

Natural killer T cells are required for the development of a superantigen-driven T helper type 2 immune response in mice

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Summary

We show, here, that one single injection or weekly injections of staphylococcal enterotoxin B (SEB), starting in 1-day-old newborn mice, induced a powerful immune response with a T helper type 2 (Th2) pattern, as judged by the isotype and cytokine profile, with the production of large amounts of SEB-specific immunoglobulin G1 (IgG1), detectable levels of SEB-specific IgE and increased production of interleukin-4 by spleen cells. These protocols also induced an increase in the levels of total IgE in the serum. Memory of SEB was transferred to secondary recipients by using total spleen cells from primed animals. The secondary humoral response in transferred mice was diminished if spleen cells from SEB-treated mice were previously depleted of CD3⁺ or Vβ8⁺ T cells or NK1.1⁺ cells. *In vivo* depletion of NK1.1⁺ cells in adult mice resulted in a marked reduction in the SEB-specific antibody response in both the primary and secondary immune responses. Additionally, purified NK1.1⁺ T cells were able to perform SEB-specific helper B-cell actions *in vitro* and *in vivo*. These results suggest that NK1.1⁺ T cells are required for the full development of humoral immunological memory, whilst making neonatal tolerance to SEB unachievable.

Keywords: immunoglobulin G; natural killer T cells; staphylococcal enterotoxin B; superantigens; tolerance

Introduction

Superantigens, such as endogenous Mls antigens and the exogenous enterotoxins produced by *Staphylococcus aureus*, bind to major histocompatibility complex (MHC) class II molecules and stimulate T cells expressing specific T-cell receptor (TCR) Vβ genes¹. The Vβ-specific stimulation of high-frequency T-cell precursors and the availability of monoclonal antibodies (mAbs) to identify superantigen-responsive T cells *in vivo* have made these antigens useful to study *in vivo* T cell-mediated immune responses.¹ For instance, it has been reported that the treatment of IE⁺ mouse strains with staphylococcal enterotoxin B (SEB) induces a marked deletion of peripheral SEB-specific responder T cells.² Moreover, the superantigen-responder T cells are anergic to further *in vitro* challenges.³ Anergy in these situations consists in the absence of both proliferation and production of cytokines, such as interleukin-2 (IL-2) and interferon-γ (IFN-γ), upon superantigen

restimulation *in vitro*.⁴ These latter findings have led to the suggestion that superantigens could induce a profound state of tolerance.⁵ This notion was extended to neonatal tolerance because, in neonatal mice, superantigens such as SEB, staphylococcal enterotoxin A (SEA) or Mls caused massive intrathymic and peripheral clonal deletion.^{6,7}

Although the *in vitro* absence of T-cell proliferation and interleukin production upon superantigen restimulation is an unequivocal finding, the *in vivo* unresponsiveness is less evident.⁸ In fact, it has been shown that CD4⁺ Vβ6⁺ T cells from BALB/c mice rendered anergic by the injection of Mls-disparate DBA/2 mouse B cells are effective helper T cells for B-cell polyclonal immunoglobulin production *in vivo* and *in vitro*, yet fail to proliferate *in vitro*.⁹ Furthermore, CD4⁺ Vβ8⁺ T cells from mice primed with SEB are able to produce IL-2 *in vivo*, yet fail to proliferate upon superantigen challenge *in vitro*.¹⁰ Even more striking was the demonstration that at the same time that it induced *in vitro* unresponsiveness, SEB primed SEB-specific T helper

Abbreviations: CEA, carcinoembryonic antigen; ICOS, inducible co-stimulator.

cells to provide help for B cells in a typical secondary immune response.¹¹ The secondary SEB immune response was characterized by high levels of anti-SEB specific immunoglobulin G1 (IgG1) and low amounts of IgG2a.¹¹ This isotype pattern is associated with a preferential activation of T helper type 2 (Th2) cells. The Th2 type of response is thought to be driven by IL-4.¹² It has been reported that natural killer (NK) T cells can be, in some experimental models, the primary source of IL-4.^{13,14} Most NK T cells express the V β 8 chain associated with an invariant V α 14-J α 281 chain,^{15,16} a large proportion of these cells being of thymic origin.¹⁷

Here we investigate the induction of neonatal tolerance to SEB. Our results indicate that NK T cells may inhibit the induction of tolerance in this model, and are of fundamental importance in maintaining the production of high levels of SEB-specific antibodies during a secondary immune response.

Materials and methods

Animals

F₁ (BALB/c $\times$ C57BL/6) mice were bred and maintained under standard conditions in the Department of Immunology, Institute of Biomedical Sciences, São Paulo. The protocols used in this study were approved by the Ethics Commission in Experimental Animal Models (Institute of Biomedical Sciences, University of São Paulo).

Antigens and immunization

We administered 50 μ g SEB (Toxin Technology, Sarasota, FL) in 50 μ l of 0.15 M phosphate-buffered saline (PBS), pH 7.2, or in PBS alone, by intraperitoneal (i.p.) injection within 24 hr of birth and once a week thereafter. Mice received a total of four injections of SEB (4 $\times$ SEB-treated). Alternatively, the mice received one injection of SEB (50 μ g) within 24 hr of birth and a second injection (50 μ g) 4 weeks later (2 $\times$ SEB-treated). Adult mice (4–6 weeks old) were immunized i.p. with 50 μ g of SEB or PBS. Secondary immunization in adult mice, with 50 μ g of SEB, was given 1 month after primary immunization. In some experiments, adult mice were immunized with ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) in Alum (1 mg/animal; Sigma-Aldrich) and secondary immunization was performed with 100 μ g of OVA i.p. diluted in PBS.

Enzyme-linked immunosorbent assay (ELISA) for OVA- or SEB-specific antibodies

Sera were obtained by bleeding mice from the tail vein at various time points after OVA, SEB, or PBS injections. ELISA for total anti-SEB immunoglobulins were performed as previously described.¹⁶ For measurements of immuno-

globulin isotypes, the following antibodies were used: biotinylated rat mAb anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, IgA and IgE (BD Biosciences-Pharmingen, San Jose, CA). These mAbs were added at an appropriate dilution and the assay was revealed as previously described.¹¹ Optical densities were measured at 492 nm in an ELISA plate reader (Titertek Multiscan MCC/340-MKII, Labsystems, Finland). For total IgE measurements, two different rat monoclonal anti-mouse IgE (Pharmingen) were used and the assay was performed according to the manufacturer's instructions. The concentration of anti-OVA antibodies in each serum sample was estimated from a standard curve generated with known amounts of OVA-specific, affinity-purified antibodies from OVA-hyperimmunized mice.

