Induction of Unresponsiveness and Impaired T cell Expansion by Staphylococcal Enterotoxin B in CD28-deficient Mice

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Summary
We used CD28-deficient mice to analyze the importance of CD28 costimulation for the response against Staphylococcal enterotoxin B (SEB) in vivo. CD28 was necessary for the strong expansion of VÎ¸B8+ T cells, but not for deletion. The lack of expansion was not due to a failure of SEB to activate VÎ¸B8+ T cells, as VÎ¸B8+ T cells from both CD28−/− and CD28+/+ mice showed similar phenotypic changes within the first 24 h after SEB injection and cell cycle analysis showed that an equal percentage of VÎ¸B8+ T cells started to proliferate. However, the phenotype and the state of proliferation of VÎ¸B8+ T cells was different at later time points. Furthermore, in CD28−/− mice injection with SEB led to rapid induction of unresponsiveness in SEB responsive T cells, indicated by a drastic reduction of proliferation after secondary SEB stimulation in vitro. Unresponsiveness could also be demonstrated in vivo, as CD28−/− mice produced only marginal amounts of TNFα after rechallenge with SEB. In addition CD28−/− mice were protected against a lethal toxic shock induced by a second injection with SEB. Our results indicate that CD28 costimulation is crucial for the T cell–mediated toxicity of SEB and demonstrate that T cell stimulation in the absence of CD28 costimulation induces unresponsiveness in vivo.

Stimulation of the T cell receptor (TCR) alone is not sufficient for optimal T cell activation. Costimulatory signals are required in addition for the induction of maximal T cell response (1, 2, 3). CD28 is probably the most important costimulatory molecule for T cells. Ligands for CD28 are B7-1 (CD80) and B7-2 (CD86), both expressed on B cells, activated monocytes, dendritic cells, and activated T cells (2). It has been shown that blocking the interaction between CD28 and the B7 molecules with monoclonal antibodies or with a CTLA-4-Ig fusion protein can abolish T cell proliferation in vitro. Furthermore, the CTLA-4-Ig fusion protein is able to reduce the immune response in different in vivo models (reviewed in reference 2), indicating that the interaction of CD28 with its ligands is crucial for a functional immune response.

Mice deficient for CD28 contain normal numbers of peripheral CD4+ and CD8+ T cells (4). Positive and negative selection appear not to be affected in either normal or TCR transgenic CD28−/− mice (4, 5). T cells from CD28−/− mice show strong impairment of proliferation in vitro after stimulation with anti-TCR antibodies or concanavalin A, after allostimulation, and after antigen specific stimulation (4, 5, 6). CD28−/− mice have normal serum levels of IgM but reduced levels of serum IgG1, indicating that T helper function, important for Ig class switching, is impaired. Consistent with this, CD28−/− mice produce normal amounts of neutralizing IgM after infection with Vesicular Stomatitis Virus, a response known to be T helper cell independent, but reduced levels of IgG, a response which is T helper cell dependent (4). Interestingly, the response against Lymphocytic Choriomeningitis Virus is not affected in CD28−/− mice. CD28 therefore seems to be differentially required for T cell responses and alternative costimulatory pathways may exist (4).

Bacterial superantigens (BSAg)1 are bacterial exotoxins secreted by staphylococci, streptococci and other bacteria (7, 8). BSAg bind to MHC class II molecules (9) and the MHC class II–BSAg complex interacts with the TCR and induces T cell activation (7, 8, 10). In contrast to lectins, BSAg display specificity for the variable domain of the TCR β chain and activate only T cells that express certain TCR β chains (11, 12). Furthermore, T cell stimulation seems to be independent of CD4 and CD8, since cells of

1 Abbreviations used in this paper: BSAg, bacterial superantigen; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B.
both T cell populations get activated by the BSAg-MHC class II complex (13). The in vivo effects of BSAg, especially of some of the staphylococcal enterotoxins (Staphylococcal enterotoxin A and B, Toxic Shock Syndrome Toxin-1), are well analyzed. Injection of BSAg induces strong T cell responses in vivo. A single injection leads to massive expansion of T cells with responsive TCRVβ-elements, that peaks at day three, followed by deletion at day 5–7, which lasts for several weeks (14, 15, 16). At these later time points T cells are in a state of unresponsiveness, as they don’t proliferate after stimulation with the particular BSAg, in spite of expressing a TCR β chain that can interact with the BSAg (14, 17, 18).

