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ICAM-1 is required for T cell proliferation but not for anergy or apoptosis induced by *Staphylococcus aureus* enterotoxin B *in vivo*

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Abstract

The response of T lymphocytes to superantigens requires expression of the appropriate TCR V_β gene products as well as the establishment of cellular interactions mediated by adhesion molecules. To study the role of intercellular adhesion molecule (ICAM)-1 in the response *in vivo* to superantigens, we have analyzed the effects induced by the bacterial superantigen *Staphylococcus aureus* enterotoxin B (SEB) in mice which have been made genetically deficient in ICAM-1. SEB treatment of wild-type mice causes proliferation, deletion and anergy of the SEB-reactive $V_\beta8^+$ T cell population. Here we show that cellular interactions mediated by ICAM-1 are not essential for the induction of anergy or for the deletion of $CD4^+V_\beta8^+$ or $CD8^+V_\beta8^+$T cells, but are required for the proliferation of these peripheral T lymphocytes. This is the first demonstration *in vivo* that the absence of the co-stimulatory signals provided by the interaction of ICAM-1 with its specific ligands impairs the proliferation of SEB-reactive T cells. Interestingly, our study showed that SEB-induced proliferation of $CD8^+V_\beta8^+$ T cells from lymph nodes (not from spleen) is independent of the interactions mediated by ICAM-1.

Introduction

Superantigens are products of infectious microorganisms which bind specifically to class II MHC molecules on APC (1–3) and interact with conserved domains of TCR V_β chains (4,5). This interaction induces activation of all T lymphocytes expressing superantigen-specific TCR V_β gene products (6). In mice, the bacterial superantigen *Staphylococcus aureus* enterotoxin B (SEB) elicits responses on peripheral T cells bearing $V_\beta8$ TCR elements (7). SEB administration *in vivo* causes cytokine release (8,9), clonal expansion of splenic and lymph node $V_\beta8^+$ T cells, and clonal deletion of part of these SEB-reactive lymphocytes via apoptosis (10). The remaining $V_\beta8^+$ T cells become anergic to superantigen stimulation (11–13). These changes affect both $CD4^+$ and $CD8^+$ T cells in peripheral lymphoid organs (10,14,15) and are thymic-independent (10). Superantigen-induced T cell activation requires, in addition to a functional and specific TCR complex, the presence of other cell-surface molecules that promote adhesion between the T lymphocytes and the APC, which presumably have a co-stimulatory role (16). These molecules include lymphocyte function-associated molecule (LFA)-1 (CD11a/CD18) expressed on lymphocytes and monocytes (17,18). Both LFA-1 and its relative, Mac-1 (CD11b/CD18), bind to intercellular adhesion molecule (ICAM)-1 (19–21). ICAM-1, expressed on all leukocytes, has been reported to be required to achieve optimal responses to superantigen *in vitro* (22). Inhibition of the interactions between LFA-1 and its counter-receptors (ICAM-1, ICAM-2 and ICAM-3) blocks allo- and xenogenic mixed lymphocyte reaction (MLR), antigen-specific and concanavalin A (Con A)-induced T cell proliferation, and T cell-dependent antibody responses (18).

Since superantigen-mediated T cell stimulation requires additional co-stimulatory signals (16) and many molecules that have been shown to provide co-stimulation are adhesion molecules (22,23), we have studied the specific role that ICAM-1 plays in superantigen-induced T cell activation using

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ICAM-1-deficient mice generated by gene targeting in embryonic stem (ES) cells (24). The study of this model *in vivo* shows that the absence of co-stimulatory interactions mediated by ICAM-1 does not affect either the elimination by apoptosis (deletion) or the induction and maintenance of antigen unresponsiveness (anergy) of the SEB-reactive T cells. However, ICAM-1 is critical for superantigen-induced T cell expansion as shown by the observation that SEB-reactive lymphocytes in ICAM-1-deficient mice do not undergo the proliferation observed in those from wild-type littermates.

Methods

Animals and in vivo treatment

Mutant and wild-type mice were bred in our specific pathogen-free mouse facility. ICAM-1-deficient mice (C57BL/6×129SVJ) (24) and wild-type littermates (8–10 weeks old) received one dose of 50 μg/mouse of SEB (Toxin Technology, Sarasota, FL) in a single i.v. injection. Control mutant (five animals per day and group for three independent experiments, totalling 100 mice for the kinetic analysis) and wild-type mice (100 mice) were injected with an identical volume (200 μl) of PBS. In one series of experiments, 1 μg/mouse of pertussis toxin (PTX) (Sigma, St Louis, MO) was co-administered with 50 μg/mouse of SEB. Mice subjected to these treatments were killed by cervical dislocation and analyzed at the indicated times.