Monoclonal antibodies and other reagents for FACS analysis

H-129 (anti-CD4), H-35 (anti-CD8), F23.1 (anti-V β 8.1, V β 8.2, V β 8.3), H-57-597 (anti-pan β -chain), PK-136 (anti-NK1.1), DX-5 (anti-NK), 2C11 (anti-CD3), PV-1 (anti-CD28), F4/80 (anti-macrophage), MR1 [anti-CD40 ligand (CD40L)], 7E.17G19 (anti-inducible co-stimulator (ICOS)) and R6-60.2 (anti-mouse IgM) mAbs, used in this study, were labelled with phycoerythrin (PE), fluorescein isothiocyanate (FITC), or biotin. For biotin-labelled mAbs, streptavidin-PE, Cychrome (Cy) or FITC were used as a second-step reagent, as indicated. All these reagents were purchased either from Pharmingen, Southern Biotechnology (Birmingham, AL) or Caltag Laboratories (Burlingame, CA).

In vivo treatment with mAbs

The treatment was performed as previously described.^{18,19} In brief, each mouse received 1 mg monoclonal anti-NK1.1⁺ cells (PK-136) by an i.p. route, three times a week, starting 1 week before any other manipulation. The treatment was maintained during the experiments. The efficacy of the mAb in depleting the animals of NK1.1⁺ or NK T cells was ascertained by flow cytometric analysis, using a different anti-NK mAb (DX-5). The reduction was invariably greater than 90% after 1 week of treatment. As a control for the PK-136 mAb treatment, purified mouse IgG2a anti-human carcinoembryonic antigen (CEA) mAbs (clone 5D11) were given at the same dose, by the same route and for the same period.

In vitro cell depletion or enrichment

Spleen cells were incubated with the following biotin-labelled mAbs: anti-NK1.1 (PK-136), anti-V β 8 (F23.1), anti-CD3 (2C11) or anti-IgM (R6-60.2). The incubation was carried out at 4° for 20 min in balanced salt solution supplemented with 10% fetal calf serum. Spleen cells were

washed a further four times. For the depletion of NK1.1⁺, V β 8⁺, CD3⁺ cells or B cells, a second incubation with streptavidin-coated magnetic Dynabeads (DynaL Biotech, Brown Deer, WI) was carried out, according to the manufacturer's instructions. After two rounds of magnetic exclusion, the remaining spleen cells were used for *in vivo* transfers. The degree of depletion was monitored by flow cytometric analysis and was found to be above 98%. For NK T-cell purification, a FITC-multisort kit from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany) was used, according to the manufacturer's instructions. First, splenic FITC- $\alpha\beta$ ⁺ cells were positively sorted. Purified $\alpha\beta$ ⁺ cells were stained with anti-NK1.1 biotin and positively sorted using streptavidin-coated microbeads from Miltenyi. The purity of NK1.1⁺ V β 8⁺ cells was usually around 80% on flow cytometric analysis. Purified splenic CD4⁺ T cells were obtained by using affinity columns from R & D Systems (Minneapolis, MN). Purified CD4⁺ T cells were further stained with biotin-labelled anti-NK1.1 (PK-136) and subjected to magnetic depletion after incubation with streptavidin-coated Dynabeads (DynaL). CD4⁺ T cells purified in this way contained no detectable NK1.1⁺ cells and their purity was above 90% in flow cytometry analysis.

In vitro cell culture

Proliferation assays. Serial dilutions of splenocytes or B-cell-depleted spleen cells (for IL-4 measurements) were cultured in triplicate in 96-microwell plates (Nalge Nunc, Rochester, NY) in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, South Logan, UT), 50 mM 2-mercaptoethanol and 1 mM HEPES. Cells were cultured at 37°C alone or in the presence of 10 µg/ml SEB (Toxin Technology, Sarasota, FL). After 72 hr, cultures were pulsed for 16 hr with [³H]thymidine (1 µCi/well; Amersham Biosciences, Chalfont St. Giles, UK), the cells were harvested and the radioactivity was measured in a liquid scintillation beta-counter.

Interleukin measurements. Total spleen cells or B-cell-depleted spleen cells from different experimental groups were cultured at a density of 10⁷ cells/well in 24-well plates (Nunc), stimulated or not with 10 µg/ml SEB, for 24 or 48 hr. Alternatively, purified T-cell populations were cultured in anti-CD3 mAb (2C11) precoated (10 µg/well) flat-bottomed 96-microwell plates (3 × 10⁵ cells/well) for 24 or 48 hr, in the presence of 2 µg/well anti-CD28 (PV-1). Supernatants were collected and frozen at -70°C.

B-cell helper assay

In short, titrated numbers of purified CD4⁺ or NK1.1⁺ $\alpha\beta$ ⁺ T cells were cultured with 4 × 10⁵ B-cell-enriched spleen cells from the indicated groups/well. The B-cell-enriched population consisted of splenic cells that were depleted of

T cells and macrophages by streptavidin-coated magnetic beads, using a mixture of biotin-labelled anti-CD3 and anti-F4/80 mAbs, as described above. Cultures were performed in triplicate, in 96-well flat-bottomed plates (Nunc), in the presence of 10 µg/ml of purified anti-CD3 mAb (2C11), in a total volume of 200 µl of complete RPMI-1640. Supernatants were collected 7 days after for detection of SEB-specific antibodies by ELISA.

Cytofluorometric studies

Staining was performed as previously described¹⁵ followed by analysis in a FACScan analyser (Becton and Dickinson, San José, CA). In all of the studies, two determinations were performed for every sample: one for total cells, to assess the absolute frequencies, and another by deliberately gating positive events for further analysis. Between 10 000 and 50 000 events were recorded per sample.

Cell transfers

Spleen cells from normal or immunized mice, depleted or not of the different T-cell subpopulations as described above, were transferred (3 × 10⁷ cells/mouse) by intravenous injection into irradiated (750 rads), thymectomized and bone marrow reconstituted F₁ (BALB/c × C57BL/6) mice. These mice were injected i.p. with 20 µg of either SEB or PBS, just after the transfers, and sera were collected 7 days later. For transfers of purified CD4⁺ or NK1.1⁺ $\alpha\beta$ ⁺ T cells, each animal received 5 × 10⁵ purified T cells, injected intravenously together with 2 × 10⁷ cells of an SEB-primed B-cell-enriched population. Control mice received no T cells.