In this study we have analyzed the response of T cells against the BSAg SEB in mice lacking CD28 and found CD28 necessary for the expansion of SEB responsive T cells but not for the deletion of this T cell population in vivo. T cells of both CD28−/− and CD28+/+ mice showed the same phenotypic changes within the first 24 h after injection of SEB, but were different in their phenotype at later time points. In addition, cell cycle analysis indicated that after 24 h an equal percentage of Vβ8+ T cells are proliferating in CD28+/+ and CD28−/− mice, whereas at later time points CD28−/− mice showed a drastic reduction in the proliferation of this T cell population, indicating that CD28 costimulation was not essential for the initial T cell stimulation with SEB. Furthermore, in CD28−/− mice, injection of SEB led to the induction of anergy in SEB responsive T cells within 24 h, which was reflected by a failure of proliferation after restimulation with SEB in vitro and by a diminished TNFα production after a rechallenge with SEB in vivo. In addition, CD28−/− mice did not succumb to lethal toxic shock after a second SEB injection.

Materials and Methods

Animals and Reagents. BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The generation of CD28−/− mice is described elsewhere (4). CD28−/− mice were back crossed into the BALB/c strain for four generations and screened for the expression of H-2k α, H-2k β and CD28 by flow cytometry. Mice are well analyzed. Injection of BSAg induces strong T cell responses in vivo. A single injection leads to massive expansion of T cells with responsive TCRVβ-elements, that peaks at day three, followed by deletion at day 5–7, which lasts for several weeks (14, 15, 16). At these later time points T cells are in a state of unresponsiveness, as they don’t proliferate after stimulation with the particular BSAg, in spite of expressing a TCR β chain that can interact with the BSAg (14, 17, 18).

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Staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB; both Sigma Chem. Co., St. Louis, MO) were dissolved in sterile phosphate buffered saline (PBS) at 1 mg/ml and further dilutions were done in sterile PBS. Mice were injected i.p. with the indicated amount of SEA or SEB in 100–200 μl of PBS.

Flow cytometry. The following mAbs were used: FITC-conjugated anti-H2-Kk (clone: AF6-88.5), FITC-conjugated anti-H2-Kd (clone: SF1-1.1), FITC- and PE-conjugated anti-TCR VB8.1, 8.2 (clone: MR.5-2), FITC- and PE-conjugated anti-TCRVβ6 (clone: RR4-7), PE-conjugated anti-CD3ε (clone: 145-2C11), biotin-conjugated anti-CD8α (clone: 53-6,7), FITC-conjugated anti-CD25 (clone: 7D4), PE-conjugated anti-CD28 (clone: 37.51), FITC-conjugated anti-CD69 (clone: H1-2F3) (all PharMingen, San Diego, CA), FITC- and PE-conjugated anti-CD4 (clone: YTS191.2) and FITC- and PE-conjugated anti-CD8 (clone: YTS169.4) (Cedarlane Laboratories, Hornby, Canada).

Mice were bled from the tail and 20 μl of blood was collected in heparinized capillary tubes (Baxter Healthcare Corp., Deerfield, IL). Blood was further diluted with 100 μl of PBS containing 20 mM EDTA. Saturation amounts of mAb were added and the cells were incubated at 4°C for 30 min. Erythrocytes were lysed by the addition of 1 ml of ammonium chloride solution and cells were washed twice with PBS and fixed with 1% paraformaldehyde in PBS.

Spleen and lymph node cells were prepared according to standard procedures. Cells (5 × 10⁶) were incubated in staining buffer (PBS, 1% bovine serum albumin, 0.1% NaN₃) with saturating amounts of mAbs for 30 min at 4°C. Cells were washed twice in cold staining buffer and resuspended in PBS containing 1% paraformaldehyde. Cells stained with biotinylated mAb were washed twice with staining buffer and incubated with saturating amounts of Streptavidin-670 (GIBCO BRL, Gaithersburg, MD) for 30 min at 4°C, washed twice with staining buffer and fixed with 1% paraformaldehyde in PBS. A total of 10,000 viable cells were analyzed using a FACSscan® flow cytometer and Lysis II software (Becton Dickinson, Mountain View, CA).

Cell Cycle Analysis. 4 × 10⁶ lymph node cells were stained with saturating amounts of either FITC-conjugated anti-VB8.1, 8.2 mAb or FITC-conjugated anti-VB6 mAb, incubated for 30 min on ice and washed twice with RPMI 1640 medium. Cells were resuspended in 1 ml of FCS and 1 ml of RPMI 1640 medium was added. Cells were fixed by slowly adding 6 ml of ice-cold ethanol (70% in H₂O). After incubating for 30 min at 4°C cells were washed with PBS and resuspended in 100 ml PBS containing 5 μg/ml RNase (Sigma). Cell suspensions were further diluted by the addition of 900 ml of PBS containing 50 mg/ml propidium iodine (Sigma) and incubated for 30 min at 4°C. DNA content of VB6+ and VB8.1, 8.2+ T cells as revealed by the uptake of propidium iodine was analyzed by flow cytometry within 3 h.

