

Resistance of CD7-deficient Mice to Lipopolysaccharide-induced Shock Syndromes

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Summary

CD7 is an immunoglobulin superfamily molecule involved in T and natural killer (NK) cell activation and cytokine production. CD7-deficient animals develop normally but have antigen-specific defects in interferon (IFN)- γ production and CD8⁺ CTL generation. To determine the *in vivo* role of CD7 in systems dependent on IFN- γ , the response of CD7-deficient mice to lipopolysaccharide (LPS)-induced shock syndromes was studied. In the high-dose LPS-induced shock model, 67% of CD7-deficient mice survived LPS injection, whereas 19% of control C57BL/6 mice survived LPS challenge ($P < 0.001$). CD7-deficient or C57BL/6 control mice were next injected with low-dose LPS (1 μg plus 8 mg D-galactosamine [D-gal] per mouse) and monitored for survival. All CD7-deficient mice were alive 72 h after injection of LPS compared with 20% of C57BL/6 control mice ($P < 0.001$). After injection of LPS and D-gal, CD7-deficient mice had decreased serum IFN- γ and tumor necrosis factor (TNF)- α levels compared with control C57BL/6 mice ($P < 0.001$). Steady-state mRNA levels for IFN- γ and TNF- α in liver tissue were also significantly decreased in CD7-deficient mice compared with controls ($P < 0.05$). In contrast, CD7-deficient animals had normal liver interleukin (IL)-12, IL-18, and interleukin 1 converting enzyme (ICE) mRNA levels, and CD7-deficient splenocytes had normal IFN- γ responses when stimulated with IL-12 and IL-18 *in vitro*. NK1.1⁺/CD3⁺ T cells are known to be key effector cells in the pathogenesis of toxic shock. Phenotypic analysis of liver mononuclear cells revealed that CD7-deficient mice had fewer numbers of liver NK1.1⁺/CD3⁺ T cells ($1.5 \pm 0.3 \times 10^5$) versus C57BL/6 control mice ($3.7 \pm 0.8 \times 10^5$; $P < 0.05$), whereas numbers of liver NK1.1⁺/CD3⁻ NK cells were not different from controls. Thus, targeted disruption of CD7 leads to a selective deficiency of liver NK1.1⁺/CD3⁺ T cells, and is associated with resistance to LPS shock. These data suggest that CD7 is a key molecule in the inflammatory response leading to LPS-induced shock.

Key words: CD7 • lipopolysaccharide • septic shock • NK1.1 • T cells

CD7 is a 40-kD member of the Ig gene superfamily that is expressed on a major subset of human peripheral T lymphocytes and NK cells (1–7). CD7 is an early T cell activation antigen in that CD7 mRNA levels rise within 15 min after initiation of a transmembrane calcium ion flux (8). CD7 can complex with CD3 and CD45 molecules (9), and CD7 signaling involves both protein kinase C and protein tyrosine kinase (6, 10). CD7 has been shown to be a functional signal-transducing molecule on resting NK cells (6, 11). Antibody cross-linking of NK cell CD7 induced increases in free cytoplasmic calcium, secretion of IFN- γ , NK cell proliferation, adhesion to fibronectin, and NK cytotoxic activity (6). Although the above studies have demonstrated *in vitro* roles for CD7 in T and NK cell activa-

tion and/or adhesion, relevant functions of CD7 *in vivo* remain unknown.

To probe *in vivo* functional roles of CD7 in the murine immune system, CD7-deficient mice were generated using homologous recombination techniques (12). We have previously shown that CD7-deficient animals had decreased *in vitro* antigen-specific IFN- γ production, and had diminished antigen-driven CD8⁺ CTL activity (12).

Use of genetically deficient mice has been instrumental in identifying the roles of cytokines in LPS-induced shock. The low-dose LPS shock model takes advantage of the increased susceptibility of mice to 1 μg of LPS after injection with 8 mg of D-galactosamine (D-gal) (13, 14). IFN- γ and TNF- α are key mediators of hepatocyte necrosis and death

in the low-dose LPS shock model (15–18). In the high-dose LPS shock model, administration of 100 mg/kg of LPS results in increased levels of serum inflammatory cytokines, neutrophil infiltration, and aggregation in liver and other tissues, and death within 3–5 d (14). Intracellular adhesion molecule (ICAM)-1, IL-1, and TNF- α are key mediators of the lethal effects of high-dose LPS (19–22).

