

Protection Against Lethal Toxic Shock by Targeted Disruption of the CD28 Gene

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Summary

Toxic shock syndrome (TSS) is a multi system disorder resulting from superantigen-mediated cytokine production. Nearly 90% of the clinical cases of TSS arise due to an exotoxin, toxic shock syndrome toxin-1 (TSST-1), elaborated by toxigenic strains of *Staphylococcus aureus*. It is clearly established that besides antigen-specific signals a variety of costimulatory signals are required for full T cell activation. However, the nature and potential redundancy of costimulatory signals are incompletely understood, particularly with regards to superantigen-mediated T cell activation in vivo. Here we report that CD28-deficient mice (CD28^{-/-}) are completely resistant to TSST-1-induced lethal TSS while CD28 (+/-) littermate mice were partially resistant to TSST-1. The mechanism for the resistance of the CD28 (-/-) mice was a complete abrogation of TNF- α accumulation in the serum and a nearly complete (90%) impairment of IFN- γ secretion in response to TSST-1 injection. In contrast, the serum level of IL-2 was only moderately influenced by the variation of CD28 expression. CD28 (-/-) mice retained sensitivity to TNF- α as demonstrated by equivalent lethality after cytokine injection. These findings establish an essential requirement for CD28 costimulatory signals in TSST-1-induced TSS. The hierarchy of TSST-1 resistance among CD28 wild-type (CD28^{+/+}), CD28 heterozygous (CD28^{+/-}), and CD28^{-/-} mice suggests a gene-dose effect, implying that the levels of T cell surface CD28 expression critically regulate superantigen-mediated costimulation. Finally, as these results demonstrate the primary and non-redundant role of CD28 receptors in the initiation of the in vivo cytokine cascade, they suggest therapeutic approaches for superantigen-mediated immunopathology.

Toxic shock syndrome (TSS) is characterized clinically by high fever, hypotension, erythroderma, skin desquamation, and in severe cases, multiple organ failure and death (1). TSS is induced by a number of bacterial superantigens (SAg) and among these SAGs, the toxic shock syndrome toxin-1 (TSST-1) is the most common cause of exotoxin-induced TSS (1, 2). TSST-1, produced by several toxigenic strains of *Staphylococcus aureus*, binds to MHC class II molecules on antigen presenting cells (3) and stimulates V β 2-bearing T cells in humans (4) and V β 3-, V β 15-, and V β 17-bearing T cells in mice (5, 6). Upon activation, these T cells proliferate and produce a surge of proinflammatory cytokines such as IL2, TNF- α , and IFN- γ (2), and it is these cytokines that eventuate in the clinical syndrome (2).

For conventional antigens to trigger T cells, two signals are needed: the first signal is delivered by the TCR-Ag-Ia complex and the second signal by the interaction between a costimulatory molecule on APC and its T cell counter-receptor (7-9). Recent observations in vitro also support a dependence on a costimulatory signal for SAg-mediated

activation of T cells (10-13). However, one study has concluded that SAg-stimulated T cell proliferation in vitro is independent of CD28 (14). Others have concluded that the requisite costimulatory signals for nominal antigen and superantigen may differ in studies using astrocytes as APC (15). Together, these data strongly suggest an in vitro requirement for costimulatory signals for TSST-1-induced T cell responses, however in vivo, a requirement for particular costimulatory signals has not yet been established. To date only one study has addressed the requirement for CD28 costimulation in vivo. Muraille and coworkers demonstrated a partial amelioration with anti-B7-2 antibodies, but not anti-B7-1 antibodies in *Staphylococcal* enterotoxin B (SEB)-induced TSS (16). Since the CD28-mediated costimulatory signal is emerging as an important regulator of both endogenous and exogenous SAg-induced T cell responses, CD28^{-/-} mice were used to assess the requirement for CD28 involvement in TSST-1-induced TSS. The CD28^{-/-} mice were entirely resistant to TSST-1-induced TSS, providing genetic evidence to indicate that

CD28 provides an essential and non-redundant costimulatory signal for TSS. Our results also demonstrated that different levels of T cell CD28 expression in CD28+/+, CD28+/- and CD28-/- mice correlated with TSST-1-induced lethality and with the serum TNF- α and IFN- γ levels. Surprisingly, TSST-1-induced elevation in serum IL-2 levels were only moderately influenced by CD28 expression. Finally, injection of TNF- α entirely abolished TSST-1 resistance of CD28-/- mice, confirming a major role of TNF- α as an effector in TSS.