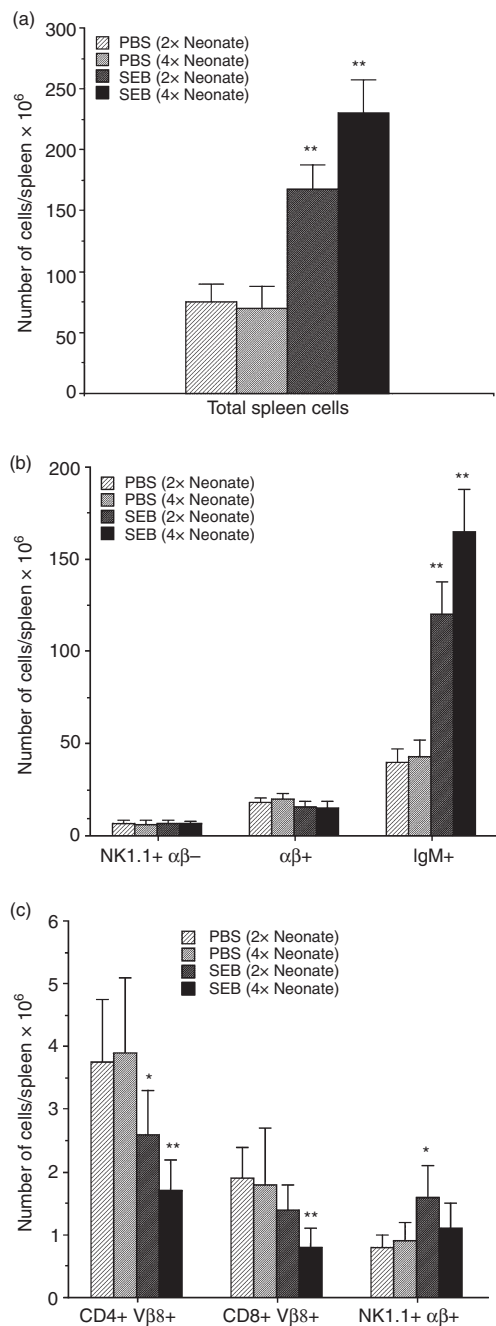
Cytokine ELISA

The levels of IL-4 and IFN- γ were quantified by two-site sandwich ELISAs. Monoclonal and polyclonal antibodies were obtained from R & D Systems. ELISAs were performed according to the manufacturer's instructions. The lower limits of detection of IL-4 and IFN- γ in the assays were 0.01 ng/ml and 0.05 ng/ml, respectively.

Results

Multiple SEB injections induce splenomegaly as a result of a large expansion of the splenic B-cell population and cause deletion of conventional V β 8⁺ T cells; yet, NK T-cell numbers are preserved

Neonatal mice were injected with 50 µg SEB within 24 hr of birth and once a week thereafter, for a total period of 4 weeks. Alternatively, neonatal mice received one single injection of 50 µg SEB in the first 24 hr after birth and a second injection when they were 6 weeks old. The total



number of spleen cells 10 days after the last SEB injection was about three times higher in the 4× SEB-treated mice, when compared to control mice that received injections of PBS and about two-fold higher in 2× SEB-treated mice (Fig. 1a). Cytometric analysis showed that the total number of αβ⁺ T cells and NK1.1⁺ αβ⁻ cells did not vary significantly between experimental and control groups (Fig. 1b). However, the total number of splenic B cells increased about 3.5- and 2.5-fold in the groups of mice that received 4× or 2× SEB injections, respectively, when compared to the control group (Fig. 1b). To follow SEB-reactive T cells, 14 days after the last SEB injection, the

Figure 1. Splenic B-cell and T-cell populations in mice treated repeated times with SEB. Spleen cells from F₁ (BALB/c × C57BL/6) mice, treated with multiple injections of PBS or SEB, were counted, stained and evaluated by FACS as described in the Materials and methods section. In (a), the columns represent the mean number of mononuclear cells in the spleen of five mice treated with PBS or multiple injections of SEB (2× or 4×). The first injection of PBS or SEB was given within the first 24 hr after birth. In (b) the mean number of splenic cells, bearing different phenotypes (NK1.1⁺ αβ⁻, αβ⁺ and IgM⁺), in four groups of five mice are represented by columns. Columns in (c) represent the mean numbers of splenic cells, bearing different phenotypes (CD4⁺ Vβ8⁺, CD8⁺ Vβ8⁺ and NK1.1⁺ αβ⁺ T cells). Mice were analysed individually. Vertical bars represent the standard error of the mean. The experiment was repeated twice with equivalent results. Student's *t*-test was used to compare experimental and control groups. A *P*-value ≤ 0.05 was considered to be significant. **P* ≤ 0.05, ***P* ≤ 0.01.

mice were killed and their spleens were analysed for the frequency of CD4⁺ Vβ8⁺, CD8⁺ Vβ8⁺ and NK T cells. Figure 1(c) shows that the total numbers of splenic CD4⁺ Vβ8⁺ and CD8⁺ Vβ8⁺ T cells in SEB-treated mice decreased considerably in relation to control PBS-injected mice. However, NK T-cell numbers were slightly increased (2× SEB) or preserved (4× SEB).

SEB-treated mice are not tolerant to SEB at the level of specific *in vivo* antibody response

Mice treated with four repeated injections of SEB from birth produced very high levels of total antibodies to the related superantigen, whereas control sera were negative for such reactivity (Fig. 2a). The same pattern could be observed in the group of mice that received only two injections of SEB during the same period (Fig. 2a). The main isotype produced was IgG1 (Fig. 2b) with low levels of IgG2a (Fig. 2c). Yet, IgG2a was produced by SEB-immunized mice, indicating that part of the Th1 immune response was preserved. Sera from 4× SEB-treated mice had detectable levels of SEB-specific IgE, whereas control sera were essentially negative (Fig. 2d). The levels of polyclonal IgE in the sera of 4× SEB-treated mice were much higher than the IgE serum levels from PBS-treated control mice (Fig. 2e).

