

## Response of T cells *in vivo* Induced by Repeated Superantigen Treatments at Different Time Intervals

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**Abstract** We have investigated the response of T cells to staphylococcal enterotoxin A (SEA) injections *in vivo*. We found that a single injection of SEA with an optimal dose of 10  $\mu$ g increased the expression of both CD4 and CD8 significantly. There was expansion of SEA-reactive T cells *in vivo* after SEA re-injection and the time interval between injections strongly influenced the responsiveness of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Anergy of T cells was observed after three SEA treatments. The time interval between injections mainly affected the unresponsiveness of CD4<sup>+</sup> T cells, not CD8<sup>+</sup> T cells. Marked deletion followed by anergy of CD4<sup>+</sup> T cells was induced at short intervals, and anergy without obvious deletion of CD4<sup>+</sup> T cells was induced at long intervals. We also found that the anergic state was reversible *in vivo*. Repeated SEA stimulation led to down-regulation of interleukin (IL)-2, and high levels of IL-10. This study showed that both CD4<sup>+</sup> and CD8<sup>+</sup> SEA-primed T cells were responsive to SEA rechallenge *in vivo*, and a third injection was needed to induce the anergy of T cells.

**Keywords** response; T cell; superantigen; time interval

Superantigens are bacterial and viral proteins that bind directly to major histocompatibility complex (MHC) class II molecules without being processed and subsequently activate T cells expressing specific T cell receptor V $\beta$  chains [1,2]. Previous studies have shown that exposure of peripheral T cells to staphylococcal enterotoxin A (SEA) or staphylococcal enterotoxin B (SEB) results in the expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and production of cytokines such as interleukin (IL)-2, interferon- $\gamma$  and tumor necrosis factor- $\alpha$  [3–5]. Superantigens also direct cytolytic T cells to kill MHC class II-expressing target cells [6,7]. Apart from their strong ability to activate T cells, superantigens were reported to induce a subsequent state of unresponsiveness characterized by anergy in the remaining T cells [8–10]. Anergy is one of the mechanisms of tolerance, typified by unresponsiveness of T cells and poor IL-2 production [11,12]. This property of superantigens strongly limits their antitumor therapy effect.

The response of T cells induced by superantigens has

been studied extensively, however, there are few published reports about the relationship between the induction of anergy and repeated superantigen treatments at different time intervals.

This study was undertaken to evaluate the responses of T cells to SEA injections at different intervals *in vivo*. We found a third injection was needed to induce the anergy of T cells, and the time interval between injections played an important role in the response of CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

## Materials and Methods

### Animals and treatment

Eight to ten-week-old C57BL/6 mice (H-2<sup>b</sup>) were purchased from the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). Mice were housed in microisolation in a dedicated, pathogen-free facility, and all animal experimentation was conducted in accordance with animal ethics guidelines. Mice were injected once intraperitoneally (i.p.) with different doses

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of SEA in 0.2 ml phosphate-buffered saline (PBS). The optimal dose of SEA was injected i.p. repeatedly to mice at different time intervals. Control mice received the same volume of PBS.

### Proliferation assays

Proliferation of spleen cells was detected by  $^3\text{H}$ -TdR assay. Spleens were removed from SEA-primed or PBS-primed mice and single-cell suspensions were prepared and cultured ( $10^6$  cells/well) in 100  $\mu\text{l}$  of culture medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 1% sodium pyruvate, 10% fetal calf serum of  $10^{-5}$  M mercaptoethanol) at 37  $^\circ\text{C}$  with 5%  $\text{CO}_2$ . After 72 h in culture, cells were labeled with [ $^3\text{H}$ ]thymidine (1  $\mu\text{Ci}/\text{well}$ ) for 6 h then harvested. Incorporation of [ $^3\text{H}$ ]thymidine was determined using a liquid scintillation  $\beta$ -counter. Data were presented as follows: SI (stimulation index)=c.p.m. experimental/c.p.m. control (cultured without SEA). Only  $\text{SI} \geq 2$  was considered to indicate significant proliferation.