In this study, we have determined the sensitivity of CD7-deficient mice to LPS-induced shock syndromes. We found that CD7-deficient mice were completely resistant to death in the low-dose LPS shock model, and they were partially resistant to death in the high-dose LPS shock model.

Materials and Methods

Animals and Treatments. Homozygous CD7-deficient mice (12) were backcrossed five generations onto the C57BL/6 background. C57BL/6 mice were obtained from The Jackson Laboratory. 10–12-wk-old sex-matched mice were studied. Phenol extracted LPS from *Escherichia coli* (Sigma Chemical Co.) was administered intraperitoneally at 100 mg/kg for the high-dose LPS shock model. For the low-dose LPS plus D-gal (Sigma Chemical Co.) shock model, animals received 1 μ g of LPS and 8 mg D-gal intraperitoneally in 0.5 ml saline. Animals were killed and livers and spleens were excised and studied as indicated below. Mouse handling and experimental procedures were conducted in accordance with the American Association of Accreditation of Laboratory Animal Care guidelines for animal care and use.

Cytokine Measurements. Quantification of murine IFN- γ and TNF- α present in sera and culture supernatants was determined using Duoset cytokine-specific ELISA kits as per the manufacturer's protocols (Genzyme Corp.).

In Vitro Splenocyte Cultures. Splenocytes were stimulated with murine recombinant (r)IL-12 (Genzyme Corp.) and murine rIL-18 (Chemicon) in RPMI 1640 with l-glutamine (GIBCO BRL) supplemented with 10% FCS, 5.5×10^{-5} M 2-ME, and 10 μ g/ml gentamicin (BioWhittaker) for 3 d at 10^6 cells/ml in tissue culture plates (Costar) at 37°C in a 5% CO₂ humidified incubator.

RNA Isolation and RNase Protection Assays. Total RNA was isolated from spleen and liver tissue using Trizol (GIBCO BRL) as per the manufacturer's protocol. Steady-state levels of specific cytokine messenger RNA in tissues were determined using the multiprobe RiboQuant RNase Protection Assay (PharMingen).

Isolation of Liver Lymphocytes and Flow Cytometry. Liver lymphocytes were prepared as previously described (23). Phenotypic analysis of liver lymphocytes (10^6 cells in 50 μ l) was performed at 4°C after an initial blocking step with 1 μ g of unlabeled anti-Fc γ R Ab (PharMingen). mAbs used included CD3 (Caltag), NK1.1 (PharMingen), and B220 (Caltag).

Statistical Analysis. Student's *t* test was used to determine significance of cytokine mRNA and protein levels. Chi-square tests were used to determine *P* values for mouse survival data.

Results

CD7-deficient Mice Are Resistant to LPS-Induced Shock Syndromes. To determine the effect of high-dose LPS treatment in CD7-deficient mice, CD7-deficient mice ($n = 30$) and C57BL/6 control ($n = 16$) mice were injected intraperitoneally with LPS (100 mg/kg) and survival was as-

sessed daily for 7 d (Fig. 1 A). C57BL/6 mice succumbed to shock between days 1 and 2 after high-dose LPS injection, with only 19% of the animals surviving on day 7. In contrast, 67% of CD7-deficient animals were alive on day 7 ($P < 0.001$), and demonstrated partial resistance to high-dose LPS shock. As additional controls, saline injected CD7-deficient ($n = 3$) and C57BL/6 control mice ($n = 3$), remained alive and healthy throughout the 7-d study (Fig. 1 A).