Proliferation Assay. Lymph node cells (1 × 10⁶) were incubated in 96-well round-bottom plates (Nunc, Kamstrup, Denmark) in 200 μl of HL-1 medium (Hycor Biomedical Inc., Irvine, CA) supplemented with 2 mM glutamine, 50 μM β-mercaptoethanol, penicillin, and streptomycin, and 2% fetal bovine serum. Cells were stimulated by the addition of 5 μg/ml SEB or 50 U/ml recombinant mouse IL-2 (Genzyme, Cambridge, MA). For stimulation with cross-linked anti-CD3 antibodies, plates were coated with polyclonal rabbit anti-hamster antibodies (Jackson Laboratories) at 10 μg/ml PBS at 4°C over night. Plates were washed with PBS and incubated for 3 h with anti-CD3ε mAb (clone: 145-2C11; PharMingen) at 1 μg/ml PBS and washed again with PBS. After the indicated time of culture, 1 μCi/well [3H]thymidine (NEN, Boston MA) was added to the cell cultures and after additional 12–18 h cells were harvested (Filtermate-196 harvester; Canberra Packard, Mississauga Canada) and [3H]thymidine incorporation determined (Matrix-96 direct beta counter, Canberra Packard).

TNFα ELISA. Peripheral blood of mice was collected in serum separator tubes (Becton Dickinson). Tubes were centrifuged for 10 min and the sera were collected and stored at −70°C. Sera were diluted 1/2 and 1/4 in PBS and TNFα was determined by ELISA (Genzyme, Cambridge, MA) according to the manual of the manufacturer.

Results

SEB Fails to Induce a Strong Expansion but Leads to Early Deletion of VB8.1, 8.2+ T Cells in CD28−/− Mice. CD28−/− and CD28+/+ mice were injected with 100 μg of SEB and the absolute numbers of VB8.1, 8.2+ and VB6+ T cells in
the spleen were determined at different time points after injection (Fig. 1). Vβ8.1 and Vβ8.2 are two variable TCRβ-elements that are recognized by SEB and Vβ6 is a variable TCRβ-element that is not. In CD28+/+ mice CD4+ Vβ8.1, 8.2+ and CD8+ Vβ8.1, 8.2+ T cells showed a strong increase in cell number at day 3, which was due to both an increase in the percentage of Vβ8.1, 8.2+ T cells and in the cellularity of the spleen (data not shown). At day 7 the numbers of both CD8+ Vβ8.1, 8.2+ and CD4+ Vβ8.1, 8.2+ T cells were comparable to the numbers in untreated animals and at day 14 both populations showed deletion. In contrast, CD28−/− mice displayed no expansion in the numbers of either CD4+ Vβ8.1, 8.2+ or CD8+ Vβ8.1, 8.2+ T cells at day 3. At day 7 cell numbers of both populations were already lower than corresponding numbers in untreated mice, and at day 14 a deletion comparable to that in CD28+/+ mice had occurred. In both CD28+/+ and CD28−/− mice, the numbers of Vβ6+ T cells showed only subtle changes during this time period.

The analysis of the percentages of Vβ8.1, 8.2+ and Vβ6+ T cells in the peripheral blood of mice injected with SEB led to similar results, confirming the impaired expansion and fast deletion of Vβ8.1, 8.2+ T cells in CD28−/− mice compared to CD28+/+ mice (data not shown).

SEB Activates Vβ8.1, 8.2+ T Cells in CD28+/+ Mice. The lack of expansion of SEB responsive T cells could reflect a failure of SEB to activate T cells in CD28−/− mice. To determine the extent of T cell activation, mice were injected with SEB and lymph node T cells were analyzed by flow cytometry for cell size and the expression of activation markers (Fig. 2). 24 h after SEB injection, most of the Vβ8.1, 8.2+ T cells in both CD28+/+ and CD28−/− mice were blasts. After 48 h the average size of Vβ8.1, 8.2+ T cells in CD28+/+ mice was decreased, but the majority were still blast cells. In contrast, in CD28−/− mice Vβ8.1, 8.2+ T cells were small, and only a few Vβ8.1, 8.2+ T cell blasts were visible at 48 h (11.7% compared to 77.7% in the CD28+/+ mice).

The surface molecule CD69 is upregulated early after T cell activation. 24 h after SEB injection CD69 was expressed both in CD28−/− and CD28+/+ mice on virtually all Vβ8.1, 8.2+ T cells. After 48 h most of the Vβ8.1, 8.2+ T cells in CD28−/− mice were CD69− whereas CD28+/+ mice still had a significant CD69+ Vβ8.1, 8.2+ T cell population (15.4% in CD28−/− mice and 47.8% in CD28+/+ mice).