Immunofluorescence analysis

Lymph node and spleen monocellular suspensions were subjected to hypotonic erythrocyte lysis, washed and stained for two- and three-color flow cytometry with mAb directed against CD4 (GK1.5) and CD8 (H02.2) from Becton Dickinson (Mountain View, CA); ICAM-1 (3E2), CD44 (Pgp-1), CD45RA (14.8) and CD3 (2C11) from PharMingen (San Diego, CA); $V_\beta 6$ (RR4-7; 25) and $V_\beta 8$ (F23.1; 26). The antibodies anti-CD3, anti-CD4 and anti-CD8 were FITC-labeled; anti-ICAM-1, anti-CD44 and anti-CD45RA were PE-labeled, and anti-$V_\beta 8$ and anti-$V_\beta 6$ were biotin conjugated and developed by means of Streptavidin–PE from Southern Biotechnology (Birmingham, AL) for two-color analysis or Streptavidin–Red 613 from Gibco/BRL (Gaithersburg, MD) for three-color analysis. Quantitative fluorometric analysis was performed on an Epics Profile flow cytometer (Coulter Electronic, Hialeah, FL) and on a FACScan instrument (Becton Dickinson).

Culture conditions

Total splenocytes (3×10^5/well and 1.5×10^5/well) were cultured for 2 days in medium (200 μl/well of RPMI 1640 supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 10 mM HEPES, 200 mM L-glutamine, 10 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 10 μg/ml SEB, 100 ng/ml PTX or 4 μg/ml Con A. For assessment of proliferation, cells were harvested after an 8 h pulse label with 1 μCi [^{3}H]thymidine (Amersham, UK).

Assessment of apoptotic DNA fragmentation

Splenic T cells were prepared by hypotonic erythrocyte lysis and subsequent B cell depletion by panning with polyclonal sheep anti-mouse Ig serum, as described (10). After culture *in vitro* (3 h at 37°C in RPMI 1640 medium supplemented with 10% FCS), cells were washed once in PBS. Dry pellets of 3×10^6 splenocytes were resuspended in 20 μl of 10mM EDTA, 50 mM Tris–HCl, 0.5% sodium lauryl sarkosinate, 0.5 μg/ml protease K and incubated for 1 h at 50°C. After addition of 10 μl of 10 μg/ml RNase A, samples were incubated for 1 h at 37°C, then for 5 min at 70°C, mixed with 10 μl of 10 mM EDTA plus 1% low-melting agarose, 0.25% bromophenol blue and 40% sucrose, and electrophoresed in 40 mM Tris–acetate with 1 mM EDTA over a 1.2% agarose gel containing 0.1 μg/ml ethidium bromide.

Cytokine measurement

The release of cytokines produced after priming with SEB was determined by ELISA. Blood was taken either at 90 min or 4 h after toxin challenge. Sera from three different animals was analyzed in each group. Serial dilutions of serum samples were assayed using commercial ELISA kits for IL-2, IL-4, IL-1α and TNF-α (Endogen, Boston, MA). Absorbance values were converted to concentrations of cytokine in the serum (pg/ml) by interpolation in the respective standard curve.

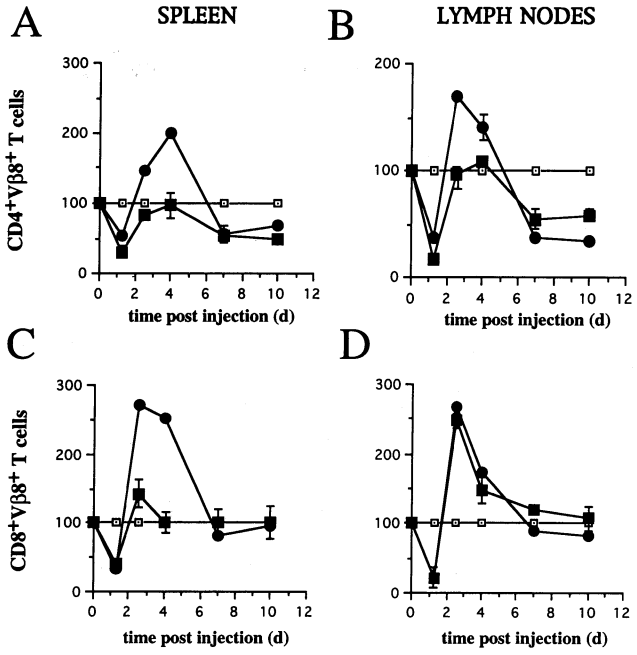

Fig. 1. Effect of SEB on the clonal expansion and deletion of peripheral $V_\beta 8^+$ T cells in ICAM-1deficient mice. Wild-type (filled circles) and mutant (filled squares) mice received a single i.v. injection of SEB and on days 1, 2.5, 4, 7 or 10 the frequency of spleen $CD4^+V_\beta 8^+$ (A) or $CD8^+V_\beta 8^+$ T cells (C) and lymph node $CD4^+V_\beta 8^+$ (B) or $CD8^+V_\beta 8^+$ T cells (D) was determined by flow cytometry as described in Methods. The variation of the percentages of cells belonging to these subsets is referred to the values obtained for the unprimed control mice (empty squares), which are considered as 100%. One representative experiment out of three independent experiments is shown here. Error bars represent the standard deviation of the values obtained for five mice used in each experiment.