### Antibodies and fluorescence-activated cell sorting analysis

The antibodies used for flow cytometry, anti-CD4-phycoerythrin (PE), anti-CD8-PE, anti-IL-2-PE, anti-IL-10-PE and anti-CD3-fluorescein-isothiocyanate, were purchased from Biologend (San Diego, USA). Samples were stained and analyzed on a FACSCalibur cytometer using CellQuest software (BD Biosciences, Becton, USA). For analysis of intracellular IL-2 and IL-10, cells were fixed with 2% paraformaldehyde and permeabilized using 0.5% saponin before staining.

### Cytokine assays

The samples of serum were collected at various time points after SEA treatments *in vivo*, and were stored at  $-70$   $^\circ\text{C}$  until tested for cytokine content. The levels of IL-2 and IL-10 in sera were measured with sandwich enzyme-linked immunosorbent assay kits (Sigma, USA) according to the manufacturer's instructions.

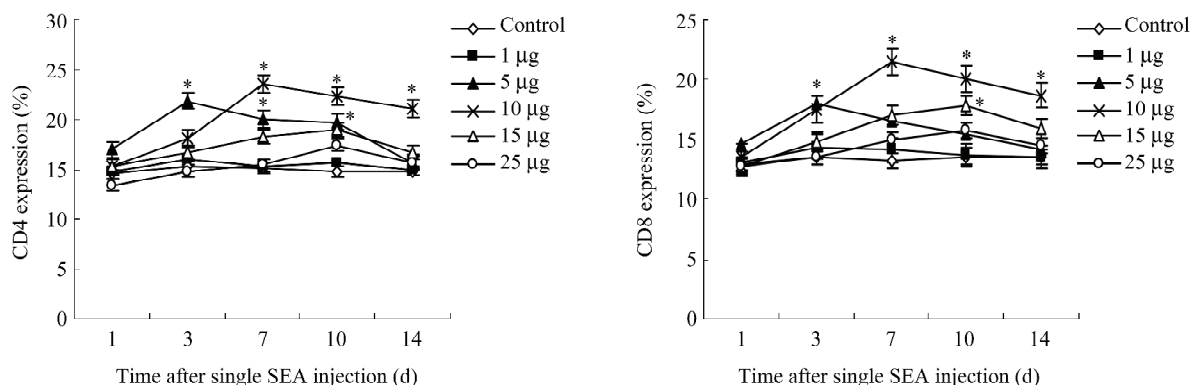
### Statistical analysis

One-way ANOVA was used to determine differences in immune responses among the various treatment groups. Newman-Keuls tests were carried out as post-hoc analysis for one-way ANOVA. A  $P$  value of less than 0.05 was considered significant.

## Results

### Responses of T-cell subsets after single SEA challenge *in vivo*

In order to choose the optimal dose of SEA *in vivo*, spleen cells were analyzed for the expression of CD4 and CD8 after mice were injected i.p. with different doses of SEA (Fig. 1). The results showed that there were obvious increases of the two subsets during the first 14 d after different doses of SEA challenge except 1  $\mu\text{g}$ . The most efficient dose of SEA was 10  $\mu\text{g}$ , which led to a significant up-regulation of CD4 and CD8 expressions from day 7 to day 14 after injection. The ratio of CD4 and CD8 after SEA challenge was similar to the ratio before SEA challenge,

