Staphylococcal enterotoxin B-induced activation and concomitant resistance to cell death in CD28-deficient HLA-DQ8 transgenic mice

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Abstract

HLA class II molecules present superantigens more efficiently than their murine counterpart. Therefore, transgenic mice expressing HLA-DQ8 with and without CD28 were used to address the role of CD28 in staphylococcal enterotoxin B (SEB)-driven immune responses. SEB-induced in vitro proliferation of naive DQ8.CD28⁻/⁻ splenocytes was comparable to DQ8.CD28⁺/⁺ cells, and was several fold higher than that of C57BL/10 and BALB/c splenocytes. SEB-activated, naive DQ8.CD28⁻/⁻ cells in vitro produced significantly less IL-2, IL-4 and IL-10 than DQ8.CD28⁺/⁺ cells, while IFN-γ and IL-6 production was comparable. SEB-induced in vivo expansion of CD4⁺ T cells and, to a greater extent, CD8⁺ T cells was compromised in DQ8.CD28⁻/⁻ mice, indicating that SEB-induced proliferation of CD8⁺ T cells is more dependent on CD28 co-stimulation. Upon re-stimulation, SEB-primed CD28⁺/⁺ T cells failed to proliferate but were capable of producing cytokines. Conversely, CD28⁻/⁻ T cells were capable of proliferation, but not cytokine production. SEB-primed CD28-deficient cells produced significantly less nitric oxide when compared to CD28-sufficient cells following re-stimulation with SEB. CD28⁺/⁺ and not CD28⁻/⁻ mice were highly susceptible to SEB-induced lethal shock characterized by significantly elevated serum IFN-γ. Thus, (i) efficient presentation of SEB by HLA-DQ8 circumvents co-stimulation through CD28, (ii) unique CD28-derived signals are mandatory for generation of certain effector functions, and (iii) absence of CD28-derived signals confers resistance to activation-induced cell death and protects mice from SEB-induced shock.

Introduction

Superantigens (SAg), a family of microbial proteins, are strong polyclonal activators of T and B lymphocytes. The T cell SAg differ from mitogens in that activation by SAg is MHC class II dependent and TCR mediated. They differ from conventional antigens in that T cell activation by SAg is MHC unrestricted and CD4/CD8 co-receptor independent. While a processed nominal antigen is presented either by MHC class I or class II molecules to CD8⁺ and CD4⁺ T cells respectively, SAg in their native form bind to MHC class II molecules outside the peptide binding groove and vigorously activate both T cell subsets bearing certain TCR Vβ families [reviewed in (1)].

In addition to TCR-mediated events, signals generated through co-stimulatory molecules are mandatory for generating a viable immune response. Of the several co-stimulatory molecules described, the CD28–B7 system is the best characterized and the major pathway used by T cells. TCR-mediated signaling in the absence of CD28 co-stimulation has been shown to induce anergy in several systems [reviewed in (2)]. On the other hand, several studies have shown that CD28-mediated co-stimulation can be bypassed by providing strong activation signals such as repeated antigenic stimulation (3) or high antigen concentration (4,5). Accessory molecules like heat-stable antigen (6), CD2 (7) or ICAM-1 (8) can mediate sufficient co-stimulation in the absence of CD28. Thus CD28-deficient animals can generate an efficient immune response (9), reject grafts/tumors (9,10) and are...
susceptible to autoimmune diseases (11,12). However, CD28-mediated co-stimulation has been shown to be crucial for SAg-mediated T cell activation (13,14), and effector functions both in vivo and in vitro (15–17).

As CD28 has been shown to be dispensable in several classical immune responses, it was intriguing as to why SAg, capable of inducing massive co-receptor independent, MHC-unrestricted T cell activation would require co-stimulation. Dependence on CD28 co-stimulation in the murine SAg models studied so far could be attributed to poor presentation of bacterial SAg by murine class II molecules. As strong antigenic stimulation can bypass the need for CD28-mediated co-stimulation (3–5), we hypothesized that the more efficient presentation of SEB by human MHC class II molecule (18) might circumvent CD28 requirement.

