-CD3ε (*C*), or a combination of PMA (10 ng/ml) and ionomycin (60 ng/ml) (*D*). Mixing experiments were performed by adding an equal number of purified T cells from SEB and NaCl-treated mice (1.5×10^5 of each cell population) to APC-containing wells. Culture supernatants were harvested following 24 h of culture and assayed for IL-2 content by bioassay. Results are expressed as cpm of [^{3}H]thymidine incorporation by the IL-2 dependent cell line CTL.L. These results are representative of three independent experiments.

(PALS) and in the marginal zone (see Fig. 7*A*). In keeping with flow cytometry analyses, cryosection studies indicated that injection of SEB led to a significant reduction in the number of CD11c$^+$ cells in both DC-rich areas (see Fig. 7*B*).

Discussion

The response to SEB is characterized by an early activation phase that includes cytokine production and clonal expansion, followed by T cell unresponsiveness mediated by both peripheral cell apoptosis (9) and induction of anergy (19). Most studies have led to the conclusion that both the early T cell response and the induction of peripheral tolerance are restricted to T lymphocytes expressing SEB-reactive Vβs, such as Vβ8 in BALB/c mice (8).

To gain better understanding of the mechanisms by which SAgs subvert the host immune response, we have performed a kinetic study of the immunocompetence status of splenocytes recovered from SEB-injected adult mice. IL-2 production has been used as a

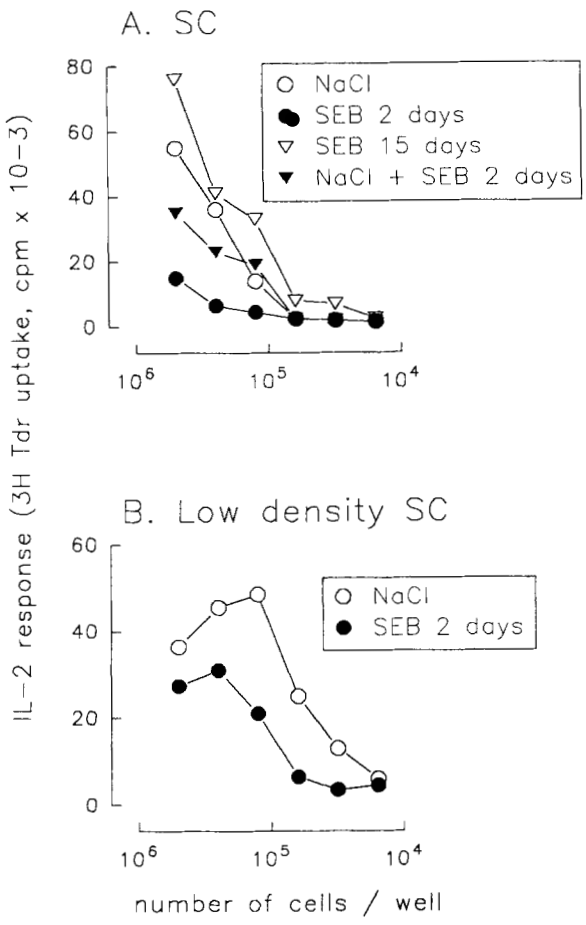

FIGURE 5. Induction of an APC defect by SEB administration. Various number of unfractionated spleen cells (*A*) or purified low density cells (*B*) from control, pyrogen-free NaCl or SEB-treated mice were irradiated and used as accessory cells to stimulate G10-nonadherent responding cells from CBA mice (3×10^5/well). Culture supernatants were harvested following 48 h of culture and assayed for IL-2 content by bioassay. Results are expressed as cpm of [^{3}H]thymidine incorporation by the IL-2 dependent cell line CTLL. These results are representative of three independent experiments.