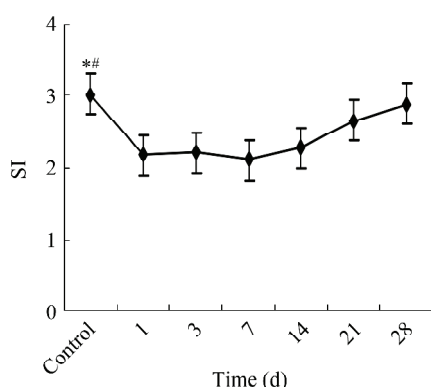


**Fig. 1** Expression of CD4 and CD8 T cells after single injection of staphylococcal enterotoxin A (SEA) *in vivo*

Mice were given an intraperitoneal injection of different doses of SEA or phosphate-buffered saline (control). Spleens were removed on different days after SEA injection and spleen cells were double stained with anti-CD4 or anti-CD8 monoclonal antibodies in combination with anti-CD3 monoclonal antibody for fluorescence-activated cell sorting analysis. The results are from three independent experiments, with three mice used in each experiment. \* $P < 0.01$  compared to the same day in the control group.

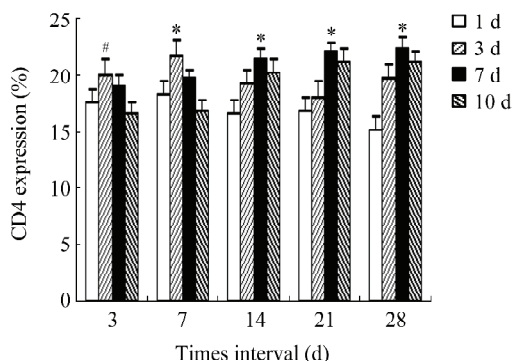
which showed the strong proliferative response of both CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells stimulated by SEA.

To examine whether the primed T cells *in vivo* have proliferative capability, spleens were removed at different days after SEA injection and the spleen cells were restimulated with SEA (100 ng/ml) *in vitro* (Fig. 2). Proliferation of the primed T cells was seen (SI>2), but it was clearly lower than that of the control. We also found that the proliferative capability could be gradually recovered. After 28 d, the responsiveness of the primed cells was similar to that of the control.



**Fig. 2** Staphylococcal enterotoxin A (SEA)-primed spleen cells in response to SEA stimulation *in vitro*

Mice were given an intraperitoneal injection of 10 µg SEA. Spleens were removed at different days after SEA injection and the spleen cells were cultured for 72 h with 100 ng/ml SEA *in vitro*. The proliferation was detected by <sup>3</sup>H-TdR assay. Only a stimulation index (SI)≥2 was considered to be significant proliferation. The control was normal spleen cells stimulated with SEA. The results are from three independent experiments, with three mice used in each experiment. \*P<0.01 as the control compared to 1–14 d after SEA injection; #P<0.05 as the control compared to 21 after SEA injection.



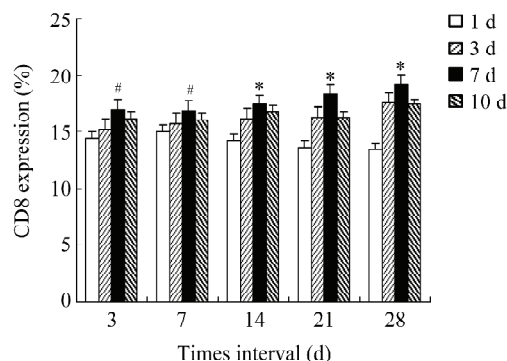
### Expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells after two SEA injections

SEA was injected twice i.p. at different time intervals, and the responses of T cells in spleen were analyzed (Fig. 3). We found that there was still expansion of SEA-reactive T cells *in vivo* after SEA re-injection. However, the interval between SEA injections resulted in different expressions of CD4 and CD8. The peak of CD4 appeared at day 3 (3 d and 7 d intervals) and at day 7 (14, 21 and 28 d intervals), whereas the peak of CD8 only appeared at day 7. The interval between two injections led to different ratios of CD4 and CD8. The ratio decreased from approximately 1.35 to 1.15 10 d after SEA injections at short intervals (3 d and 7 d), but the ratio was not changed at long intervals (such as 28 d).