HLA transgenic mice serve as valuable tool to dissect the immunopathogenesis of several human diseases (19). In addition, other laboratories have successfully used HLA transgenic mice to analyze the response to SAg (20–22). Moreover, HLA class II transgenic mice resemble more closely the human situation than the conventional mice strains (23,24). Therefore, we used mice transgenically expressing HLA class II molecule DQ8 and lacking endogenous class II to address the roles of CD28 in staphylococcal enterotoxin B (SEB)-driven immune response.

Methods

Mice

BALB/c and C57BL/10 mice originally came from Jackson Laboratories (Bar Harbor, ME). Generation of HLA-DQ8 transgenic mice has been described earlier (25). HLA-DQ8.CD28−/− mice were generated as follows. CD28−/− mice (Courtesy of Dr T. Mak, Toronto) on a B6 × 129/J background were mated with HLA-DQ8+ mice. The heterozygous offspring were intercrossed; CD28±/± littermates expressing DQ8 were selected and intercrossed to produce DQ8.CD28±/± mice. Similarly, the CD28+/+ siblings expressing DQ8 were also selected and intercrossed to generate the DQ8.CD28+/+ mice which were used as controls in this study. These two lines were maintained by intercrossing. As the CD28+/+ and CD28±/± mice used in this study are siblings, they have similar genetic background. These HLA transgenic mice do not express any endogenous mouse class II antigens. Mice bred in the barrier facility were moved to conventional facility following weaning.

Antibodies and reagents

The following anti-TCR Vγ antibodies [2 (B20.6), 4 (KT4–10), 5.1.2 (MR9-4), 6 (44-22-1), 7 (TR310), 8.1, 2, 3 (F23.1), 9 (MR2-10), 11 (RR3-13), 14 (14.2) and 17 (KJ23a)] and anti-HLA-DQ8 (IVD12, HB-14) were from ATCC (Rockville, MD). FITC-conjugated secondary antibodies were from Accurate Chemicals and Scientific Corp. (Westbury, NY). Anti-CD28 (37.51), anti-CD4 (RM 4.5), anti-CD8 (53.67), anti-CD95 (Jo2), anti-CTLA-4 (9H10) and OKX40 (clone OX86) were from PharMingen (San Diego, CA). Recombinant murine IL-2 was obtained from Atlanta Biologicals (Atlanta, GA).

5-(6-)Carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling and monitoring

Splenocytes were labeled with CFSE (Vybrant CFDA SE cell tracer kit, V-12883; Molecular Probes, Eugene, OR) as per the supplier’s instructions. Briefly, 5 × 10^7 cells/ml were incubated with 5 μM of CFSE for 10 min with intermittent shaking. Labeling was terminated by adding equal volume of FCS and cells washed thrice with serum-containing medium. CFSE-labeled cells were then cultured with indicated stimulant in 24-well plates for 72 h and subsequently the dye levels were analyzed by a flow cytometer (Becton Dickinson) using CellQuest version 3.3 software.

Determination of cell viability by Trypan blue dye exclusion

Splenocytes from SEB-primed mice were cultured as described above in a total volume of 100 μl in the presence of 1 μg/ml of SEB or left unstimulated. After 24 h, an equal volume of Trypan blue was added to each well, mixed and the total numbers of dead/live cells were enumerated using a hemocytometer.

Cytokine analysis

Splenocytes were cultured in 24-well plates with SEB or left unstimulated. Cytokines present in the culture supernatants were quantified by sandwich ELISA as described earlier (26). Cytokines present in the sera were determined by routine ELISA as per published protocols (27). The test sera were diluted to 25% in PBS containing 0.05% Tween 20 and the standards were reconstituted in 25% pooled normal mouse sera in PBS/Tween.

Measuring nitrite concentration in tissue culture supernatants

The stable oxidative end-product of nitric oxide (NO) present in the culture supernatant was measured using Greiss reagent as per published protocols (28).
**Induction of toxic shock with SEB**

Mice received two i.p. injections of SEB (100 μg in 200 μl PBS) 48 h apart and were closely monitored for the symptoms of shock.

**Statistics**

The statistical significance of the results was determined by using the software GraphPad Prism (version 3.0a; San Diego, CA).