gauge in this study, since it allows a direct comparison between in vitro and in vivo responses. T cell mitogens used in the in vitro secondary responses included a broad range of TCR agonists such as SEB, a third party SAg SEA (known to interact with nonoverlapping sets of Vβs), anti-CD3ϵ Abs, and a lectin (Con A). When splenic lymphocytes were assayed 2 wk following in vivo treatment, a selective deficiency in the SEB response was observed (see Fig. 1). In marked contrast, splenocytes recovered 48 h after SEB-administration failed to respond to all T cell mitogens tested (Fig. 1). Similarly, although in vivo administration of SEB under our experimental conditions appears to selectively activate Vβ8-expressing cells (not shown), in vitro unresponsiveness affected both Vβ6$^+$ and Vβ8$^+$ cells in response to plastic-absorbed anti-TCRβ Abs (see Fig. 3). Note that in all in vitro experiments reduced IL-2 production corresponded to a defective proliferative response (not shown).

Cells from SEB treated mice respond to pharmacologic agents bypassing the early steps of signal transduction, indicating that they had retained an adequate enzymatic machinery for IL-2 production (see Fig. 4*D*). This form of generalized unresponsiveness was also observed in vivo, as depicted in Figure 2. Because failure to produce IL-2 could not be overcome by addition of naive APC (see Fig. 5), we conclude from this work that administration of SEB leads to an early and transient phase of generalized, non-Vβ-restricted T cell unresponsiveness, followed by a state of long term, Vβ-specific anergy.

Although the molecular basis for this generalized unresponsiveness has not been addressed in this study, two hypotheses can be considered to explain these observations. It is conceivable that activation of Vβ8-expressing lymphocytes by SEB leads to the production of a factor/cytokine able to down-regulate the immune responsiveness of T cells bearing non-SEB reactive TCRs (the "innocent bystander" hypothesis). This soluble mediator could be directly produced by activated, Vβ8$^+$ T cells and may represent a previously identified cytokine with known suppressive activity (IL-4, IL-10, TGF-β, and others) or be indirectly induced following the excessive in vivo inflammatory response, as described for endogenous steroids (25). Note however that production of endogenous glucocorticoids is generally characterized by a rapid onset and decay, with no detectable biologic activity at 48 h following injection (25). The production of APC-derived, suppressive factors cannot be excluded, as multimeric binding of SAgs to APC-expressed cell surface molecules (MHC encoded or not; see Refs. 26 and 27, respectively) could in principle lead to the secretion of immunomodulatory molecules.

Alternatively, unresponsiveness may reflect the ability of SEB to interact with multiple Vβs, with distinct affinities. A low affinity interaction between SEB and non-Vβ8 TCRs may not be sufficient to induce a detectable in vivo response, but may lead to clonal unresponsiveness (the "SEB-dependent, sterile T cell activation" hypothesis, formerly put forward by H. Liu et al., Ref. 28). The observation that injection of low doses of SEB (0.1 μg, data not shown) that do not appear to induce the systemic release of cytokines is sufficient to induced a generalized form of unresponsiveness, is in favor of the latter hypothesis.

The identification of the mechanisms responsible for this novel form of immunodeficiency has been hampered by the finding that SEB administration also affects APC functions. It is clear from the in vitro analysis that spleen cells recovered 2 days following SEB administration display a reduced ability to present allo-MHC determinants to naive T cells. The flow cytometric analysis of spleen cell populations revealed that neither B cells (identified as CD45R$^+$ cells) nor macrophages (identified as CD11b^{high} cells) were significantly affected by SEB injection. In contrast, a selective deficiency in splenic DC (identified as CD11c^{high} cells) numbers was consistently observed. This finding was further supported by an histochemical study that revealed a change in splenic DC numbers/distribution following SEB administration. Note that a similar reduction in splenic DC numbers was observed following SEB injection in LPS responsive (C3H/HeN) and unresponsive mice (C3H/HeJ), arguing against a role for contaminating endotoxins in this in vivo model (data not shown). DC recovered from SEB-treated mice expressed both I-E, CD24, CD54, CD80 and CD86 molecules at control levels, indicating that DC that have escaped SEB-mediated in vivo down-modulation displayed a normal phenotype (data not shown). It has been previously demonstrated (29) that bacterial SAgs induce a dose-dependent depletion of Langerhans cell from murine epidermis, thus strengthening the conclusion that bacterial SAgs modulate APC distribution in vivo.