Materials and Methods

Mice. Female C57BL/6 (H-2^b) mice (age 6–8 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME). CD28-/- mice were generated as previously described (17). A male CD28+/- mouse, as produced by backcrossing the originally described CD28-/- mice to a C57BL/6 mouse, was bred with a female C57BL/6 mouse to produce heterozygous offspring. The heterozygous litter mates were intercrossed to generate CD28+/+, CD28+/- and CD28-/- mice. These mice were further backcrossed to the C57BL/6 background for five generations. The mice were phenotyped for CD28 expression by FACS analysis of peripheral blood T cells and genotyped by a polymerase chain reaction (PCR) from genomic DNA obtained by tail biopsy using primers specific for either wild-type or knock-out CD28 constructs (17). The mice used as controls were the wild-type and heterozygous litter mates of the CD28-deficient mice. The experiments described herein were conducted according to the principles set forth in the Guide for The Care and Use of Laboratory Animals, NIH Publication, 85–23 (1985).

Determination of CD28 Phenotype by Flow Cytometry. The CD28 phenotype was determined by using a FITC-conjugated anti-CD3 mAb (PharMingen, San Diego, CA), a phycoerythrin-conjugated anti-CD28 mAb. Background staining levels were determined by use of a FITC- or phycoerythrin-conjugated Leu-4 mAb (PharMingen). For phenotyping, peripheral blood was collected from (C57BL/6 \times CD28 KO) F5 mice and PBMC were isolated by gradient centrifugation. 10⁶ PBMC were incubated with a FITC-conjugated anti-CD3 mAb followed by phycoerythrin-conjugated anti-CD28 mAb. The stained cells were analyzed by an Epics flow cytometer (Coulter Inc., Hialeah, FL) and an Excel-based curve smoothing program was used to describe the histogram.

Monoclonal Antibodies and Reagents. Fluorochrome-conjugated anti-CD3 and anti-CD28 antibodies were procured from PharMingen. TSST-1 was purchased from Toxin Technology (Sarasota, FL). D-galactosamine (D-Gal) was purchased from Sigma Chemical Co. (St. Louis, MO).

Mortality Assay. CD28-deficient mice or their control wild-type and heterozygous litter mates were injected with 20 μ g of TSST-1 in their hind foot pads 30 min after intraperitoneal injection of D-Gal.

Sera Preparation. The CD28-deficient or control mice, 12 in each group, were treated with D-Gal and TSST-1 as described above. Two mice from each group were euthanized at each of the indicated time-points and their blood was clotted on ice, then centrifuged at 10,000 rpm. The supernatants were collected and stored at -70°C for subsequent lymphokine assays.

Lymphokine Assay. Using appropriate dilutions of sera, TNF- α , IL-2, and IFN- γ were assayed with Endogen (Cambridge, MA) ELISA kits according to the manufacturer's instructions.

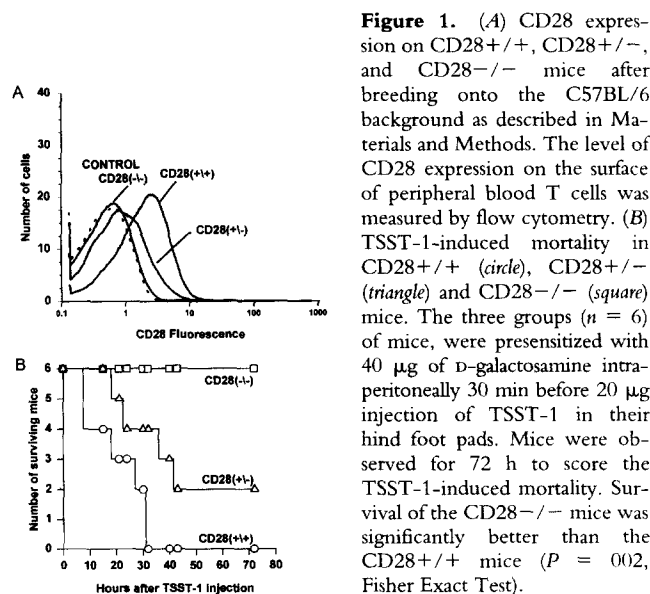
Statistical Analysis. Each experiment was performed two to

four times and representative data are presented. Differences between experimental groups in the mortality assay were analyzed using Fischer's exact test. ELISAs were analyzed using Student's *t* test.