Spleen cells from SEB-treated mice underwent proliferative anergy to *in vitro* SEB re-stimulation

Spleen cells from 4× or 2× SEB-treated mice could not proliferate in the presence of SEB *in vitro* (Fig. 3a). In addition, total spleen cells from SEB-treated mice produced much lower levels of IFN-γ and higher amounts of IL-4 when compared to PBS-treated control mice upon 48 hr of *in vitro* re-stimulation with SEB (Fig. 3b,c). Moreover, spleen cells derived from SEB-primed mice produced higher levels of IL-4 in non-stimulated culture conditions when compared to spleen cells derived from

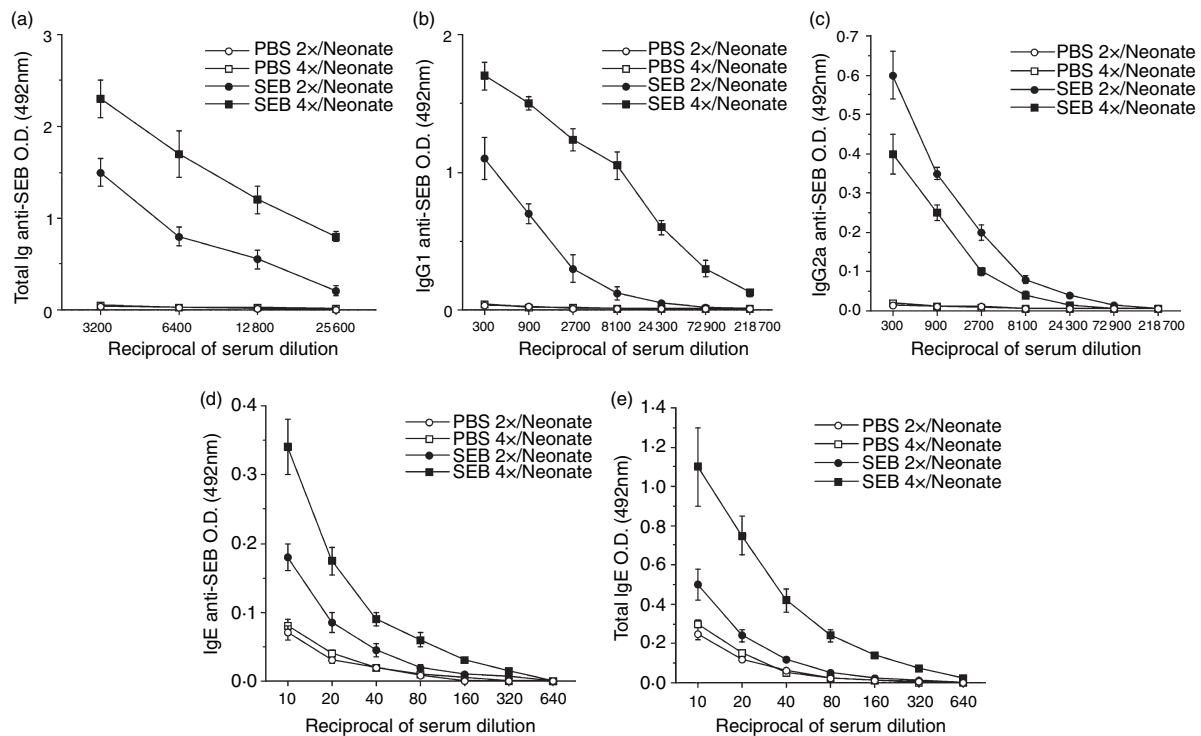


Figure 2. SEB-treated mice are not tolerant to SEB at the level of specific antibody response. F_1 (BALB/c $\times$ C57BL/6) mice, repeatedly injected with PBS or SEB (2 $\times$ or 4 $\times$), were bled 14 days after the last injection. Sera were evaluated for the levels of anti-SEB-specific antibodies and total levels of non-specific IgE by ELISA, as described in the Materials and methods section. Results are shown as the mean OD (492 nm) obtained for each serum dilution from three to five mice. The vertical bars represent the standard errors of the mean. The levels of total immunoglobulin (IgG + IgM), IgG1, IgG2a and IgE anti-SEB are shown in (a), (b), and (c,d), respectively. The levels of non-specific IgE are shown in (e). Note the differences in y-axis between (a), (b), (c,d) and (e). Titration curves were compared by two-way analysis of variance test. P -values < 0.05 were considered to be significant.

PBS-treated mice. These results are consistent with the above findings where 4 $\times$ or 2 $\times$ SEB-treated mice showed high production of SEB-specific IgG1 and low levels of SEB-specific IgG2a. The production of IL-4 was also investigated in purified $\alpha\beta^+$ NK1.1 $^+$ T cells from naive versus 4 $\times$ SEB-treated animals. The purified NK T cells from 4 $\times$ SEB-treated animals produced higher levels of this cytokine than did control naive cells, upon stimulation *in vitro* (Fig. 3d).

The transfer of an SEB-specific secondary humoral immune response to mice was affected by removal of NK1.1 $^+$, CD3 $^+$ or V β 8 $^+$ cells

Thirty million spleen cells from mice injected 4 $\times$ or 2 $\times$ with SEB were transferred to thymectomized and irradiated syngeneic mice. Total spleen cells, or spleen cells depleted *ex vivo* by magnetic negative selection of NK1.1 $^+$, CD3 $^+$ or V β 8 $^+$ cells, were given intravenously to different groups of recipient mice, as described in the Materials and methods section. Figure 4 shows the levels of anti-SEB IgG antibodies 7 days after the transfers. Total spleen cells from SEB-primed mice could transfer a typical secondary response to recipient mice, whereas the

production of anti-SEB antibodies was impaired in the groups that received SEB-primed spleen cells previously depleted of CD3 $^+$ cells. A similar effect was observed when the SEB-primed spleen cells were depleted of NK1.1 $^+$ or V β 8 $^+$ cells.

In vivo depletion of NK1.1 $^+$ cells led to a reduced humoral immune response to SEB

To evaluate the role of NK1.1 $^+$ T cells in the humoral immune response to SEB, we used the PK-136 monoclonal antibody to deplete NK1.1 $^+$ cells. This depletion of NK1.1 $^+$ cells decreased anti-SEB antibody production during the primary and secondary immune response in SEB-immunized adult mice (Fig. 5a). The primary or secondary humoral immune response to OVA in adult mice was not modified by the *in vivo* depletion (Fig. 5b).

$\alpha\beta^+$ NK1.1 $^+$ T cells from SEB-primed mice are able to deliver SEB-specific B-cell helper activity *in vitro* and *in vivo*

Purified $\alpha\beta^+$ NK1.1 $^+$ or CD4 $^+$ NK1.1 $^-$ T cells were obtained from SEB-primed mice and were added at