Results

The lack of ICAM-1 in homozygous mutant mice impedes the SEB-induced clonal expansion of peripheral CD4$^+$ T cells but does not affect the superantigen-driven clonal deletion by apoptosis of these lymphocytes

The response of CD4$^+$ T lymphocytes in wild-type mice to the i.v. injection of SEB consists of (i) an early (12–24 h after SEB administration) deletion phase of CD4$^+$V$_\beta$8$^+$ peripheral T cells via apoptosis (27), (ii) a transient proliferative expansion of these T lymphocytes which reaches a maximum level (2- to 3-fold initial levels) at ~3–4 days after SEB treatment, and (iii) a second deletion of the CD4$^+$V$_\beta$8$^+$ subset which is clearly present 10 days after enterotoxin injection and lasts over 30 days (10,28,29). In mutant mice treated with SEB, the early deletion of 50–80% of CD4$^+$V$_\beta$8$^+$ T cells is indistinguishable from that observed in SEB-primed wild-type controls (Fig. 1A and B). However, the proliferation of CD4$^+$V$_\beta$8$^+$ T cells observed in the spleen and lymph nodes of wild-type individuals is diminished in the ICAM-1-deficient animals. Around day 3 in the lymph nodes and day 4 in the spleen, SEB-primed wild-type mice show a 2-fold increase in the percentage of CD4$^+$V$_\beta$8$^+$ T cells when compared with that of PBS-injected mice (Fig. 1A and B). However, 4 days after injection of SEB the size of the peripheral CD4$^+$V$_\beta$8$^+$ T cell subset from ICAM-1-deficient mice is not different than that of unprimed animals. Despite the suboptimal proliferation of the CD4$^+$V$_\beta$8$^+$ population in ICAM-1 mutant mice, this population is reduced during the second deletion phase as much as the same population in the wild-type animals. This second deletion response is maximum between days 8 and 10 after SEB injection (Fig. 1A and B). The death of SEB-reactive CD4$^+$ T cells from ICAM-1-deficient mice presumably occurs by apoptosis. In fact, the same degree of oligonucleosomal DNA fragmentation is present in spleen T cells from SEB-primed wild-type and ICAM-1-deficient mice (Fig. 2A) during the first and second deletion phases (around day 1 and 4 respectively). These results show that ICAM-1 is not involved in the signals that induce apoptosis and subsequent reduction of splenic and lymph node CD4$^+$V$_\beta$8$^+$ T cells mediated by SEB *in vivo*, but has a chief role in the SEB-driven proliferation of these lymphocytes. The lack of ICAM-1 in homozygous mice does not affect lymphoid development (24). The percentage of thymic and mature T cells bearing the different V$_\beta$ TCR elements is comparable in wild-type and ICAM-1-deficient mice (data not shown). To rule out that in the absence of ICAM-1 the action of SEB was less specific, we examined the effects of SEB on the V$_\beta$6$^+$ population in ICAM-1 mutant mice. As expected, since TCRV$_\beta$6$^+$ elements are not SEB-reactive (30), we did not detect any significant responses (proliferation, deletion) of V$_\beta$6$^+$ T lymphocytes to SEB neither in ICAM-1-deficient mice nor in wild-type mice (data not shown).

SEB-induced proliferation of spleen, but not lymph node, CD8$^+$ T cells is dependent on ICAM-1

Superantigens bind to MHC class II molecules on APC to stimulate T cells (2). However, this response is not MHC-restricted in a classical way since the CD8$^+$ T cell compartment is also able to respond to superantigens. The administration *in vivo* of SEB to wild-type mice exerts dual effects on the splenic and lymph node superantigen-reactive CD8$^+$ T cell population. In these lymphoid organs, activation of CD8$^+$V$_\beta$8$^+$ T cells can be followed as a proliferative response which takes place 2–4 days after SEB injection (10,28) (Fig. 1C and D), and which is 2-fold when compared with that of CD4$^+$ T cells (Fig. 1). This proliferative phase is preceded by an almost complete elimination (early deletion) of this subset and followed by a late deletion phase in which CD8$^+$V$_\beta$8$^+$ T cells decrease from 3-fold to the basal levels present before SEB injection (considered as 100%). In both deletion phases, the programmed cell death (PCD) of these cells results in