**Results**

**Response of naive splenocytes to SEB**

SEB-induced in vitro proliferation of splenocytes from DQ8 transgenic mice was several fold higher than that of B10 mice (Fig. 1A). Proliferative response of splenocytes from DQ8.CD28+/+ mice was comparable with (at times even higher than) CD28+/− mice and several-fold higher than that of B10 mice. On the other hand, Con-A-induced proliferation of DQ8.CD28−/− splenocytes was consistently low when compared to B10 and DQ8.CD28+/+ mice. Similar results were obtained with varying doses of SEB at different cell concentrations tested (data not shown). As B10 mice do not express functional H-2E molecule, we compared the proliferative potential of DQ8.CD28+/+ and DQ8.CD28−/− splenocytes with that of BALB/c, which expresses both H-2A and H-2E molecules. BALB/c splenocytes responded more vigorously to Con A than HLA-DQ8 transgenic mice (Fig. 1B), but the response to SEB was significantly lower except at a very high concentration of 10 μg/ml (Fig. 1C). While the response to Con A was consistently low in DQ8.CD28−/− (Fig. 1B), the SEB response was similar to DQ8.CD28+/+ mice (Fig. 1C).

Expression of DQ8 and percentage of CD4+ and CD8+ T cells was equivalent between DQ8.CD28+/+ and DQ8.CD28−/− transgenic mice, thereby ruling out differences in SEB presentation/response between these two groups (data not shown). These results show that (i) HLA-DQ8 can present SEB more efficiently to murine T cells than H-2b and H-2d-derived class II molecules, and (ii) presentation of SEB by HLA-DQ8 can induce extensive in vitro proliferation of T cells even in the absence of CD28 co-stimulation.

**Monitoring in vitro cell division with CFSE**

Splenocytes from DQ8.CD28+/+ and DQ8.CD28−/− mice were labeled in vitro with the fluorescent dye CFSE and stimulated with either Con A or SEB. CD4 and CD8 gated T cells were subsequently analyzed by flow cytometry to quantify the dye levels, which would indicate cell division. As SEB-reactive T cells constitute the majority of the entire T cell population in HLA-DQ8 transgenic mice (see below), we used the entire CD4/CD8 gated T cell population to monitor cell division instead of just TCR Vβ8-bearing T cells alone. As shown in Fig. 2(A–D), we could not find any appreciable difference in the dye dilution pattern between SEB-stimulated CD4+ and CD8+ gated cells from CD28+/+ and CD28−/− mice. Consistent with previous data, Con A-induced cell division was less in CD28−/− mice (Fig. 2B and D). There was very little difference in cell size between SEB-activated CD28+/+ and CD28−/− mice as reflected by similar forward scatter profiles (data not shown). The results of CFSE-dilution study further confirmed that CD28−/− T cells can proliferate as efficiently as CD28+/+ cells following activation by SEB in vitro.

![Fig. 1. Response of naive splenocytes to SEB. Splenocytes from DQ8.CD28+/+ and DQ8.CD28−/− mice along with C57BL/10 (A) or BALB/c (B and C) were cultured in 96-well flat-bottomed tissue culture plates (1 × 10⁶/well) with either medium alone or with indicated stimulant for 48 h. [³H]Thymidine was added during the last 18 h of culture and thymidine incorporation was measured using a β-counter. Mean ± SD of at least four experiments is given.](image-url)
These results indicate that naive CD28±/± T cells in this system could proliferate vigorously to the same extent as CD28+/+ T cells (for as long as 72 h). This is in contrast to the earlier study in which strong TCR ligation without co-stimulation induced rapid and early (within 18 h) apoptosis of naive murine T cells (30).