The α-chain of the IL-2R (CD25) represents another T cell activation marker. In untreated mice only a minute portion of Vβ8.1, 8.2+ T cells were CD25+. At 24 h after SEB injection, the majority of Vβ8.1, 8.2+ T cells in both CD28+/+ and CD28−/− mice were CD25+. However, activated T cells in CD28−/− mice expressed a 10-fold lower amount of CD25. After 48 h almost all Vβ8.1, 8.2+ T cells in the CD28+/+ mice were CD25+. In contrast, CD28+/+ mice displayed still a significant CD25+ Vβ8.1, 8.2+ T cell population (5.2% in CD28−/− versus 31.3% in CD28+/+ mice). Vβ6+ T cells showed no significant changes in either cell size or expression of activation markers during the observed period (data not shown).

Blast transformation indicated that the Vβ8+ T cells had started to proliferate. To prove that this really was the case we did cell cycle analysis of Vβ8+ and Vβ6+ T cells after

![Figure 1. SEB fails to induce expansion of Vβ8.1, 8.2+ T cells, but leads to deletion of these cells in the spleen of CD28+/+ mice. Mice were injected intraperitoneal with 100 μg SEB. After the indicated time periods mice were killed and numbers of splenic cells were determined. Spleen cells were stained with FITC-conjugated anti-CD4 or anti-CD8 mAbs and with PE-conjugated anti-Vβ6 or anti-Vβ8.1, 8.2 mAbs and analyzed by flow cytometry. Cell numbers were calculated from the total number of spleen cells and the percentages of the different cell populations. Each bar represents the mean value of three mice.](image-url)
In vivo response of CD28-deficient mice to Staphylococcal Enterotoxin B

**Figure 2.** SEB is able to activate Vβ8.1, 8.2+ T cells in vivo. Mice were injected i.p. with 100 μg SEB and killed after 24 and 48 h. Pooled brachial, axillary, and inguinal lymph node cells were stained with PE-conjugated anti-Vβ6 or anti-Vβ8.1, 8.2 mAbs and with FITC-conjugated anti-CD69 or anti-CD25 mAbs and analyzed by flow cytometry using a gate for viable cells.

**Figure 3.** Induction of proliferation of Vβ8.1, 8.2+ T cells by SEB in vivo. Mice were injected intraperitoneal with 100 μg SEB and killed at the indicated time points. Pooled brachial, axillary, and inguinal lymph node cells were stained with FITC-conjugated anti-Vβ6 or anti-Vβ8.1, 8.2 mAbs, fixed with ethanol and the DNA content was analyzed after propidium iodine staining by flow cytometry using a gate for viable Vβ6+ or Vβ8.1, 8.2+ T cells. The experiment shown is representative of two independent experiments. Black bar, % Vβ6+ or Vβ8.1, 8.2+ T cells in S-phase; dotted bar, % Vβ6+ or Vβ8.1, 8.2+ T cells in G2-phase or mitosis.

In summary, SEB was able to activate Vβ8.1, 8.2+ T cells in both CD28−/− and CD28+/+ mice, as determined by an increase in cell size and expression of the activation markers CD69 and CD25. In addition, in both mice strains an equal percentage of Vβ8.1, 8.2+ T cells had started to proliferate. However, at 48 h almost all activated T cells in CD28−/− mice had returned to a small, CD25− CD69− phenotype, whereas a significant proportion of Vβ8.1, 8.2−

**Figure 4.** Proliferative response of T cells from CD28+/+ and CD28−/− mice to SEB in vitro. Lymph node cells (1 × 10⁵/well) were incubated in medium alone (circles) or in medium containing 5 μg SEB/ml (triangles). [3H]thymidine was added at the time points indicated and cells were harvested after additional 18 h. Each data point represents the mean value of triplicates. White symbols, CD28+/+ lymph node cells; black symbols, CD28−/− lymph node cells.
T cells in CD28+/+ mice were still CD25+ CD69+ blasts. This difference was also reflected in the reduced number of proliferating cells after 48 h. Similar phenotypic changes were also seen after injection of 20 μg of SEB and in splenocytes (data not shown).

Early Onset of Unresponsiveness against SEB in CD28−/− Mice. The different activation patterns prompted us to analyze the responsiveness of T cells at different time points after SEB injection. In in vitro assays CD28−/− and CD28+/+ T cells displayed equivalent proliferation after SEB stimulation during the first 48 h, but at later time points CD28−/− T cells showed markedly decreased proliferation compared to CD28+/+ T cells (Fig. 4).

Mice were injected with SEB and the in vitro response of lymph node T cells to SEB and IL-2 was determined at 24, 48 and 72 h after SEB injection (Fig. 5). Proliferation was measured 48 h after the start of the culture. At this time point any difference in the proliferative response should reflect a difference in the state of responsiveness of the T cells in vivo and not a difference of the in vitro proliferation capacity between CD28+/+ and CD28−/− T cells.