### Energy of CD4<sup>+</sup> and CD8<sup>+</sup> T cells induced by multiple SEA injections

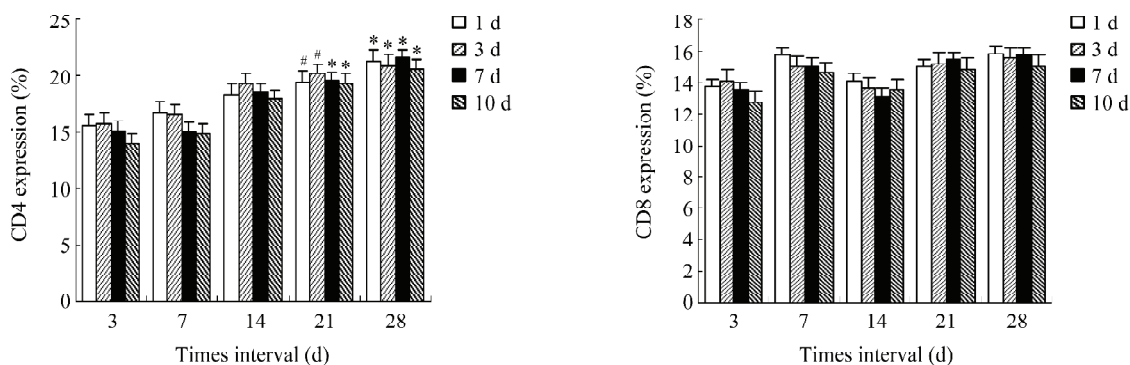
To analyze the influence of the number of SEA injections on the induction of T cell energy, mice were injected i.p. three times at different time intervals (Fig. 4). Although unresponsiveness of T cells was observed following SEA treatments, CD4 expressions at short intervals were much lower than those at long intervals. The expression of CD8 was not significantly affected by the interval. These data suggested that the time of interval between injections mainly affected the unresponsiveness of CD4<sup>+</sup> T cells, not CD8<sup>+</sup> T cells. Marked deletion followed by anergy of CD4<sup>+</sup> T cells was induced at short intervals, and anergy without obvious deletion of CD4<sup>+</sup> T cells was induced at long intervals.

In an attempt to evaluate the changes of the unrespon-



**Fig. 3** Effects of time interval on T cells after two staphylococcal enterotoxin A (SEA) injections

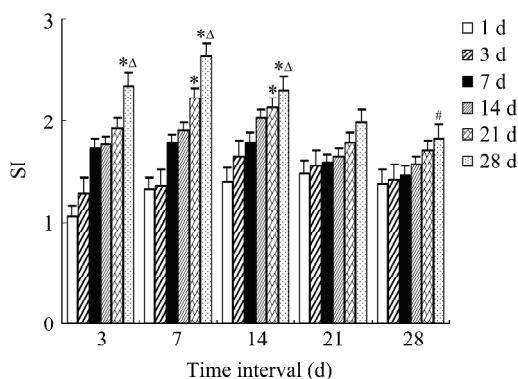
Mice were injected twice intraperitoneally with 10 µg of SEA at different time intervals. Spleens were removed on different days after last injection and spleen cells were double stained with anti-CD4 or anti-CD8 monoclonal antibodies in combination with anti-CD3 monoclonal antibody for fluorescence-activated cell sorting analysis. The results are from three independent experiments, and three mice were used in each experiment. \*P<0.01 and #P<0.05 compared to day 1 in the same group.