SEB-induced cytokine production

Once the proliferative capacity of CD28±/± cells was established, their in vitro cytokine production was studied. IL-4 and IL-10 (as markers of Th2 response) and IFN-γ (as a marker of Th1 response) were measured in addition to IL-2 and IL-6. As depicted in Fig. 3(A), CD28+/+ mice produced very low levels of IL-2, IL-4 and IL-10 (P < 0.01), while levels of IL-6 and IFN-γ were comparable (P = NS) with DQ8.CD28+/+ mice. To rule out any difference in the time kinetics of SEB-induced cytokine production between these two groups, culture supernatants were collected at 24 h intervals for 3 days and analyzed by ELISA. CD28+/+ cultures had high IL-2 levels at 24 h following activation. At 48 h, it remained at similar levels and by 72 h the level decreased (Fig. 3B). Although IL-2 production by CD28±/± cells followed a similar pattern, quantitatively they were significantly less at all time points than CD28+/+ cells (Fig. 3B). In CD28+/+ cultures, IL-4 levels steadily increased from 24 h onwards and was highest at 72 h while CD28±/± cultures had negligible amount of IL-4 at all time points (Fig. 3C). The IL-10 production pattern was similar to that of IL-4 in both CD28+/+ and CD28±/± cultures (Fig. 3D). Splenocytes from both CD28+/+ and CD28±/± mice produced comparable amounts of IFN-γ, which increased steadily from

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**Fig. 2.** Monitoring in vitro cell division with CFSE. Splenocytes from DQ8.CD28+/+ and DQ8.CD28±/± mice were labeled with CFSE as described in Methods, and were cultured in the presence of medium alone or the indicated stimulant (Con A 2.5 μg/ml, SEB 1μg/ml). After 72 h, the cells were collected and the dye levels in CD4+ (A and B) or CD8+ (C and D) gated cells were quantified using a flow cytometer. (A and C) DQ8.CD28+/+. (B and D) DQ8.CD28±/±. The result from one representative experiment out of four is given. The gray shaded area indicates cells cultured with medium alone; thin and thick solid lines indicate cells cultured with Con A and SEB respectively.
24 h onwards (Fig. 3E). Overall, CD28-deficient splenocytes produced less IL-2, IL-4 and IL-10, and comparable levels of IL-6 and IFN-γ when compared to CD28-sufficient splenocytes following activation with SEB.

**SEB-induced expansion of T cells in vivo**

The classical *in vivo* response to any SAg consists of an initial proliferative phase during which the SAg-reactive T cells expand and reach maximal number by day 3. Following the SAg-induced proliferative phase, massive AICD ensues during which the activated cells are deleted, resulting in reduction in the SAg-reactive T cell numbers below the pre-treatment levels (usually by day 7) (31). To study *in vivo* T cell activation, TCR Vβ usage in the expanded population and to study the role of CD28 in this *in vivo* response in DQ8 transgenic mice, splenocytes from SEB-primed mice were collected at different

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**Fig. 3.** SEB-induced cytokine production of naive cells. Splenocytes from DQ8.CD28+/+ and DQ8.CD28−/− mice were cultured in 24-well plates (10 × 10⁶/well) either in the presence of medium alone or SEB (1 μg/ml) for 72 h. Cytokines present in the supernatants were quantified by sandwich ELISA. (A) Total cytokines at 72 h. (B–E) Time kinetics of SEB-induced cytokine production. Each bar/data point represents mean ± SD of SEB-induced cytokine production over the medium controls from at least four individual mice (*P* < 0.01, DQ8.CD28+/+ versus DQ8.CD28−/−).
time points and screened by flow cytometry. SEB caused massive activation resulting in expansion of both CD4+ and CD8+ T cells bearing the TCR V\textsubscript{b}8 family in HLA-DQ\textsubscript{8}.CD28+/+ transgenic mice (Fig. 4A and C). There was a 5-fold increase in both CD4+ and CD8+ T cells bearing TCR V\textsubscript{b}8 by day 3 post-SEB injection. The percentage of TCR V\textsubscript{b}8-bearing CD4+ cells remained elevated (3-fold) even by day 7, while the percentage of TCR V\textsubscript{b}8-bearing CD8+ T cells was only slightly above the pre-treatment values at day 7 (Fig. 4A and B). Although CD28\textsuperscript{−/+} T cells proliferated very efficiently \textit{in vitro}, their \textit{in vivo} expansion was comparatively less. Even though the T cell numbers in naive CD28-deficient mice was similar to that of CD28-sufficient mice, there was only a 2- or 3-fold increase in TCR V\textsubscript{b}8-bearing CD8+ and CD4+ T cell numbers respectively at day 3 post-SEB injection in CD28-deficient mice. At day 7, in CD28-deficient mice the percentage of TCR V\textsubscript{b}8-bearing CD4+ T cell remained elevated (2-fold) while the percentage of TCR V\textsubscript{b}8-bearing CD8+ T cell were below the pretreatment values (Fig. 4C and D). TCR V\textsubscript{b}7-bearing T cells also showed expansion in both CD28\textsuperscript{+/+} and CD28\textsuperscript{−/−} mice (Fig. 4A–D). As