T cells from CD28−/− and CD28+/+ mice without prior exposure to SEB had a similar proliferative response to SEB in vitro. One day after SEB injection, T cells from CD28+/+ mice displayed an increase in the in vitro SEB response. But this effect reverted within the following 48 h and T cells became unresponsive at 72 h after SEB injection. In contrast, at 24 h after SEB injection T cells from CD28−/− mice had already lost their ability to respond in vitro to SEB and remained in this state at 48 and 72 h.

T cells from untreated CD28−/− and CD28+/+ mice showed some proliferation when incubated with IL-2. At 24 h after SEB injection both cell populations displayed an increased response to IL-2 which decreased at 48 and 72 h. Overall, cells from CD28−/− mice had a lower response to IL-2, which corresponded to the lower expression level of IL-2Rα/CD25 (Fig. 3 for 0, 24 and 48 h; and data not shown for 72 h). Stimulation with SEB in the presence of IL-2 resulted in similar proliferation of T cells from both CD28−/− and CD28+/+ mice. The proliferative response to anti-CD3 mAb showed no drastic changes, indicating that the changes seen in the proliferation to SEB were restricted to the SEB-responsive T cell population.

CD28−/− Mice Are Resistant to Lethal Toxic Shock Induced by a Second SEB Injection. To analyze the secondary response against SEB in vivo, mice were injected with 100 μg of SEB and rechallenged with the same SEB dose after 48 h. After the primary injection both CD28−/− and CD28+/+ mice showed signs of illness (rough fur and decreased mobility). Both strains usually recovered well and had a healthy appearance by 48 h. The rechallenge with SEB 48 h after the primary injection had a profound effect on CD28+/+ mice, as all animals displayed severe signs of illness and were dead or moribund after a further period of 24 h (Table 1). In contrast, all CD28−/− mice survived the SEB rechallenge and had a healthy appearance.

CD28−/− Mice Produce only Low Amounts of TNFα after Rechallenge with SEB. One of the mediators of SEB-induced toxic shock is TNFα. SEB induces high TNFα serum levels that peak 1 to 2 h after injection and are produced mainly by T cells (20, 21). To analyze the TNFα-production we injected mice with SEB and rechallenged them with SEB or SEA 48 h after the primary injection. 90 min after the rechallenge the BALB/c mice showed no signs of toxicity and were dead or moribund after a further period of 24 h. In contrast, all CD28−/− mice survived the SEB rechallenge and had a healthy appearance.

Table 1. CD28−/− Mice Are Resistant to Lethal Toxic Shock Induced by a Second SEB Injection

| SEB injection | Day 0 | Day 2 | Dead/group no. |
|---------------|------|------|----------------|
| CD28−/−       | 100 μg | 0/6  |
| CD28+/+       | 100 μg | 0/6  |
| CD28−/−       | 100 μg | 5/6* |

Mice were injected i.p. with 100 μg SEB and rechallenged with the same dose of SEB after 48 h. Numbers represent data from two independent experiments with three mice per group in each experiment. No. survival was scored 24 h after the second SEB injection.

*The one mouse alive after 24 h was moribund and therefore killed.
primary SEB injection both CD28−/− and CD28+/+ mice had comparable amounts of TNFα in the serum (Fig. 6). It seemed therefore that CD28 was not necessary for the induction of TNFα in a primary in vivo response. After the rechallenge with SEB, CD28+/+ mice had serum TNFα levels comparable to the levels after the primary injection. In contrast, CD28−/− mice produced only marginal amounts of TNFα. After SEA injection both CD28+/+ and CD28−/− mice produced equal amounts of TNFα, and this TNFα production was not affected by the pretreatment of the mice with SEB.

Discussion

In CD28+/+ mice, SEB induced strong expansion of both CD4+ Vβ8+ and CD8+ Vβ8+ T cells in peripheral lymphoid organs, whereas CD28−/− mice failed to show this strong expansion. Closer examination revealed that the lack of expansion was not simply due to the failure of SEB to stimulate this cell population. Injection of SEB induced similar phenotypic changes of Vβ8+ T cells in both CD28−/− and CD28+/+ mice. After 24 h equal percentages of Vβ8+ T cells were blast cells, expressed the early T cell activation marker CD69, and more important an equal number of Vβ8+ T cells had entered the cell cycle. In CD28+/+ mice, a significant proportion of Vβ8+ T cells retained this activated proliferating phenotype after 48 h, whereas only a few of those cells could be detected in CD28−/− mice at this time point. It seems that stimulation of T cells by SEB in the absence of CD28 costimulation leads to T cell activation, but this T cell activation is not maintained compared to the activation in the presence of CD28, and therefore does not support the large expansion of SEB responsive T cells seen in CD28+/+ mice. This result resembles the differences seen in proliferation after SEB stimulation in vitro (Fig. 4). It is also in accordance with published results from in vitro proliferation assays, where T cells from CD28−/− mice showed a strong impairment in their proliferative response after stimulation with allogenic cells or with peptide antigen (5, 6). In these experiments the initial response of CD28−/− and CD28+/+ T cells was similar, but the proliferation of CD28−/− T cells was not sustained. An important reason for this difference was the low amount of IL-2 produced by CD28−/− T cells in these assays, which is probably also a reason for the lack of T cell expansion seen after SEB injection in our in vivo experiments. In summary, CD28 costimulation is not necessary for the initial phase of T cell activation, but is essential for ongoing proliferation in vitro and, as demonstrated here, after T cell stimulation with SEB in vivo.