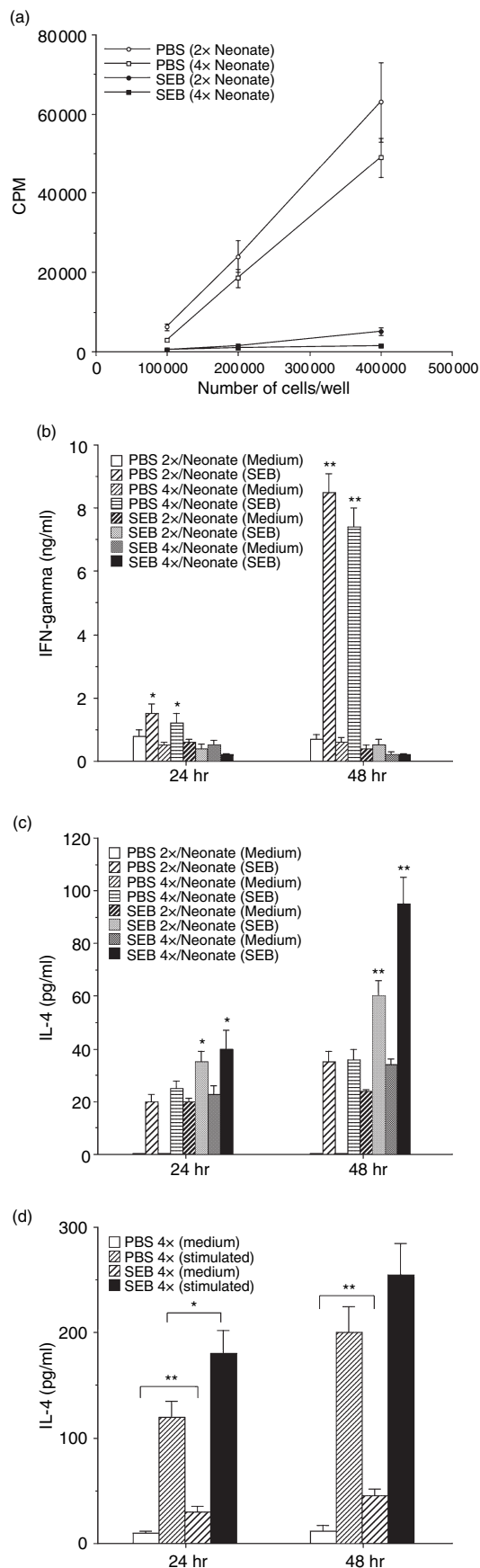


Figure 3. Spleen cells from SEB-treated mice underwent proliferative anergy to *in vitro* restimulation. Spleen cells from mice, repeatedly treated with PBS or SEB (2× or 4×) were cultured *in vitro* in the presence of medium alone or in the presence of 10 µg/ml of SEB. Three to four spleens from each group of mice were pooled and used to prepare the cell suspensions. Proliferation assays were performed as described in the Materials and methods section. (a) Shows the results of thymidine incorporation obtained in one representative experiment out of three. The data are shown as counts per min (c.p.m.; mean of triplicate cultures ± standard deviation of the mean). The background c.p.m. (non-stimulated cultures) was always less than 2000 c.p.m. (b,c) Show the levels of IFN-γ or IL-4 production by spleen cells from the different experimental groups after 24 or 48 hr of culture. Spleen cells were cultured with medium alone or 10 µg/ml of SEB. IFN-γ and IL-4 were measured by ELISA, as described in the Materials and methods section. Columns represent the mean of the amount of IFN-γ (ng/ml) or IL-4 (pg/ml), and the vertical bars show the standard deviation of the mean of triplicate cultures. The data are from one of two similar experiments. (d) Shows the production of IL-4 by αβ⁺ NK1.1⁺ T cells from naive or from previously SEB-stimulated animals. The animals were injected four times with SEB, starting within 24 hr after birth, as described in the Materials and methods section. Cells from the spleens of 10 mice were pooled purified, cultured and stimulated as described in the Materials and methods section. Columns represent the mean of the amount of IL-4 (pg/ml), and the vertical bars represent the standard deviation of the means of triplicate cultures. The data are from one of two similar experiments. Wilcoxon matched pairs and Kruskal-Wallis tests were used. A *P*-value ≤ 0.05 was considered significant. **P* ≤ 0.05, ***P* ≤ 0.01.

different ratios in relation to the naive or SEB-primed B-cell-enriched population. An anti-CD3 mAb was used to bridge both T and B cells. Primed NK1.1⁺ T cells were more effective in providing SEB-specific B-cell helper activity than SEB-primed CD4⁺ NK1.1[−] T cells as they induced higher levels of SEB-specific antibodies *in vitro* (Fig. 6a). To test the functional activity *in vivo*, purified SEB-primed CD4⁺ NK1.1[−] or αβ⁺ NK1.1⁺ T cells were transferred, together with SEB-primed B-cell-enriched population, to thymectomized, irradiated and bone marrow reconstituted adult mice. Figure 6(b) shows that recipient animals made a typical secondary humoral immune response. In addition, mice transferred with αβ⁺ NK1.1⁺ T cells were able to maintain high levels of SEB-reactive IgG for longer than mice transferred with SEB-primed CD4⁺ NK1.1[−] T cells.

NK1.1⁺ αβ⁺, NK1.1⁺ Vβ8⁺, NK1.1⁺ CD4⁺ and NK1 T cells are not deleted after SEB injection

Multiple injections (2×) of SEB in F₁ (BALB/c × C57BL/6) adult mice led to the maintenance of the percentages of splenic αβ⁺ T cells expressing NK1.1 marker 5.3 ± 1.3% (mean ± SD) when compared to control mice receiving PBS 4.5 ± 2.2% 2 weeks after the last SEB or

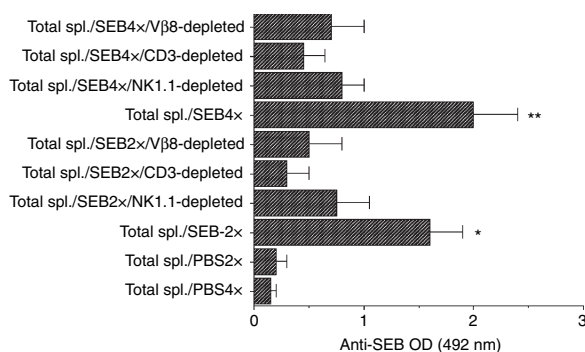


Figure 4. The transfer of an SEB-specific secondary humoral immune response to mice was affected by removal of NK1.1⁺, CD3⁺ or Vβ8⁺ T cells. The anti-SEB antibody production in thymectomized and irradiated mice, 7 days after reconstitution with different spleen cell populations and immunization with SEB is shown. Either 3×10^7 total spleen cells or the same number of spleen cells depleted of distinct cell populations (CD3⁺ T cells, NK1.1⁺ cells, Vβ8⁺ T cells) from mice repeatedly treated with PBS or SEB were transferred to thymectomized and irradiated syngeneic mice, as described in the Materials and methods section. The columns represent the mean of the OD (492 nm) obtained from five mice, and the horizontal bars correspond to standard errors of the mean of the anti-SEB antibody levels. Sera were diluted 1/2000 in PBS and used in an ELISA. Kruskal–Wallis test was used and a P -value ≤ 0.05 was considered significant. Groups of mice that received total spleen cells from 2× or 4× SEB-treated mice versus the other respective groups were compared. * $P \leq 0.05$, ** $P \leq 0.01$.