In spite of extensive efforts, we have been unable to identify the mechanism by which SEB affect APC function in vivo. Recent work performed in our laboratory suggests that several soluble

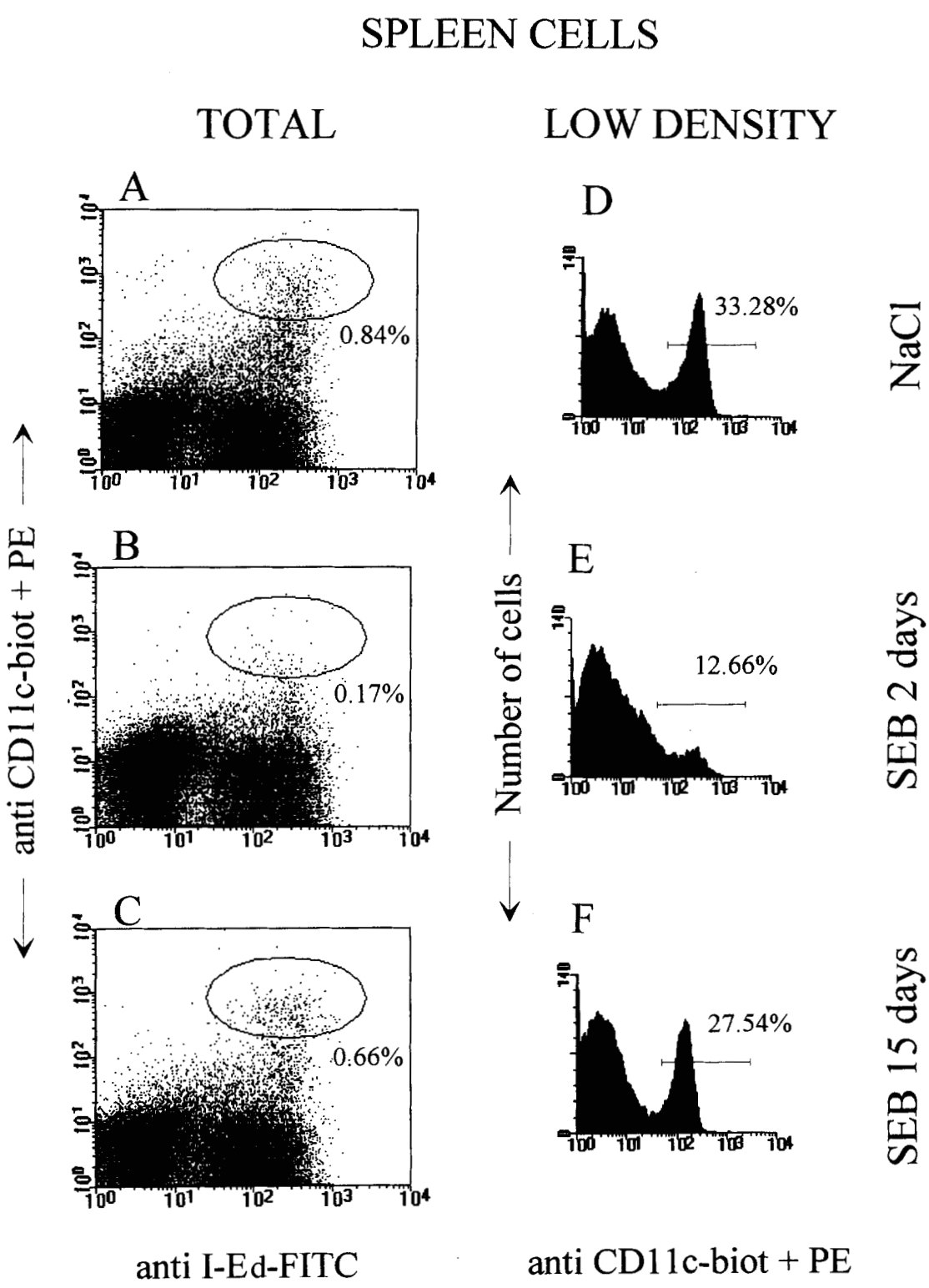

FIGURE 6. Effects of SEB administration on DC. BALB/c mice were treated on day 0 with pyrogen-free NaCl or SEB (50 μg) and analyzed on day 2 or 15 for I-E^d and CD11c expression. Pooled spleen cells from three mice in each group were analyzed by two-color immunofluorescence following staining with FITC-labeled anti-I-E^d and biotin-labeled anti-CD11c and streptavidin-phycoerythrin-avidin (*A-C*). Pooled low density spleen cells were analyzed for CD11c expression following staining with biotin-labeled anti-CD11c and streptavidin-phycoerythrin-avidin (*D-F*).

factors, probably acting in a redundant fashion, are able to modulate in vivo DC numbers/distribution. We have for example recently observed that in vivo administration of glucocorticoids (30), recombinant TNF-α (T. De Smedt et al., data not shown), LPS (31) or anti-CD3ϵ mAbs (E. Muraille et al., manuscript in preparation) lead to a profound inhibition of the APC splenic function and a significant reduction in splenic DC cells numbers. Neither blocking Abs to TNF-α, nor a glucocorticoid-receptor antagonist

Table I. *SEB administration results in a loss of CD11c-expressing cells from the spleen*

	Expt. 1[a]		Expt. 2		Expt. 3	