**Fig. 4** Effects of three staphylococcal enterotoxin A (SEA) injections at different time intervals on T cells

Mice were injected three times intraperitoneally with 10  $\mu$ g of SEA at different time intervals. Spleens were removed on different days after the last injection and spleen cells were double stained with anti-CD4 or anti-CD8 monoclonal antibodies in combination with anti-CD3 monoclonal antibody for fluorescence-activated cell sorting analysis. The results are from three independent experiments, with three mice used in each experiment. \* $P$ <0.01 and # $P$ <0.05 compared with the same day after SEA injections at 3 d and 7 d intervals.

siveness of T cells *in vivo*, spleens were removed at different days after the last injection, and the proliferative capability of anergic T cells *in vitro* was examined by  $^3$ H-TdR assay (Fig. 5). We found that the partial responsiveness appeared at day 28 after the last injection with the 3 d interval, and at day 21 with the 7 d and 14 d interval. However, there was no significant proliferative response (SI<2) until 28 d after the last injection with the 21 d and 28 d interval.



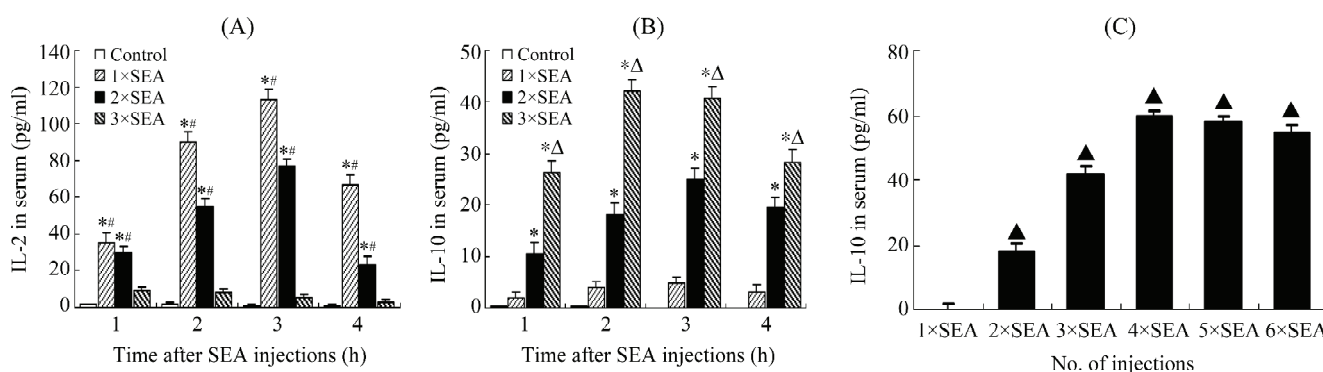
**Fig. 5** Response of spleen cells *in vitro* after mice were injected three times with staphylococcal enterotoxin A (SEA)

Mice were injected three times intraperitoneally with 10  $\mu$ g of SEA at different time intervals. Spleens were removed on different days after the last injection. Spleen cells were cultured with 100 ng/ml SEA, and the proliferation was examined by  $^3$ H-TdR assay after 72 h. A stimulation index (SI) $\geq$ 2 was considered to be significant proliferation. The results are from three independent experiments, with three mice used in each experiment. \* $P$ <0.01 and # $P$ <0.05 compared to day 1 in the same group.  $\Delta$  $P$ <0.05 compared with the same day after SEA injections at 21 d and 28 d intervals.

### IL-2 and IL-10 levels after repeated SEA injections

It is well established that T-cell anergy is associated with a failure to produce IL-2 [9,13] and early studies have suggested that anti-inflammatory cytokine IL-10 was completely dominating the immune response after repeated SEA injections [14,15], which led us to examine the levels of IL-2 and IL-10 after SEA treatments and describe the anergic state *in vivo*. Large amounts of IL-2 were released in serum and maximal production was observed 3 h after a single SEA injection, whereas IL-10 in serum was almost undetected. The second SEA challenge given 3 d later resulted in lower levels of IL-2 in serum, but significant levels of IL-10 were observed. Higher IL-10 levels and almost no IL-2 production were seen after the third stimulation [Fig. 6(A,B)]. To investigate the relationship between the IL-10 production and the times of SEA treatments, we analyzed the levels of IL-10 in serum after repeated SEA injections. High IL-10 production was induced by repeated SEA injections, and the fourth SEA challenge resulted in the highest level of IL-10 [Fig. 6(C)].