PBS injection. The percentage of Vβ8⁺ T cells expressing NK1.1 in the spleens of SEB-treated mice was slightly higher ($7.1 \pm 0.8\%$) when compared with the PBS-injected control group ($4.9 \pm 0.5\%$). The percentage of spleen CD4⁺ T cells expressing NK1.1 was increased in the SEB-treated experimental group ($4.5 \pm 0.6\%$), compared with controls ($2.7 \pm 0.4\%$). In addition, the percentage of CD4⁺ NK1.1⁺ T cells expressing the Vβ8 chain increased 2 weeks after the last SEB injection ($58 \pm 1.3\%$ in SEB-treated mice versus $50.9 \pm 1.5\%$ in the PBS-treated control group). Figure 7 shows representative plots of one animal from each group. The same pattern could be observed up to 4 weeks after the last injection (data not shown).

Expression of CD40L and ICOS in splenic NK1.1⁺ T cells from naive or SEB-primed mice

Figure 8 shows that a considerable proportion of splenic αβ⁺ NK1.1⁺ T cells express CD40L in naive mice ($28.2 \pm 4.8\%$ of the total spleen αβ⁺ NK1.1⁺ T cells). Surprisingly, there was a reduction in the percentage of these cells expressing CD40L 14 days after SEB priming ($15 \pm 3.7\%$ of the total αβ⁺ NK1.1⁺ cell population). A similar pattern was observed for CD4⁺ NK1.1⁺ T cells: $55.8 \pm 12.1\%$ of this subpopulation was positive for

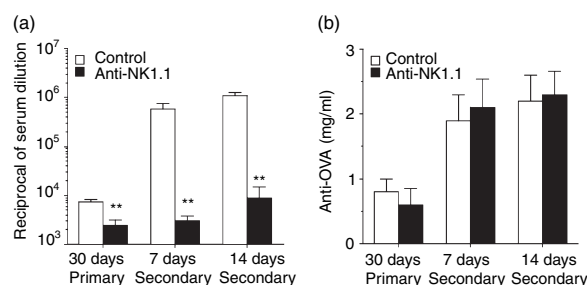


Figure 5. *In vivo* depletion of NK1.1⁺ cells led to a reduced humoral immune response to SEB in mice. (a) Shows the primary (30 days after priming) and secondary (7 and 14 days after booster) immune responses to staphylococcal enterotoxin B (SEB) in mice treated or not with anti-NK1.1 mAb or control IgG2a mAb (see Materials and methods section). Adult mice were immunized i.p. with 50 μg of SEB diluted in PBS and boosted with an equal amount of antigen i.p., 30 days after priming. Anti-SEB antibody levels are shown as titres, in which the average of the optical densities obtained with sera from control mice (non-immunized), diluted 1 : 100, was considered the end-point. The optical densities for each dilution of experimental sera were compared with control values. The titre for each sample was expressed as the reciprocal of the highest dilution that presented an absorbance value higher than the cut-off value. The columns represent the geometric means of serum titres (reciprocal of serum dilution) of six to eight mice for each experimental group, and the vertical bars represent the standard errors of the means. (b) Shows the primary (30 days after priming) and secondary (7 and 14 days after booster) immune responses to ovalbumin (OVA) in mice treated or not with anti-NK1.1 mAb or control IgG2a mAb (see Materials and methods section). The results (columns) represent the amount of OVA-specific antibodies (mg/ml) (see Materials and methods section) for each experimental group, and the vertical bars represent standard deviation of the means. Groups were of six mice each. Wilcoxon signed rank test was used (SEB). A P -value ≤ 0.05 was considered significant. Control and anti-NK1.1-treated groups were compared at each time-point. * $P \leq 0.05$, ** $P \leq 0.01$.

CD40L in the spleens of naive mice, whereas this percentage dropped ($44.6 \pm 8.2\%$) in the spleen cells from SEB-primed mice (data not shown). These results prompt us to investigate the expression of another molecule involved in T-cell helper function, namely ICOS. In contrast to the decreased percentage of CD40L⁺ NK T cells found after SEB immunization, the percentage of αβ⁺ NK1.1⁺ T cells expressing ICOS did not diminished upon SEB administration ($33.5 \pm 6.7\%$) of the total αβ⁺ NK1.1⁺ cell population in PBS-treated mice versus $41.5 \pm 10.1\%$ in SEB-treated mice.

Discussion

Neonatal tolerance is an established phenomenon that has been recognized for years.²⁰ Its establishment appears to be confined to a special phase during fetal and early development, when the immune system is prone to tolerate antigens rather than making classical immune

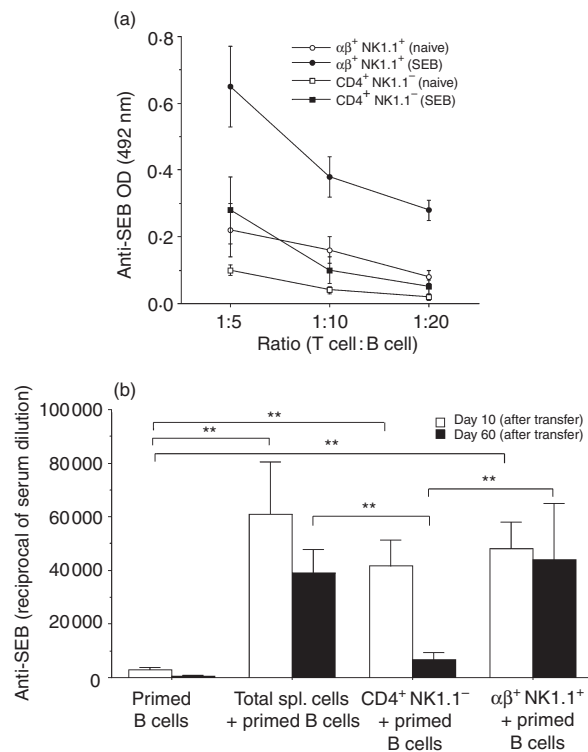


Figure 6. Purified $\alpha\beta^+$ NK1.1⁺ T cells from SEB-primed mice are able to deliver SEB-specific B-cell helper activity *in vitro* and *in vivo*. (a) Shows the levels of SEB-specific antibodies in culture supernatants. Purified $\alpha\beta^+$ NK1.1⁺ or CD4⁺ NK1.1⁻ T cells from naive or SEB-primed adult mice were titrated in cultures containing a SEB-primed B-cell-enriched population, in the presence of soluble anti-CD3, mAb as described in the Materials and methods section. After 7 days, supernatants were assayed for the presence of SEB-specific antibodies by ELISA. Results express the mean $\pm$ SD of ODs, obtained from triplicate cultures. (b) Shows the levels of SEB-specific antibodies in thymectomized, irradiated and bone marrow-reconstituted adult animals transferred with total spleen cells from SEB-primed animals or different purified T-cell subsets from SEB-primed animals together with SEB-primed B-cell-enriched populations, as described in the Materials and methods section. Anti-SEB antibody levels are shown as titres in which the average of the optical densities obtained with sera from control mice (non-immunized), diluted 1 : 100, was considered the end-point. The optical densities for each dilution of experimental sera were compared with control values. The titre for each sample was expressed as the reciprocal of the highest dilution that presented an absorbance value higher than the cut-off value. The columns represent the geometric means of serum titres (reciprocal of serum dilution) of six to eight mice for each experimental group, and the vertical bars represent the standard errors of the means ($n = 4$ mice/group). Wilcoxon signed rank test or Kruskal–Wallis test was used. A P -value ≤ 0.05 was considered significant. * $P \leq 0.05$, ** $P \leq 0.01$.