![Fig. 4. SEB-induced expansion of T cells in vivo. DQ8.CD28\textsuperscript{+/+} (A and C) and DQ8.CD28\textsuperscript{−/−} (B and D) mice received a single i.p. injection of either PBS alone or SEB (50 \textmu g in 200 \textmu l PBS). Spleens were collected either 3 or 7 days later and the percentage of CD4+ (A and B) or CD8+ (C and D) T cells expressing the respective TCR V\textsubscript{b} families among the total splenic mononuclear cells were quantified by two-color FACS analysis. (E) The percentage of CD4+ or CD8+ T cells expressing the TCR V\textsubscript{b}8.1, 2 and 3 families from a different set of mice. Each bar represents mean ± SD from at least four mice.](image-url)
they constitute only a small percentage, only T cells expressing TCR V\(_{b}\)\(_8\) were analyzed in subsequent experiments. Figure 4(E) depicts the changes in TCR V\(_{b}\)\(_8\)-bearing CD4\(^+\) and CD8\(^+\) T cells from an independent set of mice. Priming even with a 5-fold lower dose of SEB resulted in similar expansion (data not shown). Overall, (i) SEB was capable of causing massive expansion of murine T cells bearing the TCR V\(_{b}\)\(_8\) family in vivo when presented by HLA-DQ8 and (ii) CD28-sufficient T cells were capable of clonal expansion, albeit to a lesser extent than CD28-sufficient cells.

We also counted the absolute splenocyte numbers at both day 3 and day 7 post-SEB injection in both groups of mice. Although the cell numbers in PBS-treated CD28-sufficient and -deficient mice were similar, CD28\(^+/+\) mice had significantly higher cells per spleen than CD28\(^{-/-}\) mice (\(P < 0.025\)) at day 3. The splenocyte numbers in CD28\(^{+/+}\) mice fell below that of PBS-treated mice by day 7, while the numbers in CD28\(^{-/-}\) mice remained the same (\(P = \text{NS}\)) (data not shown).

**SEB-induced anergy/activation-induced cell death (AICD)**

It is widely accepted that the T cells obtained from SAg-primed mice are incapable of proliferation/cytokine production (anergic) following re-stimulation with the same SAg (1,31,32).

Therefore, we studied the secondary proliferative responses of T cells from both CD28-sufficient and -deficient mice primed earlier at different time points with two objectives: (i) to study if the recall responses in HLA transgenic mice follow a similar pattern as that of conventional mice and (ii) to study the role of CD28 co-stimulation in this process. Proliferative responses of both CD28\(^{+/+}\) and CD28\(^{-/-}\) splenocytes collected 3 days after the primary SEB injection were comparable and several fold less than PBS-treated mice of the same group (Fig. 5A). This is probably due to AICD rather than anergy. As the T cells are already in a high activation state at this time point (as indicated by the peak in the expanded population), reactivation with a strong stimulus (such as SEB) might have rapidly induced apoptosis (31,33). Interestingly, the CD28\(^{-/-}\) splenocytes collected 7 days post-SEB priming responded more vigorously than similarly treated CD28\(^{+/+}\) counterparts, albeit the response was less than that of naive mice (Fig. 5A). To rule out the possibility that the low thymidine incorporation seen in CD28\(^{+/+}\) cells is not due to failure to detect early proliferation, cells were pulsed with \(^{3}\text{H}\)thyidine at different time points following activation and harvested 18 h later. Incorporated radioactivity was consistently lower in CD28\(^{+/+}\) splenocytes than CD28\(^{-/-}\) cells at all time points tested (Fig. 5B). Re-
stimulation of splenocytes from DQ8.CD28+/+ mice as late as 9 days following priming showed that the proliferative potential was still suppressed in these mice (data not shown). Priming with lower doses of SEB (10 μg/mouse) also induced similar proliferation defects in DQ8.CD28+/+ mice (data not shown).