	NaCl	SEB	NaCl	SEB	NaCl	SEB
Splenocytes[b]	1.1×10^8[c]	1.6×10^8	1.5×10^8	2.1×10^8	1.2×10^8	1.6×10^8
Low density cells[d]	2×10^6	3.2×10^6	1.8×10^6	2.3×10^6	2.4×10^6	3.8×10^6
$CD11c^+$ cells[e]	7.7×10^5 (0.7)[f]	5.3×10^5 (0.3)	9.1×10^5 (0.6)	3.1×10^5 (0.14)	8.6×10^5 (0.7)	4.1×10^5 (0.25)

[a] Mice (three animals in each group) were injected with saline or 50 μg of SEB and spleen cells recovered 48 h later.
[b] Mononuclear cells recovered from collagenase-treated spleen cells.
[c] Number of cells (mean value, $n = 3$).
[d] Low density spleen cells obtained following separation over a BSA gradient.
[e] Number of $CD11c^+$ cells estimated by flow cytometric analysis of low density cells stained with FITC-coupled N418 mAb.
[f] Percentage of $CD11c^+$ cells/spleen cells.

Table II. *Effect of SEB on splenic cell population*

Phenotype	Expt. 1		Expt. 2	
	NaCl	SEB[a]	NaCl	SEB
Group A				
$CD11b^{high}CD11c^{low}$	5.7[b]	6.6	13.9	13.8
$CD11b^{low}CD11c^{high}$	14.7	3.3	10.4	2.2
Group B				
$CD11c^-CD24^+$	74.2	71.6	52.6	48.5
$CD11c^+CD24^+$	14.0	3.5	11.3	2.1
Group C				
$I\text{-}E^{d-}CD45R^-$	32.4	51	49.6	55.3
$I\text{-}E^{d+}CD45R^-$	14.5	3.2	11.2	2.4

[a] Mice were injected with 50 μg of SEB and spleen cells recovered 48 h later.
[b] Percentage of cells expressing a given phenotype among DC-enriched, low density cells, obtained following separation over a BSA gradient of pooled spleen cells from three mice in each group.