Within 12 h of intraperitoneal injection of low-dose LPS (1 μ g) and D-gal (8 mg), only 20% of control C57BL/6 animals ($n = 10$) survived (Fig. 1 B), consistent with previously reported data for this model (13). In contrast, no deaths were observed in CD7-deficient mice ($n = 10$) up to 72 h after injection with LPS ($P < 0.001$). Because of the total resistance of CD7-deficient mice to death in the low-dose LPS shock syndrome model, we studied this model further.

Elevated Serum Levels of TNF- α and IFN- γ in the Low-Dose LPS Shock Model Are Dependent on CD7 Expression. Studies using IFN- γ R-deficient (15) and TNFR1-deficient (16, 18) mice have clearly demonstrated the importance of IFN- γ and TNF- α as dominant cytokines that induce hep-

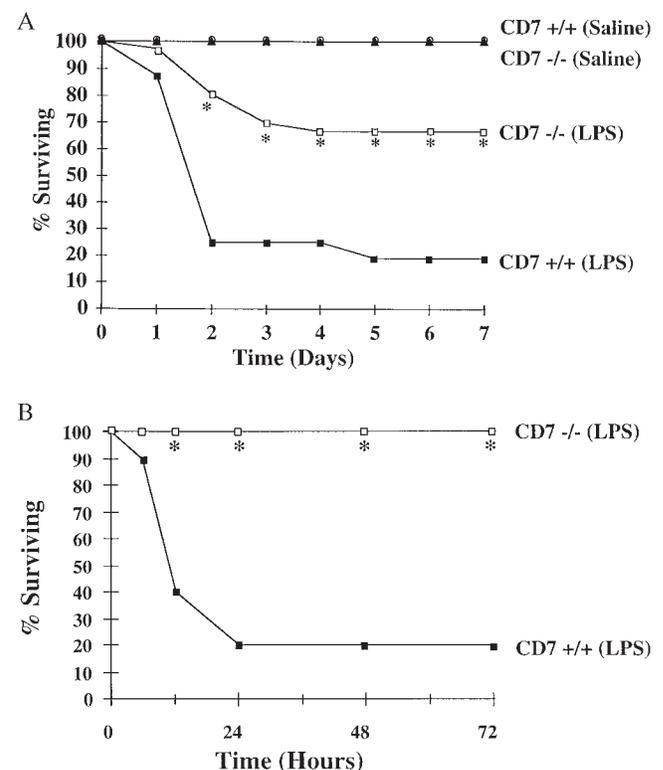


Figure 1. Survival of CD7-deficient mice treated with LPS. (A) High-dose shock model. C57BL/6 control (■, $n = 16$) and CD7-deficient mice (□, $n = 30$) were injected intraperitoneally with LPS (100 mg/kg). Control C57BL/6 (○, $n = 3$) and CD7^{-/-} mice (▲, $n = 3$) were injected intraperitoneally with saline. Mortality was assessed daily for 7 d. (B) Low-dose LPS plus D-gal shock model. C57BL/6 control mice (■, $n = 10$) and CD7-deficient mice (□, $n = 10$) were injected intraperitoneally with LPS (1 μ g) and D-gal (8 mg) in saline. Mortality was assessed for 3 d; * $P \leq 0.001$ when comparing survival of C57BL/6 control mice to CD7-deficient mice.

atitis and death in the low-dose LPS shock model (14). Therefore, serum levels of TNF- α and IFN- γ were measured in control C57BL/6 and CD7-deficient mice at multiple time points after injection of LPS and D-gal (Fig. 2). There was a sharp increase in serum TNF- α levels in C57BL/6 animals 1 h after LPS plus D-gal treatment, that fell to baseline after 4 h of treatment (Fig. 2 A). In contrast, a blunted peak in TNF- α secretion was observed in CD7-deficient mice 1 h after LPS plus D-gal treatment, with a rapid return to baseline 2 h after treatment. Peak TNF- α serum levels in CD7-deficient animals were $55 \pm 7\%$ of that of control animals ($P < 0.001$) with a decrease in serum TNF- α levels 2 h after LPS plus D-gal treatment.