Results

The Level of CD28 Expression Is a Critical Determinant for TSST-1-induced Lethal Shock. To assess the role of the CD28 costimulatory receptor in TSST-1-induced shock, mice homozygous for the disrupted CD28 gene (CD28-/-) were compared to their wild-type homozygous (CD28+/+) or CD28 heterozygous (CD28+/-) litter mates. These mice were obtained by intercross of mice subsequent to backcross done five times of the original CD28 heterozygote by C57BL/6 strain. The CD28 genotype was defined by PCR against genomic DNA using primers specific for wild-type or disrupted CD28 (17) (data not shown). In preliminary studies, we could not detect differences in the peripheral T cell repertoire of the CD28-/- and CD28+/- mice as determined by flow cytometric analysis of V β 3, V β 6, and V β 8 expressing T cells (data not shown), confirming previous studies that have not revealed CD28-mediated differences in thymic selection for nominal and endogenous SAg (17, 18). The phenotypic expression of CD28 on the surface of resting T cells in peripheral blood was assessed by flow cytometric analysis (Fig. 1 *a*). It was found that the surface staining with anti-CD28-PE of T cells from CD28-/- mice had background fluorescence intensity while T cells from CD28+/+ mice had a much higher fluorescence intensity. T cells from CD28+/- mice demonstrated an intermediate staining intensity with anti-CD28. These results are consistent with the idea that CD28 surface expression on T cells is controlled by a gene-dosage effect.

To test the effect of CD28 expression on TSST-1-induced lethal shock, CD28+/+, CD28+/- and



CD28^{-/-} mice, 6 per experimental group, were injected with D-Gal and TSST-1 as described previously (2). D-Gal was used to abolish the natural resistance of the rodents to enterotoxins (2). All CD28^{+/+} mice showed signs of TSST-1-induced illness including shivering, piloerection, cessation of movement and heaping up together within 2 h of TSST-1 injection. In striking contrast, none of the six CD28^{-/-} mice tested showed any sign of illness. While six out of six CD28^{+/+} mice died within 36 h of TSST-1 injection, all CD28^{-/-} mice survived (Fig. 1 b) indicating that functional expression of the CD28 gene is required for TSST-1-induced TSS. Interestingly, CD28^{+/-} mice displayed an intermediate pattern of disease susceptibility and showed a delayed death—of the six mice injected, four died and two survived (Fig. 1 b). These mice also developed symptoms from TSS later, with onset ~4 h after TSST-1 injection. This pattern of CD28-dependent TSST-1-resistance was reproduced in four independent experiments. Since CD28^{+/-} mice have intermediate T cell surface expression of CD28 between CD28^{+/+} and CD28^{-/-} mice, the level of CD28 expression likely determined the severity of symptoms and clinical course of the disease. It has been shown that CTLA4, a related molecule that shares the same ligands with CD28 (19), is expressed in CD28^{-/-} mice (20). The association between CD28 expression and TSST-1 lethality indicated that B7:CTLA4 interactions are not sufficient for the induction of TSS.

Secretion of Proinflammatory Cytokines Is Dependent on the Level of CD28 Expression. It has been shown previously that a cytokine cascade mediates the immunopathology of TSS. Therefore, the role of CD28 in cytokine production, particularly TNF- α , IFN- γ , and IL-2 production during TSS was assessed (Fig. 2 a). Serum levels of TNF- α were measured at multiple time points after TSST-1 injection. While a sharp increase was observed within 1 h after TSST-1 injection in CD28^{+/+} mice, CD28^{-/-} mice had a profound defect in TNF- α serum levels. The complete abrogation of TNF- α accumulation at all time points in the CD28^{-/-} mice was in contrast to the level of TNF- α in CD28^{+/-} mice that was nearly half of that observed in CD28^{+/+} mice (Fig. 2 a). Given the intermediate level of CD28 expression in these mice, these results indicate that the level of CD28 expression determines the level of TNF- α secretion after TSST-1 injection.