Cytokine production by SEB-primed splenocytes
To study if the SEB-primed T cells in CD28+/+ mice are anergic and therefore do not proliferate efficiently following re-stimulation with SEB, culture supernatants from SEB-re-stimulated cultures were screened for the presence of IL-2, IL-4, IL-10 and IFN-γ. Interestingly, CD28+/+ T cells produced significant quantities of cytokines, especially IL-2, IL-10 and IFN-γ, although they did not proliferate upon re-stimulation with SEB. On the other hand, CD28−/− T cells did not produce any of these cytokines even though they proliferated more efficiently than CD28+/+ cells (Fig. 5C). The kinetics of cytokine production was similar to that of naive mice, but quantitatively SEB-primed mice produced less than the naive mice (data not shown).

NO production by SEB-primed splenocytes
As NO has several immunomodulatory properties, the capacity of SEB to stimulate NO production by primed splenocytes and to study the role of CD28 co-stimulation on NO production, SEB-primed splenocytes from B10 mice and DQ8 transgenic mice with and without CD28 were re-stimulated in vitro with SEB and the culture supernatants were tested for the presence of nitrite, a stable oxidative end-product of NO. Compared to B10 cultures which had undetectable levels of nitrite, DQ8.CD28−/− cultures had higher levels of nitrite while the DQ8.CD28+/+ cultures had the highest quantities of nitrite (Fig. 5D). Overall these results indicate that (i) NO production by SEB-primed B10 mice is minimal, probably due to poor presentation of SEB by mouse MHC class II, and (ii) efficient NO production by DQ8 transgenic mice is probably dependent on CD28-derived signals.

Fas expression and cell death in SEB-primed splenocytes
As Fas–Fas ligand (CD95–CD95 ligand) play a major role in AICD, we looked for Fas expression in SEB-primed CD28+/+ and CD28−/− T cells following in vitro re-stimulation with SEB. As shown in Fig. 6(A), CD28−/− T cells expressed significantly less CD95 than CD28+/+ T cells at all time points. To measure cell death following reactivation, the number of viable cells remaining at 24 h following re-stimulation with SEB was determined by Trypan blue dye exclusion. As shown in Fig. 6(b), addition of SEB did not increase significantly the recovery of viable CD28+/+ cells. On the other hand, addition of SEB did increase significantly (P < 0.009) the recovery of viable CD28−/− cells, thus confirming the thymidine incorporation results. Moreover, SEB-stimulated CD28+/+ cultures had more dead cells than CD28−/− cultures (P < 0.03).

Fig. 6. Apoptosis following SEB reactivation. (A) Splenocytes from SEB-primed (50 μg/mouse) CD28−/− or CD28+/+ mice were collected on day 7 and were re-stimulated in vitro with SEB (1 μg/ml). The percentages of cells expressing Fas (CD95) at different time points following re-stimulation were determined by FACS. (B) The number of live/dead cells remaining in culture at 24 h in (A) was enumerated by Trypan blue dye exclusion. Each set represents mean data from five mice. The number of live cells in SEB-stimulated cultures was not different from the cells cultured with medium alone in CD28−/− cells (P = 0.18), while it was significant in CD28+/+ cells (P < 0.009). SEB-stimulated CD28+/+ cultures had significantly higher number of dead cells than similarly stimulated CD28−/− cells (P < 0.03) (C). Recombinant murine IL-2 was added at different concentrations to the splenocyte cultures as in (A) and (B), and cell proliferation was determined by thymidine incorporation. Each bar represents mean ± SD of at least three mice.
To study the modulatory role of exogenously added IL-2 on recall responses to SEB, splenocytes from SEB-primed mice were cultured with varying concentrations of rmIL-2. As shown in Fig. 6(C), addition of exogenous IL-2 did not enhance cell proliferation significantly in either CD28-sufficient or -deficient cultures as measured by thymidine incorporation.