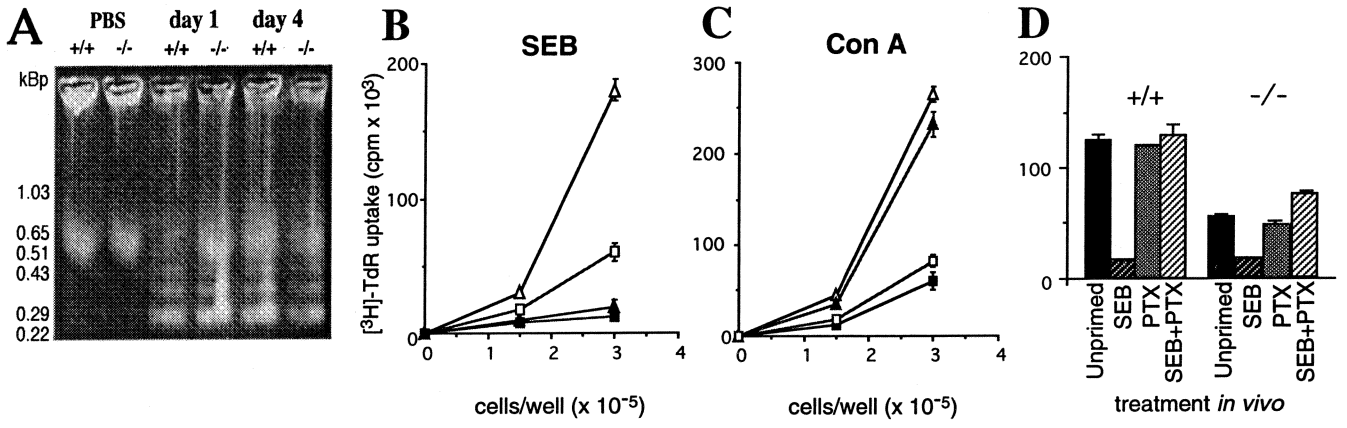

Fig. 2. SEB-induced clonal deletion and anergy of splenic T cells from ICAM-1-deficient mice. (A) DNA fragmentation patterns in T cells from wild-type (+/+) and mutant (–/–) mice treated with either PBS or SEB. Analysis was made on day 1 (early deletion) and day 4 (second deletion) after treatment. (B) and (C) Splenocytes were recovered from wild-type (triangles) or ICAM-1-deficient (squares) mice 10 days after a single injection of SEB (solid symbols) or PBS (empty symbols). [^{3}H]Thymidine incorporation was determined after culture at different cell densities in microtiter wells containing SEB (B) or Con A (C) for 48 h. (D) Effect of PTX on SEB-induced anergy in splenic V$_\beta$8$^+$ T cells. Wild-type (+/+) and mutant (–/–) mice were injected i.v. with SEB and/or PTX. Ten days later, total splenocytes were recovered, cultured in the presence of SEB and [^{3}H]thymidine incorporation was determined.

apoptotic DNA fragmentation (31). The study of mutant mice revealed that the ICAM-1 deficiency does not affect the elimination of peripheral $CD8^+V_\beta8^+$ T cells, since the early deletion of the subset (18 h after injection) as well as the late deletion (around day 3), although less marked in the mutant mice, occur in both groups of primed animals (Fig. 1C and D). This was further confirmed by the observation that in ICAM-1-deficient mice oligonucleosomal DNA fragmentation of spleen T cells after injection of SEB *in vivo* is not affected (Fig. 2A). Following a similar behavior demonstrated for the $CD4^+V_\beta8^+$ fraction, the SEB-induced expansion of splenic $CD8^+V_\beta8^+$ T cells is impaired in ICAM-1-deficient mice (Fig. 1C) suggesting that the interactions provided by ICAM-1 with its specific ligands are essential for the proliferative response to SEB *in vivo*. However, there is no difference in the proliferative response induced by SEB on lymph node $CD8^+V_\beta8^+$ T cells in the ICAM-1-deficient mice compared with wild-type littermates (Fig. 1D). Thus, surprisingly the SEB-induced expansion of this cell population seems to be independent of interactions mediated by ICAM-1.

Co-stimulation with ICAM-1 is required for the activation *in vitro* of human $CD4^+CD45RA^+$ T cells by low concentration of *S. aureus* enterotoxin A (SEA) but is not required for the activation of $CD4^+CD45RO^+$ T lymphocytes (32). CD45RA and CD44 expression characterize naive and activated cells respectively (33–35). To determine if, in ICAM-1-deficient mice, the difference in proliferative response to SEB between spleen and lymph node $CD8^+$ T cells correlates with a different expression pattern of the above markers, we studied their expression among the $CD8^+$ T population in these lymphoid organs. The level of CD45RA and CD44 expression was similar in both spleen and lymph node $CD8^+$ T cells. Expression levels remained comparable even after stimulation with SEB (data not shown).