In contrast to the early 2 h rise in TNF- α , serum IFN- γ levels in C57BL/6 mice peaked 6 h after injection of LPS plus D-gal (Fig. 2 B). Importantly, a near complete absence of serum IFN- γ was observed in CD7-deficient mice at the same time point ($P < 0.001$), and throughout the entire 12-h study period. Thus, disruption of the CD7 gene resulted in a partial block in serum TNF- α production, and in a complete block in serum IFN- γ production in the low-dose LPS-induced shock model.

IFN- γ and TNF- α Cytokine mRNA Expression Was Decreased in Liver from LPS-Treated CD7-deficient Mice. Next, cytokine gene expression in liver and spleen tissue from C57BL/6 and CD7-deficient mice was quantified by RNase protection assays. C57BL/6 ($n = 3$) and CD7-deficient ($n = 3$) animals were treated with low-dose LPS plus D-gal for either 0 or 6 h. The expression of various proinflammatory cytokine genes in liver and spleen are shown in Fig. 3.

Marked increases in TNF- α , IFN- γ , and IL-6 steady-state mRNA levels were observed in liver tissues from C57BL/6 control mice treated with LPS plus D-gal for 6 h ($P < 0.05$; Fig. 3 A). As seen in the control mice, expression of TNF- α and IL-6 mRNA were also significantly el-

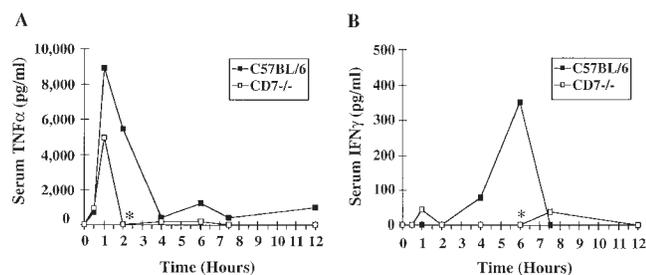


Figure 2. CD7-deficient animals have diminished *in vivo* cytokine serum levels in response to treatment with low-dose LPS and D-gal. (A) Time course of TNF- α serum concentrations after injection of C57BL/6 control mice (■) or CD7-deficient mice (□) with LPS (1 μ g) and D-gal (8 mg). (B) Time course of IFN- γ serum concentration after injection of C57BL/6 control mice (■) and CD7-deficient mice (□) with LPS and D-gal. Serum cytokine levels, determined by ELISA from one mouse per time point are presented. Data shown are from an experiment that is representative of three separate experiments performed. Statistical significance was determined for each time point by combining data from three experiments; * $P \leq 0.001$ when comparing C57BL/6 control mice to CD7-deficient mice.