To determine whether the serum levels were in accordance with the content of T cells, intracellular levels of both cytokines were analyzed. An obvious up-regulation of intracellular IL-2 could be observed as early as day 3 after the SEA injection, but the expression of IL-10 in T cells was very low. The level of IL-2 in cells went on increasing and the peak appeared at day 7 following two injections; at the same time intracellular IL-10 began to be gradually up-regulated. Three SEA treatments led to quick reduction of intracellular IL-2 expression and higher levels of IL-10, which could be maintained for at least



**Fig. 6 Interleukin (IL)-2 and IL-10 concentrations in sera after staphylococcal enterotoxin A (SEA) treatment**

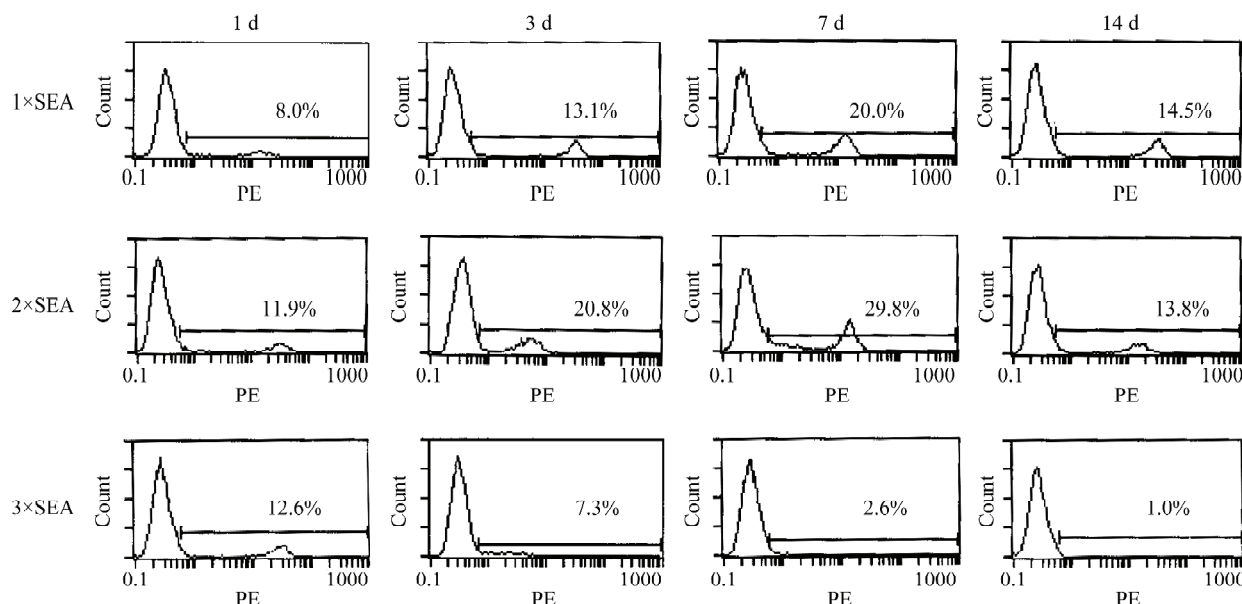
Mice were injected intraperitoneally various times at 3 d intervals with 10 µg of SEA or phosphate-buffered saline (control). Sera were collected at the indicated time points for analysis of IL-2 and IL-10 content. The results are from three independent experiments, with three mice used in each experiment. (A) IL-2 production in serum. (B) IL-10 production in serum. (C) IL-10 production in serum collected 2 h after multiple injections. \**P*<0.01 compared to the same time in the control group; #*P*<0.01 compared to the same time in the 3xSEA group; Δ*P*<0.01 compared to the same time in the 2xSEA group; ▲*P*<0.01 compared with the 1xSEA group.

14 d (Figs. 7 and 8).