In studying possible differences between the lymph node and the spleen $CD8^+V_\beta8^+$ population we observed that in SEB-primed wild-type mice, the level of ICAM-1 on the surface of lymph node $CD8^+$ T cells increased only half as much as on the same $CD8^+$ T population derived from the spleen, or on $CD4^+$ T cells from the spleen or lymph nodes (Fig. 3A). The study of ICAM-1 expression in the $CD8^+V_\beta8^+$ subset revealed a weak increase in ICAM-1 levels in spleen and lymph node $CD8^+V_\beta8^-$ cells from SEB-primed mice (Fig. 3B), probably due to SEB-induced cytokine production *in vivo* (9,36). We also observed an increase in the expression of this adhesion molecule in the splenic $CD8^+$ T cells bearing the SEB-specific $V_\beta8$ elements [mean channel of fluorescence (m.c.f.) 30.1 ± 1.9 versus 1.2 ± 0.2 in unprimed wild-type mice]. The change in ICAM-1 expression is not observed in the lymph node $CD8^+V_\beta8^+$ T cells as marked as it is in the spleen population (12.7 ± 2.0 versus 1.4 ± 0.2, Fig. 3B). The increase in the level of ICAM-1 expression in $CD4^+V_\beta8^+$ T cells is similar in both peripheral lymphoid organs (data not shown).

To further examine the mechanisms underlying the suboptimal activation of these $V_\beta8^+$ T cells, we studied if the production of IL-2, IL-4, IL-1 and TNF-α was altered after SEB immunization *in vivo*. Our results show that at different time points after SEB injection, the production of cytokines controlling T cell proliferation was 6-fold decreased for IL-2 and

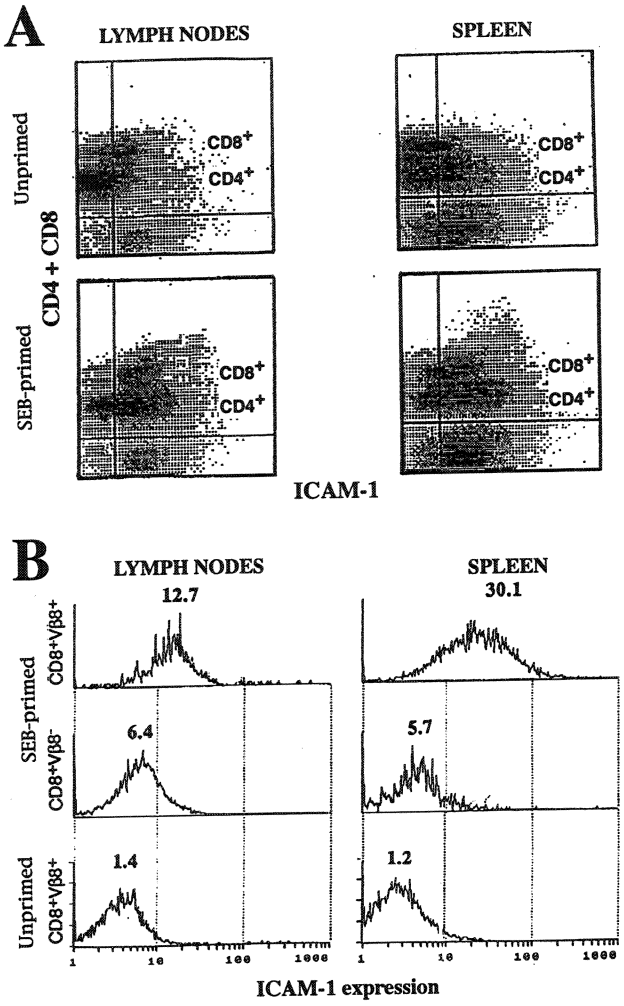

Fig. 3. Effect of SEB on ICAM-1 expression in peripheral T cells. (A) At 48 h after superantigen administration, $CD4^+$ and $CD8^+$ spleen and lymph node T cells from SEB-primed wild-type mice and PBS-injected controls (unprimed) were analyzed by flow cytometry to determine the expression of ICAM-1 in these subsets. (B) To examine variations in the intensity of ICAM-1 expression in $CD8^+V_\beta8^+$ T lymphocytes from spleen and lymph node, three-color inmunofluorocytometry analysis was performed as described in Methods. The figure shows the intensity of ICAM-1 expression in the subpopulations indicated in each histogram before and after SEB priming of wild-type mice. The value of the m.c.f. is also indicated in each histogram.

undetectable for IL-4, in the ICAM-1-deficient mice (Fig. 4A and B). Furthermore, 90 min after superantigen administration, the production of IL-1α and TNF-α was half in the mutant mice when compared with wild-type littermates (Fig. 4C and D).