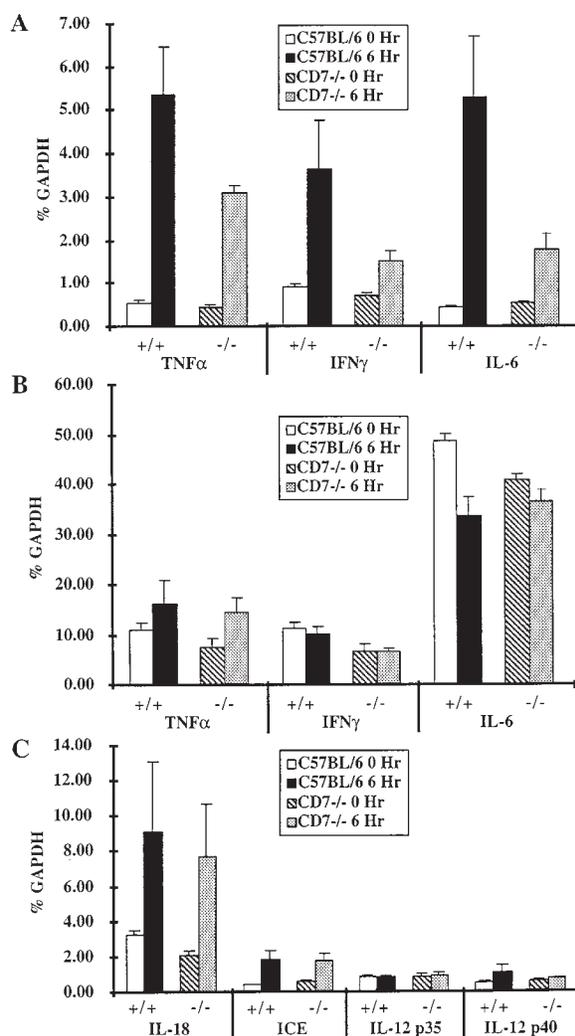


Figure 3. Steady-state cytokine mRNA levels in the low-dose LPS shock model. Total RNA was extracted from liver (A and C) and spleen (B) from C57BL/6 control (+/+, $n = 3$) and CD7-deficient (-/-, $n = 3$) mice 0 and 6 h after injection with low-dose LPS and D-gal. Specific cytokine mRNA levels were quantified by RNase protection assays and reported as percentage of GAPDH signal. Data represent the mean \pm SEM of results obtained from three mice at each time point.

evated in liver tissue from CD7-deficient mice exposed to LPS plus D-gal for 6 h, compared with pretreatment levels ($P < 0.05$; Fig. 3 A). However, there was no significant increase in the steady-state level of IFN- γ in 6-h-treated CD7-deficient mice versus the 0 h mice. In addition, liver from LPS plus D-gal-treated CD7-deficient animals had lower steady-state mRNA levels of TNF- α and IL-6 mRNA compared with those seen in C57BL/6 liver tissues ($P < 0.05$). In contrast, spleen of both C57BL/6 and CD7-deficient mice had no significant differences in TNF- α , IFN- γ , or IL-6 steady-state mRNA levels after treatment with LPS plus D-gal for 6 h (Fig. 3 B).

Deficient IFN- γ production in the liver could be due to a lack of induction of the IFN- γ inducing factors IL-12 and IL-18 or ICE. To address these possibilities, IL-12 (p35,

p40), IL-18, and ICE steady-state mRNA expression in liver was determined by RNase protection assays. IL-18 and ICE mRNA expression in liver (Fig. 3 C) was determined after 6 h of exposure to LPS plus D-gal. There was no difference between C57BL/6 control mice ($n = 3$) and CD7-deficient mice ($n = 3$) in constitutive or LPS-induced steady-state levels of IL-18 and ICE mRNA expression. At the 6-h time point no significant induction in p35 or p40 IL-12 mRNA was detected in CD7-deficient or control mice. In addition, no difference in constitutive or LPS-induced steady-state levels of either IL-12 mRNA was observed between C57BL/6 control mice and CD7-deficient mice.

CD7-deficient Splenocytes Responded Normally to IL-18 with IFN- γ Production. Splenocytes from C57BL/6 control mice ($n = 3$) and CD7-deficient mice ($n = 3$) were cultured in vitro for 72 h with a wide dose range of IL-12, IL-18, and IL-12 plus IL-18 (Fig. 4). As shown in Fig. 4 A, IL-12 alone did not induce IFN- γ production by splenocytes from either C57BL/6 control or CD7-deficient mice. IL-18 alone induced a low level of IFN- γ production in splenocyte cultures from both C57BL/6 control and CD7-deficient mice ($P = \text{NS}$) (Fig. 4 B). When IL-12 and IL-18 were combined to stimulate IFN- γ production (Fig. 4 C), an enhanced induction in IFN- γ production was observed for both C57BL/6 splenocytes and CD7-deficient splenocytes. There was no significant difference in the level of IFN- γ stimulated by both cytokines in splenocyte cultures from C57BL/6 versus CD7-deficient mice.