Interestingly, SEB was able to induce peripheral T cell deletion in both CD28+/+ and CD28−/− mice. CD28 therefore is not necessary for the induction of the program that leads to deletion of T cells after their activation (37). CD28 costimulation has rather a protective function for activated T cells since CD28−/− mice showed an earlier onset of deletion. It has been shown that T cell stimulation induces expression of the Bcl-xL molecule which is able to protect cells from apoptosis and that CD28 costimulation enhances Bcl-xL expression (22). In addition, CD28 costimulation leads to an increase in the transcription and the stabilization of IL-2 mRNA and therefore to an increased and prolonged IL-2 production (23, 24, 25), which has also been demonstrated for T cell stimulation with SEB in vitro (26). IL-2 supports the proliferation and expansion of T cells and has in addition a protective function against apoptosis (27). Furthermore, it has been postulated that TCR ligand density and affinity is important for the dependence of T cells on CD28 costimulation (3). During the in vivo responses against allogenic and virus infected cells, where antigen is present for several days and leads to continuous activation of T cells, TCR stimulation alone is probably sufficient to induce mechanisms that support proliferation and protect activated T cells from apoptosis, therefore leading to normal responses even in the absence of CD28 costimulation. In our system SEB is injected only once and is cleared within a short period of time. T cells are therefore confronted with a short period of high ligand density followed by a rapid decline of the ligand concentration. The mechanisms which protect T cells from apoptosis and prolong the lifetime of T cells after T cell activation are therefore especially challenged. The lack of CD28 must necessarily lead to the aborted proliferation and the early onset of deletion of T cells seen in CD28−/− mice.

In spite of the similarities in the phenotype, there was a striking difference between T cells from CD28+/+ and CD28−/− mice 24 h after SEB injection. Vβ8+ cells in CD28−/− mice were already unresponsive to a rechallenge with SEB. Induction of unresponsiveness by SEB is a well-analyzed phenomenon. It has been shown that after the initial proliferation after SEB injection, T cells enter a state, where they fail to proliferate to subsequent SEB exposure (14, 15, 17, 18). Our results confirm these observations, as we saw a strong reduction of the in vitro proliferation of T cells from CD28+/+ mice 72 h after SEB injection. T cells...
from CD28−/− mice were already unresponsive 24 h after injection. The addition of IL-2 could rescue the response confirming that the T cells are anergic. Interestingly, Vβ8+ T cells of both CD28+/+ and CD28−/− mice had the same phenotype 24 h after SEB injection. Therefore, the state of unresponsiveness did not correlate with the appearance of T cells and was not restricted to resting T cells as at this time point most of the Vβ8+ T cells in CD28−/− mice were blasts and a significant proportion in cell cycle. Since T cells from both CD28+/+ and CD28−/− mice respond to IL-2 or SEB and IL-2, the lower expression level of IL-2Rα/CD25 was probably not responsible for the anergy. Our in vivo data resemble initial in vitro results, where it was shown that T cell stimulation via TCR in absence of costimulation leads to induction of anergy that can be prevented by CD28 costimulation (28, 29, 30).

An important question was, whether the unresponsiveness seen in vitro had also relevance in vivo. We addressed this question by rechallenging CD28−/− mice with SEB 48 h after the primary injection. The result was striking. All CD28+/+ mice were dead or moribund within 24 h, whereas CD28−/− mice did not even show signs of illness. It has been shown that T cells are responsible for the SEB induced toxicity mainly by the production of large amounts of IFNγ and TNFα (20, 21, 31, 32). At a later phase of the SEB response, TNFα is also produced by macrophages (21). It has been described that CD28 costimulation of T cells leads to an increase in the production of certain lymphokines, including TNFα and IFNγ (23, 24). We therefore can not exclude that lower or not maintained production of cytokines at both injections was responsible for the protection of CD28−/− mice against lethal toxic shock after the SEB rechallenge. However, with the SEB dose used, both CD28−/− and CD28+/+ mice showed the same signs of illness after the first injection. But after the second injection CD28−/− mice showed no signs of illness at all. In addition, we could not observe a difference in the serum levels of TNFα 90 min after the primary SEB injection, which represents the peak of T cell TNFα production (20, 21). But we detected a difference after the rechallenge with SEB. At this time point the number of Vβ8+ T cells in CD28−/− mice was roughly the same as that after the initial SEB injection. Thus, in vivo unresponsiveness of T cells of CD28−/− mice might account for the decrease in the TNFα production.