IFN- γ is also known to be involved in the acute inflammation and tissue damage characteristic of TSS pathology and therefore, serum levels of IFN- γ from TSST-1-injected mice were examined (Fig. 2 b). In contrast to the early accumulation of TNF- α , the appearance of IFN- γ in the serum was delayed. CD28^{+/+} mice displayed a pronounced rise in the serum level of IFN- γ 4 h after TSST-1 injection, whereas only a marginal elevation of IFN- γ was observed in CD28^{-/-} mice. Similar to TNF- α , CD28^{+/-} mice displayed intermediate IFN- γ levels, indicating that the level of CD28 expression also determined TSST-1-induced IFN- γ production.

Previous reports indicated that CD28 can provide costimulation for SEB to result in increased IL-2 gene transcription in vitro (10). Consistent with this, serum IL-2 elevation in vivo was significantly lower in CD28^{-/-} mice as compared to CD28^{+/+} and CD28^{+/-} mice after TSST-1 injection (Fig. 2 c). In contrast to the CD28 gene dosage differences shown above for TNF- α and to a lesser extent for IFN- γ production, the level of IL-2 in CD28^{+/-} mice was not different from that found in CD28^{+/+} mice. Apparently low-level CD28 expression is sufficient for full IL-2 secretion while the higher CD28 levels found in CD28^{+/+} mice are required for full TNF- α . Furthermore, the reduced but still appreciable IL-2 elevation in the CD28^{-/-} mice indicates the presence of CD28-independent mechanisms of costimulation for IL-2, in contrast to an absolute requirement for CD28 in the case of TNF- α (Fig. 2, a vs c).

TNF- α Is an Essential Cytokine Required for TSST-1-Induced Shock Syndrome. TNF- α has been proposed to be a major cytokine in TSS as it is the first cytokine to peak after TSST-1 injection, it effects the subsequent production of other cytokines and their receptors, and anti-TNF- α antibodies prevent TNF-induced lethality (2). Thus, the TSST-1-resistant phenotype of the CD28^{-/-} mice could be explained by the lack of TNF- α secretion. Alternatively, it was possible that the downstream cascade of TNF-mediated events was also disrupted in the CD28^{-/-} mice, and therefore, that CD28^{-/-} mice might be resistant to TNF- α . To distinguish between these possibilities, TNF- α was injected at a sublethal dose along with TSST-1 in CD28^{+/+} and CD28^{-/-} mice. As shown in Fig. 3 a, the TSST-1-resistant CD28^{-/-} mice succumb to TSST-1-induced

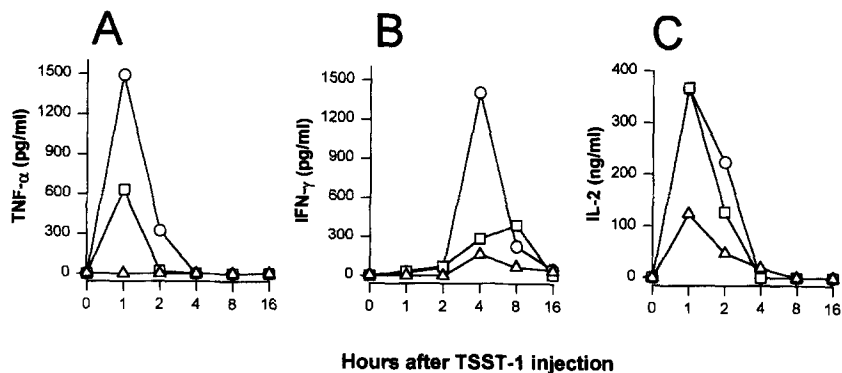


Figure 2. Serum concentrations of TNF- α (A), IFN- γ (B), and IL-2 (C) at different time points after injection of TSST-1 in CD28^{+/+} (circle), CD28^{+/-} (square), and CD28^{-/-} (triangle) mice. These mice, 12 in each group, were treated with D-galactosamine and TSST-1 as described in Fig. 1. Two mice from each group were euthanized at each of the indicated time-points and the sera were collected and stored at -70°C for subsequent cytokine assay. The cytokines were assayed by ELISA (Endogen, Inc., Boston, MA) and the mean value from the paired sera depicted.