The lack of ICAM-1 does not affect the SEB-induced anergy of peripheral $V_\beta8^+$ T lymphocytes

$V_\beta8^+$ T cells recovered from SEB-injected animals are anergic, as assessed by their low proliferative response *in vitro* to SEB (11). Accordingly, in wild-type mice, 10 days after the i.v. administration of SEB, $V_\beta8^+$ T cells that have not been

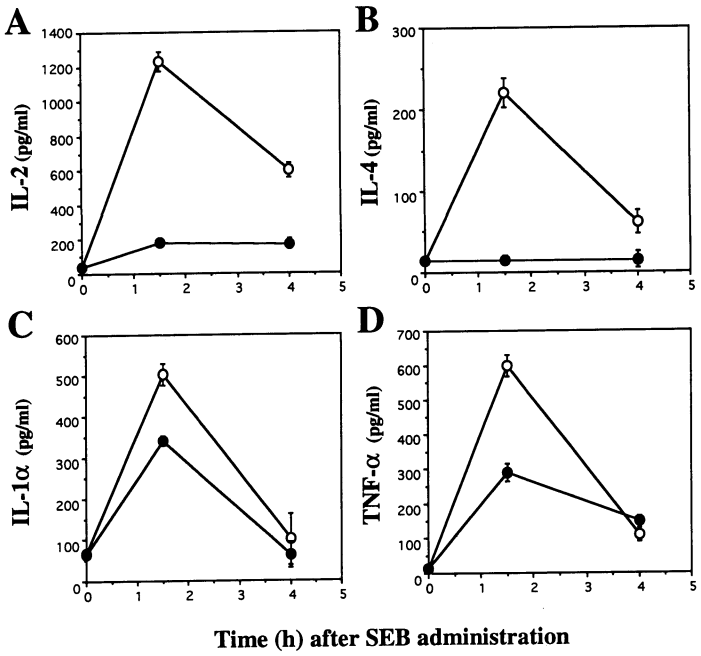

Fig. 4. Effect of ICAM-1 deficiency on serum cytokine levels after SEB administration. The systemic release of IL-2 (A), IL-4 (B), IL-1α (C) and TNF-α (D) was measured after a single dose (50 μg i.v./mouse of SEB). Wild-type (empty symbols) or ICAM-1-deficient mice (solid symbols) were bled from the retro-orbital plexus with heparinized capillaries at 90 min and 4 h after challenge with SEB. Within each group, sera from three animals were measured by ELISA in duplicates.

eliminated by apoptosis *in vivo* are impaired in their ability to mount a proliferative response to SEB *in vitro* (Fig. 2B). To assess whether or not co-stimulatory signals mediated through ICAM-1 are required for the induction of SEB-induced anergy, we tested the proliferative response to SEB *in vitro* of splenocytes from ICAM-1-deficient mice after SEB priming *in vivo*. Spleen $V_\beta8^+$ T cells from SEB-primed mice—mutant or wild-type—fail to proliferate to the superantigen *in vitro* (Fig. 2B). Thus, ICAM-1 is not essential for the induction or maintenance of anergy of the SEB-reactive T cells. Spleen T lymphocytes from unprimed wild-type mice showed a greater proliferative response *in vitro* to SEB (Fig. 2B) or Con A (Fig. 2C) than that of splenic T cells from PBS-injected ICAM-1-deficient mice. This confirms that ICAM-1 also plays an essential role in the proliferation of T lymphocytes *in vitro* to superantigens. Furthermore, an increase in the dose of SEB *in vivo* and *in vitro* (100 μg/mouse and 20 μg/ml respectively) does not modify the suboptimal T cell activation observed with lower doses. It is worth noting that in the absence of ICAM-1 there is still a significant *in vitro* response to SEB or Con A. This indicates that other molecules like ICAM-2 and ICAM-3 are sufficient for an incomplete response, but only in the presence of ICAM-1, these cells can achieve an optimal proliferative response. Also, we observed that $V_\beta8^+$ T cells from SEB-immunized ICAM-1-deficient mice and wild-type mice were able to respond to Con A, albeit to a lesser degree than cells from unprimed mice. This defective response does not compromise the specificity of the anergy observed in the wild-type or ICAM-1-deficient mice. In this context, the observation that PTX blocks this effect is a critical piece of information supporting the specificity of this effect (see below).

The deficient proliferative response to SEB observed in ICAM-1-deficient mice is not rescued by the co-injection of PTX

PTX, produced by *Bordetella pertussis*, is a non-superantigenic exotoxin (37) which acts as T cell mitogen *in vitro* (38,39). *In vivo*, PTX also prevents the induction of peripheral T cell anergy, enhances the T cell response (37) and inhibits the superantigen-induced deletion of peripheral T cells (31). To study if PTX could rescue *in vivo* the defective expansion of SEB-reactive T cells in ICAM-1-deficient mice, we co-injected PTX with SEB in wild-type and mutant mice. The effects that the injection of PTX induces on $CD4^+V_\beta8^+$ peripheral T lymphocytes from wild-type and ICAM-1-deficient mice are shown in Table 1. These effects have been analyzed at day 1 (early deletion), day 3 (maximum expansion) and day 10 (second deletion) after simultaneous administration of both toxins. Intravenous co-injection of PTX results in a prevention of both clonal deletion phases of $CD4^+V_\beta8^+$ T lymphocytes in wild-type mice. In ICAM-1 mutant mice, the co-administration of PTX does not rescue the proliferation of $CD4^+V_\beta8^+$ T cells, which is impaired due to the lack of ICAM-1. However, it almost prevents completely the deletion of these cells in response to SEB (Table 1). Splenic $CD8^+V_\beta8^+$ T cells show a similar response to the co-injection of PTX and SEB (data not shown), indicating that PTX cannot revert the block in the proliferative response to SEB imposed by the lack of ICAM-1.