**SEB-induced toxic shock**

To study if transgenic expression of HLA-DQ8 rendered otherwise resistant mice susceptible to SEB-induced shock and to understand the role of CD28-mediated co-stimulation in this process, DQ8.CD28+/+ and DQ8.CD28−/− mice were injected with SEB alone without d-galactosamine pretreatment. All of the CD28+/+ (six of six) mice succumbed within 24 h of second SEB injection, implying HLA molecules rendered mice susceptible to SEB-induced toxic shock even at low doses of SEB. Surprisingly, none of the CD28−/− (zero out of six) mice died suggesting an important role for CD28 in SEB-induced toxic shock. SEB-induced in vivo cytokine production was determined by measuring serum tumor necrosis factor (TNF)-α and IFN-γ at different time points by ELISA. We could not detect any TNF-α even in sera of DQ8.CD28−/− mice which succumbed to SEB-induced shock (data not shown), while we could detect very high levels of IFN-γ in these sera from 90 min onwards (Fig. 7). CD28-deficient mice had significantly less IFN-γ in the sera than CD28-sufficient mice implying a possible pathogenic role for IFN-γ in SEB-induced lethal shock.

**Discussion**

Based on the observations that HLA class II molecules present SEB more efficiently than their murine counterpart (18), we attempted to rescue the defective SEB-induced T cell proliferation of CD28-deficient T cells (13–17) by transgenic expression of HLA-DQ8. While superior proliferation of splenocytes from DQ8 transgenic mice compared to that of B10 and BALB/c set the stage, comparable proliferation of D28-deficient cells to that of CD28-sufficient cells confirmed our hypothesis. This is analogous to the situation where strong (4,5) and repeated antigenic stimulation (3) can drive T cell proliferation and effector functions in the absence of CD28 co-stimulation. However, expression of DQ8 on the antigen-presenting cells did not rescue the well-documented defects in Con A-induced proliferation of CD28−/− cells, as T cell activation by Con A is independent of class II molecules, but dependent on antigen-presenting cells (7,34). Even though CD28-mediated co-stimulation was dispensable for in vitro SEB-induced T cell proliferation and production of IL-6 and IFN-γ (9,35,36), certain effector functions seemed to be absolutely dependent on unique CD28 signals as reported earlier (37–40). This includes production of IL-2 (41), IL-4 (42) and IL-10 (43).

SAg-driven in vivo T cell expansion was also compromised in CD28-deficient mice even though these T cells proliferated very efficiently in vitro. This could be due to diminished IL-2 production by CD28−/− T cells. Secondly, the presence of large quantities of cytokines such as IL-2 and more importantly IL-4 in CD28-sufficient mice but not in CD28-deficient mice in vivo could have protected CD28−/− cells from SAg-activated death (44,45). Thirdly, Bcl-XL-dependent (46) and -independent (47) antiapoptotic pathways operational in CD28-sufficient mice also explain to an extent higher cell recovery. CD28 dependence was more pronounced in CD8+ T cells than CD4+ T cells in our model. This could be either due to differential requirement for co-stimulation between CD4+ and CD8+ T cells (48,49) or distinct CD28-derived biochemical signaling pathways between CD4+ and CD8+ T cells (50).

The prevalent ‘two-signal model of T cell activation’ suggests that TCR-mediated primary activation in the absence of CD28-mediated co-stimulation would result in anergy (2). Therefore, theoretically SEB-primed CD28−/− T cells should be ‘more anergic’ than CD28+/+ T cells (due to absence of CD28-mediated co-stimulation, signal 2 during primary response) and hence should be more refractory to reactivation. Surprisingly, primed CD28−/− T cells but not CD28−/− T cells, failed to proliferate upon recall with SEB. Nevertheless, only the former produced large quantities of IL-4, IL-10 and IFN-γ, including IL-2 upon re-stimulation with SEB. As defective cytokine production by CD28−/− cells was evident even during primary response to SEB, low cytokine production during secondary responses is not unexpected. However, the reason(s) for very low IFN-γ production by primed CD28−/− cells is not clear. Probably, IFN-γ production by primed T cells is more dependent on CD28 co-stimulation as reported earlier (11,51).