TSS when TSST-1 was accompanied by a sublethal dose of TNF- α . Neither reagent alone reproduced the lethal effects of TSS in CD28 $^{-/-}$ mice.

The TNF- α add-back experiment in the CD28-deficient mice was used to determine whether the TSS that occurred in these mice was associated with reconstitution of IFN- γ secretion. Wild type or CD28 $^{-/-}$ mice were injected with TSST-1 with or without TNF- α as described above and sera were collected 4 h after TSST-1 injection, based upon the expected kinetics determined in Fig 2. IFN- γ levels were about threefold lower in normal mice given TNF- α injections as compared to TSST-1 injection. CD28 $^{-/-}$ mice given TSST-1 only had levels sevenfold less than wild-type mice. It was found that TNF- α co-injection with TSST-1 restored the levels of IFN- γ in CD28 $^{-/-}$ mice nearly to the level of CD28 $^{+/+}$ mice treated with TSST-1 (Fig. 3 b). Thus, conditions associated with eventual lethality had high IFN- γ levels while the conditions associated with ultimate survival had lower IFN- γ levels. These results are consistent with the notion that the lethal effect of TNF- α as observed in TSS is mediated through induction of cytokines including IFN- γ . Therefore we concluded that CD28-transduced signals are essential for production of TNF- α in the context of TSST-1 stimulation and further, that TNF- α can bypass the CD28-mediated signaling deficit to reconstitute the clinical features of TSS in CD28 $^{-/-}$ mice.

Discussion

Since costimulation through CD28 plays a critical role in a variety of T cell responses against nominal foreign antigens, auto-, allo-, or xeno-genetic transplantation antigens (9, 18, 21), we have studied the role of this costimulatory signal in superantigen-driven T cell activation by using CD28 $^{-/-}$

mice. The concordance of the level of T cell surface CD28 expression with the severity and lethality of TSS confirm the pivotal role of the CD28 molecule in TSST-1-induced TSS. The CD28 $^{-/-}$ mice had a striking defect in the ability to secrete TNF- α , and reveal that there are no redundant costimulatory molecules for this form of superantigen-mediated T cell activation. Furthermore, serum levels of TNF- α and IFN- γ were also observed to be correlated to the CD28 surface expression and lethality.

The pathogenesis of TSS by bacterial SAg is mediated by T cells as SCID mice are entirely resistant to TSST-1 and SEB but are rendered susceptible when reconstituted with syngeneic T cells (2, 22). The T cells which express the SAg-reactive V β TCRs, e.g., V β 3, V β 15, and V β 17 for TSST-1, V β 8 for SEB are required for the onset of TSS. Although SAg can bind directly the MHC class II molecules and activate the pertinent V β TCR-bearing T cells by direct interactions with TCR, recent studies have demonstrated that ICAM-1-deficient mice are resistant to SEB-induced lethal TSS (23), indicating the importance of adhesion molecules in TSS. Therefore, it is now evident that in addition to MHC class II, TCR and adhesion molecules, costimulatory molecules also play important roles in SAg-induced T cell responses.

The most likely explanation uncovered for the TSST-1-resistant phenotype of the CD28-deficient mice was the impairment of TNF- α secretion. It is notable that while the serum levels of TNF- α and IFN- γ in TSST-1-injected CD28 $^{+/-}$ mice were almost half the level of those in CD28 $^{+/+}$ mice, the IL-2 levels were not different between CD28 $^{+/+}$ and $^{+/-}$ mice. Three different possibilities may account for this difference in TNF- α and IL-2 regulation. First, the threshold of CD28-mediated signaling required for the production of these cytokines may differ and thus, signaling through V β -TCR and engagement of fewer CD28 molecules on T cells would be sufficient for triggering IL-2 but not TNF- α and IFN- γ . The second possible mechanism would involve CD28-mediated stabilization of cytokine mRNA (24). Since TNF- α is transcribed at a substantial rate in resting T cells (25) but steady state mRNA levels of TNF- α remain low due to rapid degradation (26), it is possible that CD28 signaling may be more important for stabilizing TNF- α mRNA than IL-2 mRNA (24). Finally, it is possible that CD28 has effects at a posttranslational level. There are two forms of tumor necrosis factor, a type II membrane protein of relative molecular mass 26 kD and a soluble, 17-kD form generated from the cell-bound protein by metalloproteinase-mediated proteolytic cleavage. Inhibition of the metalloproteinase protects mice from endotoxic shock (27, 28). It is possible that the activity of this enzyme which has been shown to be essential for the release of TNF- α from its precursor is in some manner regulated by CD28. Further studies will be required to define the mechanism of the TNF- α defect in CD28-deficient mice.