As described above, after the phase of SEB-induced proliferation and death *in vivo*, the remaining $V_\beta8^+$ T cell population from ICAM-1 mutant mice fails to proliferate to a subsequent exposure to SEB *in vitro*. To examine if PTX is

Table 1. Effect of PTX on the SEB-induced proliferation and deletion of peripheral $CD4^+V_\beta8^+$T cells: percentage of positive cells[a]

Treatment[b]	Spleen			Lymph nodes		
	day 1	day 3	day 10	day 1	day 3	day 10
PBS	100	100	100	100	100	100
+/+ SEB	30 ± 1.2	160 ± 5.1	42 ± 1.5	26 ± 2.0	165 ± 9.2	51 ± 7.0
+/+ SEB + PTX	92 ± 3.9	209 ± 0.9	99 ± 0.4	91 ± 6.6	190 ± 4.1	112 ± 0.3
+/+ PBS + PTX	99 ± 0.7	100 ± 0.2	102 ± 0.6	100 ± 1.1	102 ± 3.2	100 ± 0.7
−/− SEB	34 ± 2.8	70 ± 10	38 ± 4.3	22 ± 5.3	89 ± 0.4	56 ± 2.8
−/− SEB + PTX	90 ± 3.7	90 ± 0.2	93 ± 1.4	82 ± 6.4	90 ± 1.0	90 ± 1.6
−/− PBS + PTX	100 ± 1.0	103 ± 2.2	98 ± 1.4	100 ± 1.9	98 ± 2.1	100 ± 1.8

[a]Determined by immunofluorescence analysis of spleen and lymph node cells. Values from toxin(s)-primed mice are given as percentages referred to the PBS-injected control percentages which are considered as 100%.

[b]Wild-type and mutant mice received one single i.v. injection of PBS (200 µl), SEB (50 µg), PTX (1 µg) or SEB + PTX (50 + 1 µg respectively). Analysis of $V_\beta8^+$T cells was performed at 1, 3 or 10 days after the treatment.

capable of rescuing the SEB-induced anergy of these T cells in the absence of ICAM-1, we measured the proliferative response to SEB *in vitro* of $V_\beta8^+$ splenocytes from ICAM-1-deficient mice 10 days after co-injection of both toxins. Our results showed that PTX co-administration blocks the SEB-induced anergy of $V_\beta8^+$ splenic T cells from both mutant and wild-type mice (Fig. 2D).

Discussion

In this report we demonstrate *in vivo*, using a 'clean' genetic deficiency, that (i) interactions mediated by ICAM-1 are not essential for the apoptotic elimination of SEB-reactive $CD4^+$ and $CD8^+$ lymphocytes from either the lymph nodes or spleen; (ii) interactions mediated by ICAM-1 are required *in vivo* for the SEB-induced expansion of peripheral $CD4^+V_\beta8^+$ and splenic $CD8^+V_\beta8^+$ T lymphocytes; (iii) ICAM-1 is not required for the response of lymph node $CD8^+V_\beta8^+$ T cells to SEB *in vivo;* (iv) that SEB-mediated expansion and anergy of SEB-reactive $V_\beta8^+$ T cells are not linked events, the first one being ICAM-1 dependent and the second one ICAM-1 independent; and (v) signals mediated by PTX either on activation or migration of $V_\beta8^+$ T cells do not overcome the effects of the ICAM-1 deficiency.