Another question raised is, why did we see this fast induction of unresponsiveness after SEB injection in CD28−/− mice, whereas the response against allogenic or virus infected cells was not impaired (3, 4)? One explanation is probably the same as that for the aborted proliferation. SEB is cleared rapidly from the organism and therefore does not induce continuous T cell stimulation. An alternative possibility is that SEB as a superantigen does not have to be processed and is probably presented by different cells and in a different environment than peptide antigens. Other costimulatory molecules that can compensate for the lack of CD28 costimulation (33, 34, 35, 36) are therefore not as efficiently triggered as in the response against antigens presented by professional antigen presenting cells.

In summary, our results show that CD28 is not necessary for T cell activation by SEB in vivo and for subsequent deletion. However, in absence of CD28 costimulation, T cell activation can not be maintained and does not support the vast expansion of Vβ8+ T cells seen in CD28+/+ mice. In addition, SEB stimulation of T cells in absence of CD28 costimulation leads to rapid induction of unresponsiveness in vivo.

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References

1. Schwartz, R.H. 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. Cell. 71:1065–1068.
2. June, C.H., J.A. Bluestone, L.M. Nadler, and C.B. Thompson. 1994. The B7 and CD28 receptor families. Immunol. Today. 15:321–331.
3. J.A. Bluestone. 1995. New perspectives of CD28-B7-mediated T cell costimulation. Immunol. 2:555–559.
4. Shabahian, A., K. Pfeffer, K.P. Lee, T.M. Kündig, K. Kishihara, A. Wakchum, K. Kawai, P.S. Ohashi, C.B. Thompson, and T.W. Mak. 1993. Differential T cell stimulation in CD28-deficient mice. Science (Wash. DC). 261:609–612.
5. Lucas, P.J., I. Negishi, K. Nakayama, L.E. Fields, and D.Y. Loh. 1995. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. J. Immunol. 154:5757–5768.
6. Green, J.M., P.J. Noel, A.I. Sperling, T.L. Walunas, G.S. Gray, J.A. Bluestone, and C.B. Thompson. 1994. Absence of B7-dependent responses in CD28-deficient mice. Immunity. 1:501–508.
7. Marrack, P., and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. *Science (Wash. DC).* 248:1066–1070.

8. Fleischer, B., and U. Hartwig. 1992. T lymphocyte stimulation by microbial superantigens. *Chem. Immunol.* 55:36–64.

9. Fraser, J.D. 1989. High-affinity binding of staphylococcal enterotoxin A and B to HLA-DR. *Nature (Lond.)* 339:221–223.

10. Fleischer, B., and H.-W. Mittrücker. 1991. Evidence for T cell receptor–HLA class II molecule interaction in the response to superantigeneric bacterial toxins. *Eur. J. Immunol.* 21:1331–1333.

11. Herman, A., J.W. Kappler, P. Marrack, and A.M. Pullen. 1991. Superantigens: Mechanism of T cell stimulation and role in immune responses. *Annu. Rev. Immunol.* 9:745–772.

12. Janeway, C.A., J. Yagi, P.J. Conrad, M.E. Katz, B. Jones, S. Vroegop, and S. Buxter. 1989. T cell response to Mls and to bacterial proteins that mimic its behavior. *Immunol. Rev.* 107:61–88.

13. Fleischer, B., and H. Schrezenmeier. 1988. T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for MHC class II antigens on accessory and target cells. *J. Exp. Med.* 167:1697–1707.

14. MacDonald, H.R., R.K. Lees, S. Baschieri, T. Herrmann, and A. Lussov. 1993. Peripheral T-cell reactivity to bacterial superantigens in vivo: The response/anergy paradox. *Immunol. Rev.* 133:103–117.

15. MacDonald, H.R., S. Baschieri, and R.K. Lees. 1991. Clonal expansion precedes anergy and death of VB8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo. *Eur. J. Immunol.* 21:1963–1966.

16. Kawabe, Y., and A. Ochi. 1991. Programmed cell death and extrathymic reduction of VB8+ CD4+ T cells in mice tolerant to staphylococcal aureus enterotoxin B. *Nature (Lond.)* 349:245–248.