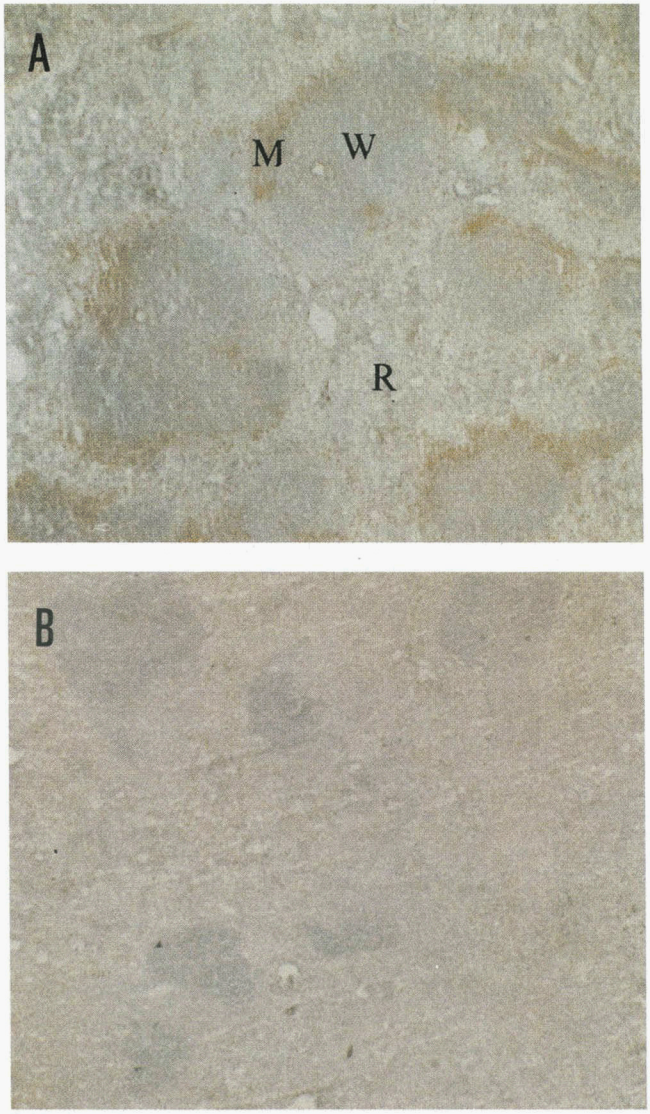

FIGURE 7. Effect of SEB on splenic DC numbers and distribution. Immunoperoxydase labeling of cryostat sections from spleens of control (*A*) and SEB (50 μg)-treated (*B*) BALB/c mice. Spleen sections were stained with anti-CD11c mAb as indicated in *Materials and Methods* and counterstained with hematoxylin. The original magnification was ×10. (M: marginal zone; R: red pulp; W: white pulp)

(RU486) were able to inhibit DC loss in SEB-treated mice (data not shown), suggesting that multiple factors with redundant regulatory properties may be operative in SEB-treated mice. Although these results do not allow a precise identification of the mechanism by which SEB affects APC function in vivo, they provide a novel mechanisms by which SAgs inhibit host responses. As inhibition in Ag presenting capacities associated with a peripheral DC loss has been observed in inflammatory response caused by both T cell (SEB) (this study and Ref. 29) and accessory cell mitogens (LPS) (31), we propose that modulation of APC function represents a natural feedback mechanisms contributing to the down-regulation of excessive in vivo inflammatory responses. Note that no T cell defect was observed in LPS-treated mice, suggesting that APC and T cell dysfunctions may occur independently (T. De Smedt, unpublished observations).