CD7-deficient Mice Have Reduced Numbers of Liver NK1.1 $^{+}$ /CD3 $^{+}$ T Cells. NK1.1 $^{+}$ /CD3 $^{+}$ T cells have been recently reported to be major producers of IFN- γ and key effector cells in the pathogenesis of lethal shock syndromes (23, 24). Thus, we isolated liver mononuclear cells from C57BL/6 control and CD7-deficient mice and performed phenotypic analysis with respect to CD3 and NK1.1 expression. Three independent experiments comparing C57BL/6 and CD7-deficient mice were performed by pooling of liver mononuclear cells from two to five animals per experiment. There was no difference in the absolute number of liver lymphocytes isolated from C57BL/6 versus CD7-deficient livers (Fig. 5). We observed a significant reduction in the percentage and number of NK1.1 $^{+}$ /CD3 $^{+}$ cells in livers from untreated CD7-deficient mice ($P < 0.05$). In contrast, the percentage and absolute number of traditional NK cells (NK1.1 $^{+}$ /CD3 $^{-}$) and T cells (NK1.1 $^{-}$ /CD3 $^{+}$) per liver in C57BL/6 control and CD7-deficient mice were similar. CD7-deficient livers also had an increase in the number of B220 $^{+}$ B cells compared with C57BL/6 controls (Fig. 5).

Discussion

In this study we have shown that CD7-deficient mice are resistant to death in both the high-dose and the low-dose LPS-induced models of shock. Using the low-dose LPS model we demonstrated decreased induction of serum TNF- α and IFN- γ , decreased liver IL-6, TNF- α and IFN- γ

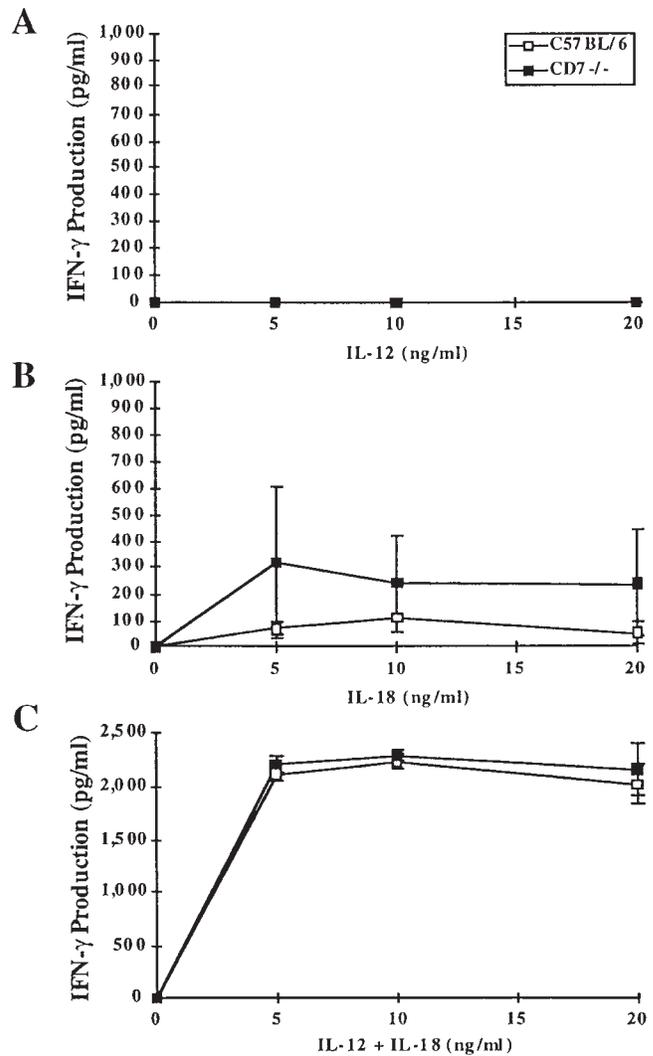


Figure 4. In vitro splenocyte IFN- γ response to stimulation with IL-12 and IL-18. Splenocytes from C57BL/6 control mice ($n = 3$) and CD7-deficient mice ($n = 3$) were isolated and incubated with a dose-curve of IL-12 (A), IL-18 (B), or IL-12 plus IL-18 (C) for 72 h. Culture supernatants were assayed for IFN- γ by ELISA. Similar results were found for IL-12 and IL-18, and IL-12 plus IL-18 induced proliferation of C57BL/6 and CD7-deficient splenocytes (data not shown). Data are the mean \pm SEM for three animals.

steady-state mRNA levels, and normal liver IL-12, IL-18, and ICE mRNA levels in CD7-deficient mice compared with control mice. Moreover, we showed that CD7-deficient lymphocytes responded normally to IL-12 and IL-18 with regard to IFN- γ production. Finally, resistance of CD7-deficient mice to LPS-induced shock was associated with a low number of resident effector NK1.1 $^{+}$ /CD3 $^{+}$ T cells in liver of CD7-deficient mice.