## Discussion

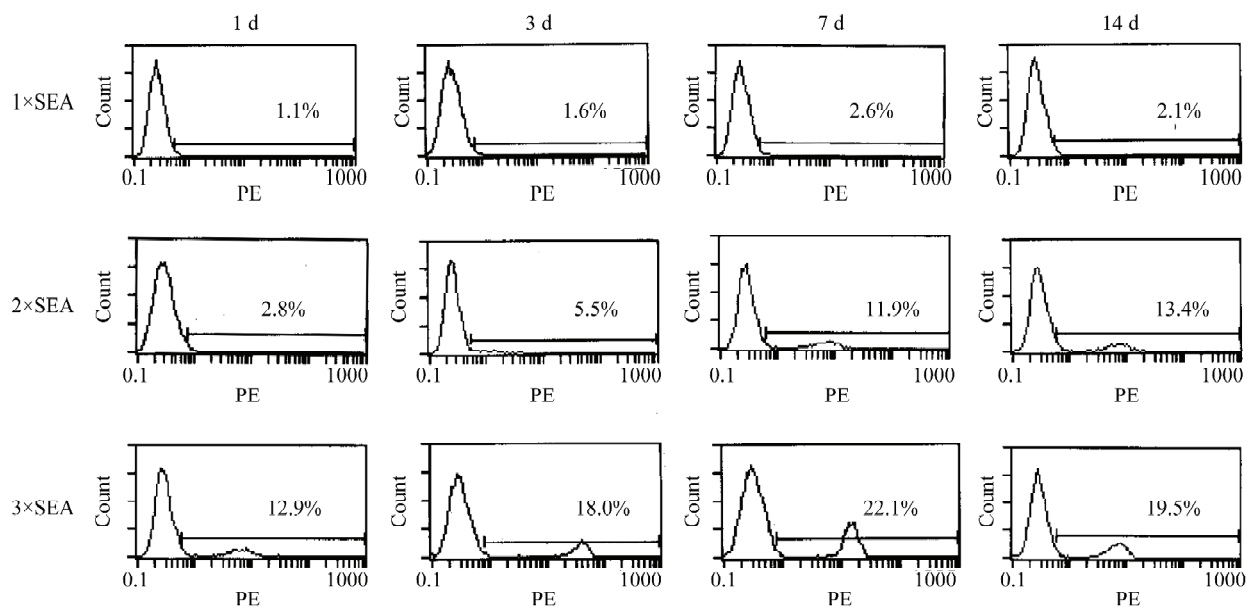
In this study, we have investigated the response of T cells to repeated superantigen treatments *in vivo*. A significant increase of T cells was seen after a single SEA

injection, with the optimal dose being 10 µg, which led to the same up-regulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Several investigators have shown that the anergic state of T cells could be induced by repeated superantigen treatments [16–18]. But controversy remained about the relationship between the number of superantigen treatments and the anergic state. Miethke *et al.* reported that SEB caused L-selectin down-regulation within 30 min and that SEB-



**Fig. 7 Fluorescence-activated cell sorting (FACS) analysis of intracellular interleukin (IL)-2 after staphylococcal enterotoxin A (SEA) treatment**

Mice were injected intraperitoneally various times at 3 d intervals with 10 µg of SEA. Splens were removed at the indicated time points after the last injection. The splenic cells were fixed with 2% paraformaldehyde and permeabilized using 0.5% saponin then stained for analysis of intracellular IL-2 by FACS. Cells were gated on forward and side scatter and 5000 cells were analyzed. Representative results of three similar experiments.