The observations reported in this study suggest that SEB administration in vivo leads to a generalized form of immunodeficiency, a conclusion that does not necessarily contradict previous reports demonstrating a Vβ-specific in vivo anergy in SEB treated mice. Indeed, most studies dealing with the phenomenon of peripheral T cell anergy have been performed 1 to 2 wk after in vivo SEB challenge. In marked contrast, early studies in which mice were treated with SEB at the time of Ag priming, demonstrated that SAgs are able to inhibit the in vivo humoral and cellular response to complex Ags such as SRBC and allo-MHC (12). Similarly, a recent report has demonstrated that mice injected with SEB became resistant to an otherwise lethal challenge with SEB or TSST-1 plus D-galactosamine (32). The authors demonstrated that this "ligand-nonspecific" in vivo resistance became manifest after 4 h and lasted for about 48 h, in striking agreement with the observations described in the present report. Finally, SEB has been recently shown to induce clonal unresponsiveness in T cell clones expressing non-Vβ8 elements such as Vβ2 or Vβ6 (28). The available evidence thus strongly suggests that the previously characterized Vβ-specific T cell anergy unfolds in vivo from a transient

state of generalized immunodeficiency, affecting both APC and T cell functions.

The question is then how this nonselective form of immunodeficiency is related to the state of long term, Vβ-specific T cell unresponsiveness. Anergy is thought to occur as a consequence of TCR stimulation in the absence of the appropriate costimulation normally provided by APC. In spite of its conceptual elegance and the extensive in vitro experimental support, the two-signal theory of anergy induction does not fully account for the in vivo induction of T cell unresponsiveness by SAgs. We and others have recently demonstrated that bacterial SAgs activate T cells in vitro (33, 34) and in vivo (23, 35) in a costimulation-dependent fashion. Attempts to increase costimulatory activity in vivo by injecting B7-up-regulating agents such as goat anti-IgD Abs or LPS or by the administration of anti-CD28 Abs have failed to modify the induction of in vivo T cell dysfunction by bacterial SAgs (36). Bacterial SAgs induce T cell unresponsiveness in B cell-deficient mice, arguing against a role for costimulatory-deficient resting B cells in the induction of T cell anergy (37). Finally, a recent in vitro study has demonstrated that the expression of costimulatory molecules does not preclude the in vitro induction of T cell unresponsiveness by SEB (38). Thus, informations acquired in recent years concerning the induction of T cell anergy in established Th1 clones, do not appear to provide a satisfactory explanation for the phenomenon of SAg-induced T cell unresponsiveness in vivo. A careful examination of the primary changes (cell surface phenotype and signal transduction capacities) occurring in unresponsive T cells in the early (Vβ-nonspecific) and late (Vβ-specific) stages of SAgs-induced immunodeficiency may be required to better understand the mechanisms by which bacteria such as SA escape immune control. Note that we have recently observed a similar form of transient, Vβ-unrestricted unresponsiveness in mice injected with TSST-1 (data not shown), suggesting that the conclusions from this study are not restricted to a single bacterial SAg. In contrast, Webb et al. recently demonstrated that T cells from minor lymphocyte-stimulating Ags (MLS) 1^a injected mice displayed an increased in vitro proliferative response when examined 2 to 4 days after treatment (39). This apparent discrepancy may be related to distinct kinetics of in vivo T cell activation between bacterial (peak proliferative response at 24 h; see Ref. 40) and viral SAgs (peak response at 4 days; see Ref. 39). The two responses also differ in their APC requirements, as the MLS response is mostly B-cell driven (41), while the response to bacterial SAgs is largely B-cell independent (37).

The observation related in this report may help to understand the role of bacterial enterotoxins during microbial infection. As stated before, the ability of these compounds to induce a selective, Vβ-restricted T cell unresponsiveness is not likely to affect the host antibacterial response in a significant manner. In contrast, the profound unresponsive state observed during the early phases following SEB administration may represent an efficient mechanism by which Gram-positive bacteria subvert the host immune response.

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