responses.²⁰ Many hypotheses have been raised to explain it,^{20,21} including that neonatal tolerance results from a T-cell inability to produce IL-2,^{22,23} from the high numbers of non-functional immature T cells and low numbers

of mature effector T cells,²⁰ from lack of professional antigen-presenting cells,^{24,25} and from the high activity of suppressor cells.²⁶ More recently, it has been proposed that T-cell migration patterns in a neonatal environment might be of importance in establishing tissue-specific tolerance at birth.²⁷ On the other hand, it has been shown that neonatal tolerance in a viral infection model is not always achieved.²⁸ In addition, it was demonstrated that the neonatal mouse immune system produces cytokines related mainly to the Th2 pathway, thus providing the conditions for inhibiting Th1 immune responses i.e. inducing immune deviation.^{29–31} Immune deviation by Th2 cells might be associated with the presence of ‘regulatory’ T cells that produce large amounts of transforming growth factor- β (TGF- β), which can inhibit lymphocyte activities.³⁰ These studies suggest that neonatal mice are able to mount a conventional Th2-type immune response and in some cases partial tolerance may be achieved by immune deviation.

In vivo administration of superantigens leads to marked deletion and complete *in vitro* proliferative anergy of the responder T-cell population.² Paradoxically, SEB administration in adult mice induces a vigorous humoral immune response to this superantigen.¹¹ It is demonstrated herein that mice injected with 50 μ g SEB within 24 hr of birth, followed by weekly injections of the same amount of SEB, developed a very pronounced splenomegaly as the result of an increase in the total numbers of B cells. On the other hand, the total numbers of splenic $\alpha\beta^+$ T cells decreased only slightly and the numbers of NK1.1⁺ $\alpha\beta^-$ cells remained constant. These findings suggest that multiple injections of SEB cause polyclonal activation of B cells and a reduction in the numbers of single positive splenic T cells as a result of the loss of V β 8⁺ conventional T cells. However, the total numbers of splenic $\alpha\beta^+$ T cells were not reduced to the same extent in this model. The latter observation indicates that these mice do show an increase in the number of splenic double-negative T cells. In fact, a small increase in the total numbers of splenic NK1.1⁺ $\alpha\beta^+$ T cells was found in these mice (Fig. 1c). These results suggest that NK1.1⁺ T cells, in contrast to conventional T cells, may be subjected to expansion rather than deletion upon repeated SEB injections. Similar results were obtained in adult mice after two injections of SEB, thus indicating that the neonatal environment was not responsible for the lack of NK T-cell deletion (Fig. 7). Comparable findings were described recently for the glycolipid antigen α -galactosylceramide.³² This observation was taken to be the result of the strong up-regulation of anti-apoptotic genes in this particular T-cell lineage.³³ However, other possibilities remain. For example, SEB or SEB-derived peptides could be presented in the context of CD1 instead of the conventional MHC class II molecules, as suggested for other superantigens.³⁴ In addition, one may not rule out that the expression of

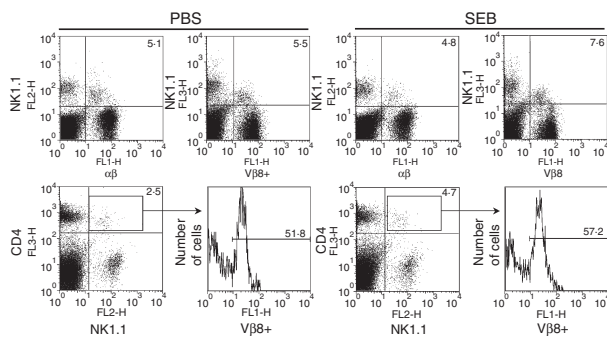


Figure 7. NK1.1⁺ αβ⁺, NK1.1⁺ Vβ8⁺, NK1.1⁺ CD4⁺ and NK1.1⁺ CD4⁺ Vβ8⁺ T cells are not deleted after repeated SEB injections. Spleen cells from adult F₁ (BALB/c × C57BL/6) mice treated with PBS or SEB were counted, stained and evaluated by flow cytometry as described in the Materials and methods section. Plots are representative of one out of five mice/group. Small numbers on the top right of each dot plot represent the percentage of each T-cell subpopulation among the population represented in the x-axis. Mice were analysed individually. Student *t*-test was used. A *P*-value ≤ 0.05 was considered to be significant.

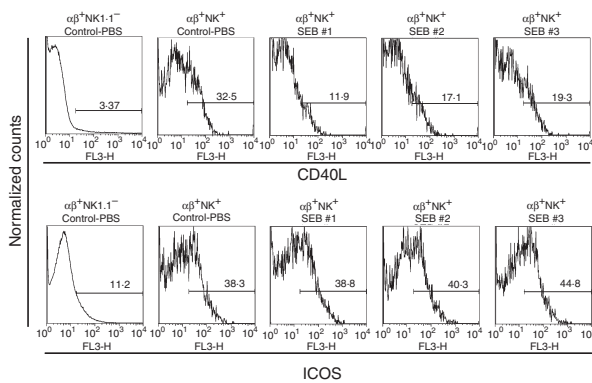


Figure 8. Expression of CD40L and ICOS in splenic NK1.1⁺ T cells from naive or SEB-primed mice. Splenic NK1.1⁺ αβ⁺ T cells from adult F₁ (BALB/c × C57BL/6) mice that received one injection of 50 µg of SEB 14 days before were gated and the percentages of CD40L⁺ cells inside this population are shown in the upper dot plots (one PBS-treated mouse and three SEB-treated mice are shown). An internal control showing the percentage of CD40L⁺ cells in gated αβ⁺ NK1.1⁻ T cells from PBS-treated mice is also shown. Lower dot plots represent gated αβ⁺ NK1.1⁺ cells and the percentages of ICOS⁺ cells inside this population (one PBS-treated mouse and three SEB-treated mice are shown). An internal control showing the percentage of ICOS⁺ cells in gated αβ⁺ NK1.1⁻ T cells from PBS-treated mice is also shown. Experiments were performed three times with similar results. Student's *t*-test was used. A *P*-value ≤ 0.05 was considered to be significant.