**Fig. 8** Fluorescence-activated cell sorting (FACS) analysis of intracellular interleukin (IL)-10 after staphylococcal enterotoxin A (SEA) treatment

Mice were injected intraperitoneally various times at 3 d intervals with 10  $\mu$ g of SEA. Spleens were removed at the indicated time points after the last injection. Spleen cells were fixed with 2% paraformaldehyde and permeabilized using 0.5% saponin then stained for analysis of intracellular IL-10 by FACS. Cells were gated on forward and side scatter and 5000 cells were analyzed. One of three similar experiments.

primed mice showed unresponsiveness to subsequent SEB challenge *in vivo* as early as 2–3 h after priming [19–21]. Sundstedt *et al.* also showed that a 7 d interval between two SEA injections resulted in proliferative hyporesponsiveness [2]. Aoki and Yoshikai showed that the second injection of SEA augmented clonal expansion of SEA-reactive T cells in mice primed with SEA 2 d previously [22]. We observed that the second injection of SEA accelerated the proliferation of SEA-primed T cells *in vivo*. Additionally, spleen cells from mice primed by SEA could proliferate in response to SEA restimulation *in vitro*. These data suggested that SEA-primed T cells retain the potential to proliferate after rechallenge with SEA. However, the responsiveness of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were affected by the time interval between injections. A short interval resulted in a quick and transitory increase of CD4<sup>+</sup> T cells and a slow increase of CD8<sup>+</sup> T cells. A long interval resulted in the same slow and prolonged proliferation of both subsets. There were important differences between other research and our own, such as the time interval between injections and the days observed. Our results showed the short interval resulted in down-regulation of CD4<sup>+</sup> T cells after 3 d and no obvious increase of CD8<sup>+</sup> T cells before 7 d after the last injection, which might lead to a different conclusion.

Previous studies showed that apart from the ability to proliferate T cells *in vivo*, superantigens induce a subsequent state of unresponsiveness, which include at least two different mechanisms, physical elimination (deletion) and functional inactivation (anergy) [23–26]. We observed that multiple SEA treatments at short intervals only resulted in a marked reduction in the number of CD4<sup>+</sup> T cells followed by an anergic state. Interestingly, CD4<sup>+</sup> and CD8<sup>+</sup> T cells appeared unresponsive without an obvious decrease in number after three SEA treatments at long intervals. These results suggested a third SEA injection was required for induction of anergy, and the time interval between SEA treatments strongly influenced the deletion of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells. The number of CD4<sup>+</sup> T cells decreased following multiple SEA injections at short intervals than those at long intervals, then the remaining SEA-reactive T cells showed failure to respond *in vitro*. However, the unresponsiveness was reversible *in vivo*. Although the proliferative capability of spleen cells from the mice injected with SEA three times was gradually restored, the period for recovery was affected by the interval between injections. That is to say, the longer the interval, the longer time the anergic state would last *in vivo*, which hinted that we might artificially control the period of anergy of T cells.

Previous studies have shown that the failure to produce normal levels of IL-2 appears to be a critical hallmark of anergy induction [27–29]. In our study, it was shown that SEA accelerated the expansion of T cells, and the activation phase was followed by unresponsiveness after repeated SEA treatments *in vivo*. In an effort to prove the anergic state, we examined the levels of IL-2. A single SEA injection gave rise to rapid production of IL-2, and two treatments triggered a second wave of release of intracellular IL-2. T cells were unable to produce IL-2 in response to three SEA treatments. IL-10 has been reported to be induced after repeated SEA challenges *in vivo*, which might act as an inhibitor to help inducing anergy [30–32]. We found IL-10 levels were opposite to the levels of IL-2. Repeated SEA challenges resulted in a stable up-regulation of IL-10 production, and high levels of IL-10 were released by anergic T cells after last injection. These results were not only in accordance with previous studies, but also suggested IL-10 was related to the maintenance of anergy.

In conclusion, this study showed that both CD4<sup>+</sup> and CD8<sup>+</sup> SEA-primed T cells have the responsiveness to SEA rechallenge *in vivo*, and a third injection was required to induce the anergy of T cells. The time interval between injections played an important role in the response of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The relationship between IL-2 and IL-10 might reflect the response of T cells to superantigens.

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