17. Rellahan, B.L., L.A. Jones, A.M. Kruisbeek, A.M. Fry, and L.A. Matis. 1990. In vivo induction of anergy in peripheral VB8+ T cells by staphylococcal enterotoxin B. *J. Exp. Med.* 172:1091–1100.

18. Baschieri, S., R.K. Lees, A.R. Lussov, and H.R. MacDonald. 1993. Clonal anergy to staphylococcal enterotoxin B in vivo: selective effects on T cell subsets and lymphokines. *Eur. J. Immunol.* 23:2661–2666.

19. Gonzalo, J.A., E. Baixeras, A. Gonzalez-Garcia, A. George-Chandy, N. Van Rooijen, C. Martinez-A., and G. Kroemer. Differential in vivo effects of a superantigen and an antibody targeted to the same T cell receptor. *J. Immunol.* 152:1597–1680.

20. Miettke, T., C. Wahl, K. Heeg, B. Echternacher, P.H. Krammer, and H. Wagner. 1992. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: Critical role of tumor necrosis factor. *J. Exp. Med.* 175:91–98.

21. Bette, M., M.K. Schäfer, N. Van Rooijen, E. Weihe, and B. Fleischer. 1993. Distribution and kinetics of superantigen-induced cytokine gene expression in mouse spleen. *J. Exp. Med.* 178:1531–1539.

22. Boise, L.H., A.J. Minn, P.J. Noel, C.H. June, M.A. Accvitti, T. Lindsten, and C.B. Thompson. 1995. CD28 stimulation can promote T cell survival by enhancing the expression of BCL-X(L). *Immunity.* 3:87–98.

23. Thompson, C.B., T. Lindsten, J.A. Ledbetter, S.I. Kunkel, H.A. Young, S.G. Emerson, J.M. Leiden, and C.H. June. 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc. Natl. Acad. Sci. USA.* 86:1333–1337.

24. Lindsten, T., C.H. June, J.A. Ledbetter, G. Stella, and C.B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science (Wash. DC).* 244:339–343.

25. Umlauf, S.W., B. Beverly, O. Lantz, and R.H. Schwartz. 1995. Regulation of interleukin 2 gene expression by CD28 costimulation in mouse T-cell clones: both nuclear and cytoplasmic RNAs are regulated with complex kinetics. *Mol. Cell. Biol.* 15:3197–3205.

26. Fraser, J.D., M.E. Newton, and A. Weiss. 1992. CD28 and T cell antigen receptor signal transduction coordinately regulate interleukin 2 gene expression in response to superantigen stimulation. *J. Exp. Med.* 175:1131–1134.

27. Boise, L.H., A.J. Minn, C.H. June, T. Lindsten, and C.B. Thompson. 1995. Growth factors can enhance lymphocyte survival without committing the cell to undergo cell division. *Proc. Natl. Acad. Sci. USA.* 92:5491–5495.

28. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science (Wash. DC).* 248:1349–1356.

29. Harding, F.A., J.G. McArthur, J.A. Gross, H.D. Raul, and J.P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature (Lond.)* 356:607–611.

30. Jerkens, M.K., D. Mueller, R.H. Schwartz, S. Carding, K. Bottomly, M.J. Stadecker, K.B. Urdahl, and S.D. Norton. 1991. Induction and maintenance of anergy in mature T cells. *Adv. Exp. Med. Biol.* 292:167–176.

31. Marrack, P., M. Blackman, E. Kushnir, and J. Kappler. 1990. The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. *J. Exp. Med.* 171:455–464.

32. Florquin, S., Z. Amraoui, and M. Goldman. 1995. T cells made deficient in interleukin-2 production by exposure to staphylococcal enterotoxin B in vivo are primed for interferon-γ and interleukin-10 secretion. *Eur. J. Immunol.* 25:1148–1153.

33. Sperling, A.L., J.M. Green, R.L. Mosley, P.L. Smith, R.J. Di paolo, J.R. Klein, J.A. Bluestone, and C.B. Thompson. 1995. CD43 is a murine T-cell costimulatory receptor that functions independent of CD28. *J. Exp. Med.* 182:139–146.

34. Liu, Y., B. Jones, A. Aruffo, K.M. Sullivan, P.S. Linsley, and C.A. Janeway. 1992. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med.* 175:437–445.

35. Naujoks, M.F., M. Morin, M.S. Anderson, M. Peterson, and J. Miller. 1993. The chondroitin sulfate form of invariant chain CD43 is a murine T cell costimulatory receptor that functions independent of CD28. *J. Exp. Med.* 175:437–445.

36. Damke, R.K., K. Klussman, P.S. Linsley, and A. Aruffo. 1995. Proliferation of CD28-deficient mice to staphylococcal enterotoxin B.