Superantigens play a potent T cell stimulatory role which is critical in infections with organisms producing these toxins. This strong T cell activation is reflected by an acute cytokine production (8,9,40) and the transient expansion of the stimulated T lymphocyte subset (10,12,28) followed by apoptotic cell death and development of anergy (12,13). Previous experiments lead to the conclusion that the expansion of these superantigen-stimulated T cells is unequivocally due to proliferation (12,14). In addition, we have demonstrated that all those lymphocytes that undergo PCD during the second deletion phase of the SEB response were part of a previous proliferative response, as measured by the incorporation of 5-bromo-2′-deoxyuridine (BrdUrd) in the DNA of the apoptotic cells (36). Here, we study superantigen-induced T cell activation in a mouse model that lacks the co-stimulatory signals provided by ICAM-1. We show that the absence of interactions mediated by ICAM-1 does not prevent the mechanisms involved in the apoptotic elimination of SEB-reactive peripheral T cells. Previous studies *in vitro* suggest that the deletion of superantigen-specific human $CD4^+$ T cells can be blocked by antibodies directed against CD11a/CD18 but not ICAM-1 or ICAM-2 (41). Nevertheless, these studies addressed the role of LFA-1 in the PCD of exclusively $CD4^+$ cells *in vitro* using blocking mAb. An alternative ligand for LFA-1 on leukocytes is ICAM-3. This adhesion molecule is more strongly expressed than ICAM-1 or ICAM-2 (42) on lymphocytes and could be the LFA-1 ligand that provides co-stimulatory signals in the SEB-driven apoptosis of mature T cells. Our study demonstrates that these co-stimulatory signals are not provided *in vivo* by ICAM-1. In addition, peripheral $CD4^+$ and $CD8^+V_\beta8^+$ T cells express high levels of LFA-1 during the early deletion period induced by SEB (data not shown). The acquisition and/or maintenance of SEB-mediated anergy is not affected by ICAM-1 as illustrated by the observation that in SEB-treated ICAM-1-deficient mice the surviving $V_\beta8^+$ T cell population fails to proliferate to SEB *in vitro*. These data demonstrate that in the absence of ICAM-1 it is possible to induce clonal deletion and anergy by SEB treatment. Both unresponsiveness mechanisms can be subverted by co-administration with PTX. However, SEB-induced cellular expansion only occurs in the presence of ICAM-1-mediated co-stimulatory signals. Several lines of evidence suggest that PTX acts directly on peripheral $V_\beta8^+$ lymphocytes. For example, the same effects can be observed in thymectomized mice (31) indicating that its effects cannot be attributed to increased maturation and export of thymocytes to the periphery, roles that have been proposed for PTX (43). On the other hand, PTX prevents *in vitro* (no migration or extravasation) the loss of $V_\beta8^+$ T cells induced by SEB (31).

One surprising finding of this study is that lymph node $CD8^+V_\beta8^+$ T cells are not dependent on interactions mediated by ICAM-1 for their expansion in response to SEB *in vivo*. This special feature of this population in the lymph node could correlate either with different co-stimulatory requirements of these cells, e.g. due to their activation status, or with different types of APC present either in lymph nodes or spleen. The finding that SEA-induced activation of human T cells requires ICAM-1 co-stimulation only for naive T lymphocytes

(CD45RA$^+$) but not for memory T cells (CD45RO$^+$) supports the former explanation. However, we failed in our attempt to identify a greater presence of activated (CD44$^+$) or non-naive (CD45RA$^-$) cells among the lymph node CD8$^+$V$_\beta$8$^+$ T cells. The only consistent difference that we have observed between lymph node CD8$^+$V$_\beta$8$^+$ T cells and spleen CD8$^+$V$_\beta$8$^+$ T cells (or CD4$^+$V$_\beta$8$^+$ from both lymphoid organs) is that in response to SEB stimulation, ICAM-1 expression is much less variable in lymph node CD8$^+$V$_\beta$8$^+$ T cells. Since ICAM-1 up-regulation is a primary event in leukocyte activation or inflammation and is critical for the progression of these processes, a lack of ICAM-1 up-regulation in this population may be the cause of the ICAM-1-independent activation by SEB. The molecular mechanisms underlying this observation remain to be elucidated.

Since ICAM-1 is also expressed on endothelial cells, it is conceivable that ICAM-1 deficiency could influence the trafficking pattern of V$_\beta$8$^+$ T cells after SEB stimulation and this effect could contribute to the final phenotypes observed here. However, several reasons argue against the significant contribution of this mechanism to the observed phenotype. (i) The expansion or deletion of V$_\beta$8$^+$ T cells occur simultaneously in spleen, lymph nodes and blood even without a functional thymus able to export new mature T cells (10). (ii) The reduction in systemic cytokine release in the mutant mice indicates a suboptimal activation of T cells and therefore correlates with the decreased proliferation of this subset. (iii) SEB induces *in vitro* an extensive proliferation of peripheral T cells bearing TCR V$_\beta$8$^+$ elements.

A strategy commonly used by a large number of infectious microorganisms to evade the immune system of their host is to modulate its immune response. Induction of clonal deletion and anergy by superantigens is one mechanism. A better understanding of the co-stimulatory role that molecules like ICAM-1 play in the control of superantigen-induced activation would facilitate the design of therapeutic procedures to prevent the immunopathological consequences induced by these toxins.

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Abbreviations

Con A	concanavalin A
ICAM	intercellular adhesion molecule
LFA	lymphocyte function-associated molecule
m.c.f.	mean channel of fluorescence
MLR	mixed lymphocyte reaction
PCD	programmed cell death
PTX	pertussis toxin
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B

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