Our results confirm previous studies using other experimental approaches indicating that TNF- α appears to be a major cytokine in TSS because not only is it the first cyto-

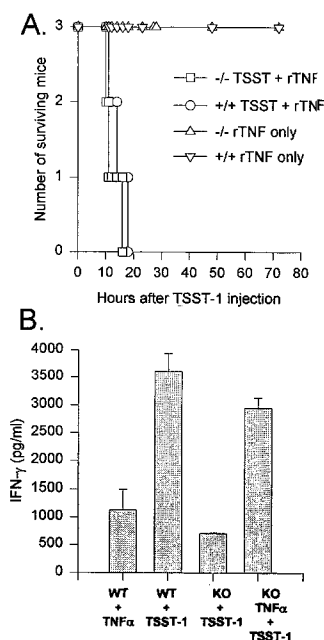


Figure 3. (A) Role of TNF- α in TSST-1-induced mortality. CD28 $^{+/+}$ (square) and CD28 $^{-/-}$ (circle) mice were injected with D-galactosamine and/or TSST-1 as described in Fig. 1. The mice also received a sublethal dose of rTNF- α (1 ng/mouse) intravenously. The control groups received only rTNF- α at the same dose (triangle). (B) Effect of TNF- α injection on serum IFN- γ concentration. Groups of mice were treated as in (A), and sera obtained 4 h after TSST-1 injection to measure IFN- γ by ELISA. The mean and range of paired serum samples is indicated.

kine to peak after TSST-1 injection, TNF- α also initiates a self-amplifying cascade of cytokine interactions that eventuates in a lethal outcome (2, 22). The cascade may start with TNF- α which stimulates T cell IL-2 receptor expression and thereby, confers IL-2 responsiveness to result in an enhanced T cell proliferation (29). In support of this, we have found that the T cells from CD28 $^{-/-}$ mice proliferate much less in response to TSST-1 than the T cells from CD28 $^{+/+}$ mice. This low T cell proliferation can be restored by TNF- α and is sensitive to anti-IL2 receptor antibody (data not shown). Thus, although CD28 $^{-/-}$ mice have significant levels of IL-2 in response to stimulation by nominal antigen as shown by Lucas and coworkers (18) and as shown here after SAg stimulation, the T cells may not be fully responsive due to low IL2-receptor expression resulting from the absence of TNF- α (data not shown). However, after being induced by TNF- α , IFN- γ , in its turn, signals through TNF- α -induced IFN- γ receptors on macrophages to augment MHC class II expression (30). This

may effect a more efficient presentation of TSST-1 to T cells as T cell proliferation is known to be directly proportional to the product of antigen concentration and the density of Ia molecules (31). The primary role of TNF- α in TSS is also supported by studies indicating that neutralizing TNF- α in vivo by injection of anti-TNF- α antibody also rescues mice from TSST-1-induced death (2) and that TNF- α receptor (p55) knock out mice are entirely resistant to SEB-induced TSS (32).

In conclusion, CD28 is essential for TSST-1 induced TNF- α production and the subsequent fatal pathological sequelae. Disruption of CD28 gene abrogates TNF- α production and thereby prevents the onset of the syndrome. Therefore, knowing that the CD28-mediated costimulatory signal is non-redundant for T cell superantigens provides a rationale for novel therapeutic interventions for toxic shock syndrome and potentially for other states of immunopathology caused by dysregulation of T cell lymphokine secretion.

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