Vα14-Jα281 by most of the NK1.1⁺ T cells diminishes the overall affinity of the Vβ8 chain for the SEB-MHC class II complex. Such experimental evidence has already been provided in other models, indicating that the Vα chain might also be of importance in such an interaction.³⁵

The data presented herein also showed a conspicuous production of SEB-specific IgG1 and IgE antibodies in mice treated repeatedly with SEB. In addition, there was an increase in the levels of total IgE in the sera of SEB-treated mice. These observations indicate that SEB treatment did not induce tolerance at the level of antibody production, in spite of the fact that SEB was given in the first 24 hr after birth and *in vitro* SEB restimulation led to a clear-cut proliferative anergy with low production of IFN-γ and increased IL-4 production by splenic cells. The production of large amounts of SEB-specific IgG1 and detectable amounts of SEB-specific IgE, as well as increased levels of non-specific serum IgE, is indicative of the production of IL-4 *in vivo*. We could show here that NK T cells from SEB-primed mice produced IL-4 when stimulated with anti-CD3 and anti-CD28 mAbs *in vitro*, indicating that SEB-primed NK T cells were not functionally anergic. To evaluate the T-cell phenotype responsible for the B-cell helper activity *in vivo*, transfers of total spleen cells or spleen cells depleted of specific T-cell subpopulations from SEB-primed mice to thymectomized, lethally irradiated and SEB-challenged mice were performed. The results of these experiments indicate that T cells bearing NK1.1 molecules in addition to the Vβ8 TCR chain confer to irradiated and thymectomized recipients the ability to mount a secondary type of humoral immune response to SEB, i.e. they participate in the help process.

It was previously suggested that NK T cells could function as helper cells.^{36–40} This hypothesis was based on the fact that NK T cells are positively selected by CD1-bearing cells in the thymus,⁴¹ on their profile of cytokine production,^{13,42} and on the ability of some NK T-cell hybridomas to recognize dendritic cells or activated B lymphocytes *in vitro*.^{43,44} The data shown here clearly suggest that NK T cells are required for B-cell helper activity upon SEB immunization, because depletion of NK cells by using anti-NK1.1 mAb administration *in vivo* strongly diminished the development of a normal primary or secondary humoral immune response to SEB. Taken together, this set of experiments gives a clear indication that in some situations, such as immunization with superantigens, NK T cells might be responsible for helping B cells to produce specific antibodies. In addition, NK T cells were not required for the development of a humoral immune response to OVA in adult mice.

It is worthwhile to point out that a secondary immune response is achieved upon immunization with SEB and, apparently, the memory response may involve the participation of conventional CD4 T cells, as shown for other experimental models.^{45,46} However, the data presented herein indicate that conventional CD4⁺ T cells are much less effective than NK T cells either *in vitro* or *in vivo* in providing help to B cells in this superantigen system. This may be because of a possible differentiation of the major-

ity of these conventional CD4⁺ T cells to Th3 pattern, with high levels of TGF- β production,^{30,47} following SEB stimulation. In fact, the high levels of SEB-specific IgG2b found during SEB immunization indicate that TGF- β is produced during immune response to SEB.¹¹ TGF- β -producing T cells are able to suppress the proliferation of T cells either *in vitro* or *in vivo*, thus arguing that this phenotype might justify, in part, the 'anergy' during *in vitro* SEB re-stimulation.⁴⁸

The majority of NK T cells already present a memory phenotype as they express high levels of CD44 molecules.⁴⁹ It was demonstrated herein and by others⁴⁰ that a considerable proportion of spleen NK T cells constitutively express CD40L. These results indicate that natural NK T cells are already able to provide B-cell help. Besides, it is known that recently thymic emigrant NK T cells secrete large amounts of IL-4 and acquire the ability to secrete IFN- γ after some time parked in lymphoid peripheral tissues.⁴² Therefore, it seems reasonable to assume that NK T cells are not committed to a given functional pattern of lymphokine production as soon as they leave the thymus, opening the possibility that different environmental antigenic experiences could lead NK T cells to secrete more IL-4 (type-2) or IFN- γ (type-1). In fact, the down-modulation of CD40L upon SEB priming could reflect 'type-2' NK T cells. It was demonstrated, previously, that the ligation of CD40L is of fundamental importance for NK T cells to secrete IFN- γ .⁵⁰ Our results would be consistent with the possibility that the down-modulation of CD40L on NK T cells by SEB activation would result in a Th2 functional pattern by the lack of CD40L coaggregation upon re-stimulation. In addition to CD40L molecules, ICOS was also described as crucial for the development and maintenance of humoral immune responses.⁵¹ Our results showed that the percentage of ICOS-expressing NK T cells did not diminish after SEB priming; thus, indicating that the NK T-cell helper activity could be mediated by ICOS instead of CD40L in this model.

In summary, and judging by the immunoglobulin isotype profile produced during the immune response to SEB, it could be hypothesized that SEB stimulation induces the differentiation of 'type 2' NK T cells. Therefore, part of the immunological memory to SEB would rely on NK T-cell functional differences rather than on clonal proliferation.

Herein, we demonstrated a possible role for NK T cells as helper cells in the immune response to a superantigen. It was clearly shown that SEB administration in neonatal mice did not result in the establishment of tolerance at the level of specific antibody production. On the contrary, repeated injections of SEB appear to induce a huge polyclonal B-cell activation and immune deviation to a Th2-type immune response. It was also shown that NK T cells are largely responsible for such

B-cell helper activity with less involvement of conventional CD4⁺ T cells. We also provide evidence that could explain the inability of inducing neonatal tolerance in some models where virus infections are used,²⁸ since it has been found that many viruses do express superantigen-like molecules.⁵² Therefore, the results reported herein indicate that superantigen-reactive NK T cells are functional in providing B-cell help *in vivo*, whilst making tolerance unachievable. This could help to explain the paradox of neonatal and adult immune responses in conditions where tolerance should be expected.^{11,25,53} These results also provide some clues for the development of vaccines and immunity to some bacteria, such as *Staphylococcus aureus*, as well as explaining the presence of large amounts of IgE antibodies to *Staphylococcus* enterotoxins in patients suffering from severe asthma, persistent allergic rhinitis or myelitic patients with hyper-IgEmia.^{54–56}

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