Absence of cell proliferation but efficient cytokine production during secondary response by CD28−/− cells could be due to AICD of responder cells. It is widely reported that even though cycling T cells upon antigen re-engagement undergo death, several of their effector functions such as lymphokine production or cytotoxicity are potently expressed [reviewed in (52)]. Therefore, as SEB-primed CD28-sufficient mice had elevated T cell numbers even at day 7, reactivation with SEB might have resulted in rapid induction of AICD. On the contrary, as CD28−/− T cells did not undergo activation to the same extent during primary activation, they were relatively resistant to AICD following re-stimulation. Elevated Fas expression in SEB-primed CD28−/− cells and increased production of cytokines implicated in AICD such as IL-2 (52)
and IFN-γ (53) might further explain increased cell death in CD28+/+ cells. CD28-dependent production of other pro-apoptotic mediators such as reactive oxygen species (54) and reactive nitrogen intermediates (55,56) may enhance apoptosis in CD28-sufficient mice. As proliferation has been shown to be a prerequisite for bacterial SAg-induced T cell apoptosis in vivo (57), CD28-derived signals which contributed to greater clonal expansion and efficient mediation of effector functions also rendered CD28+/+ cells more susceptible to the homeostatic control of immune response (by AICD) (58). Superior secondary response of cells deprived of CD28-derived signals during their primary activation has been reported earlier in a SAg model (59,60). Observations such as blocking CD28-mediated co-stimulation exacerbated spontaneous or experimental autoimmune diseases (39,61,62), CD28+ but not CD28-null human T cells show preferential sensitivity to AICD in patients with systemic lupus erythematosus (63), increase in the CD28-null T cell population in patients with rheumatoid arthritis due to their increased survival (64) and CD28-deficient T cells in multiple sclerosis patients are resistant to apoptosis (65) all support our hypothesis.

SAg-induced toxic shock is the major pathology seen in human (66). Mice are generally considered resistant to SEB-induced toxic shock unless pretreated with D-galactosamine (D-Gal) which sensitizes hepatocytes to apoptosis induced by TNF-α (67). TNF-α and IFN-γ are considered to be the key cytokines responsible for D-Gal-sensitized SAg-induced shock. CD28-deficient mice are resistant to D-Gal sensitized, toxic shock syndrome toxin-induced shock (15,16). It was unclear whether the protection of CD28-deficient mice from SAg-induced shock was due to overall failure of CD28+/+ cells to respond to SAg per se (15) or due to low production of IFN-γ and TNF-α (15,16). The results of the present study clearly show that protection of CD28-deficient mice is not due to their inability to respond to SEB, but probably due to low IFN-γ production as reported in other models (68,69). Surprisingly, we could not detect any TNF-α in HLA-DQ8 transgenic mice even though they were highly susceptible to SEB-induced shock. This questions the role of TNF in SEB-induced shock. In support of our finding, recent studies have shown that blocking by CTLA-4–Ig did not inhibit TNF production (17), and that there is no correlation between serum TNF levels and lethality in SEB-induced shock (67).

Although it is tempting to question why cells from CD28-deficient mice capable of producing high levels of IFN-γ in vitro during the primary response produced less IFN-γ in vivo, it should be borne in mind that the in vivo response might not completely mimic the in vitro scenario. As the antigen-presenting cells, T and B cells are generally distributed in distinct anatomical regions in the lymphoid organs in vivo while they are mixed together during the in vitro assay, differences in their responses are not unexpected. Moreover, CD28-deficient mice are known to have defective germinal center formation and altered T/B cell homing (70,71) thereby affecting cell–cell interaction during an immune response. This might explain the poor in vivo T cell expansion and cytokine production in CD28-deficient mice.

In conclusion, SAg (SEB)-mediated T cell activation/proliferation are not absolutely dependent upon CD28 co-stimulation, while some of the effector functions are compromised in the absence of CD28 co-stimulation and CD28-mediated co-stimulation also renders the responder cells susceptible to AICD.

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Abbreviations

AICD activation-induced cell death
CFSE 5-(6)-carboxyfluorescein diacetate succinimidyl ester
Con A concanavalin A
D-Gal d-galactosamine
NO nitric oxide
SAg superantigen
SEB staphylococcal enterotoxin B
TNF tumor necrosis factor

References

gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. J. Exp. Med. 192:123.