The CD7-deficient mouse is unique among homologous recombinant mouse strains with respect to its responses in low- and high-dose LPS-induced shock syndromes. Similar to ICAM-1 deficient, ICE-deficient, and TNFR2-deficient mice (19, 20, 22), CD7-deficient mice are partially resistant to LPS-induced death in the high-dose shock

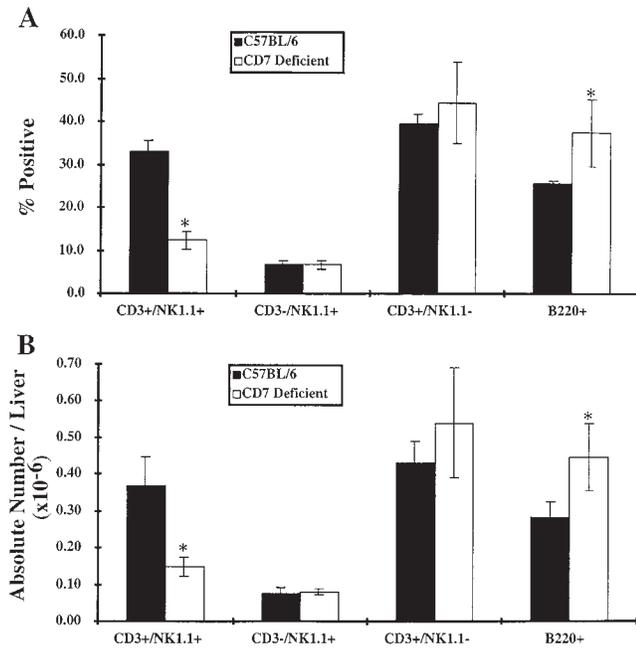


Figure 5. CD7-deficient animals have decreased percentages and absolute numbers of liver NK1.1⁺/CD3⁺ T cells. Flow cytometric analysis of isolated liver mononuclear cells from C57BL/6 and CD7-deficient mice was performed as described in Materials and Methods. A minimum of 10⁴ lymphocyte-gated events were analyzed per sample. Panel A shows percentages of CD3⁺/NK1.1⁺ T cells, CD3⁻/NK1.1⁺ NK cells, CD3⁺/NK1.1⁻ T cells, and B220⁺ B cells. In panel B, the total number of mononuclear cells isolated per liver was determined and used to calculate the absolute number per liver of CD3⁺/NK1.1⁺ T cells, CD3⁻/NK1.1⁺ NK cells, CD3⁺/NK1.1⁻ T cells, and B220⁺ B cells. Data are the mean \pm SEM from three experiments with liver mononuclear cells from two to five animals pooled per group, per experiment. * $P < 0.05$ when comparing C57BL/6 control mice to CD7-deficient mice.

model. Moreover, CD7-deficient mice are fully resistant to LPS-induced death in the low-dose shock model, similar to IFN- γ R-deficient and TNFR1-deficient mice (14, 18) (Table I).

Recent studies have reported that murine liver contains a unique population of α/β T cells that have intermediate TCR expression and are positive for the NK1.1 antigen (23). These NK1.1⁺/CD3⁺ T cells are phenotypically and functionally distinct from NK 1.1⁻ T cells, which are high in TCR expression, and from CD3⁻ NK cells, which are TCR negative (23). This population of NK1.1⁺/CD3⁺ liver T cells has been suggested to be autoreactive (25, 26), a major source of IFN- γ production, and key effector cells in the pathogenesis of lethal shock syndromes (23, 24, 27). These studies further revealed that LPS activates NK1.1⁺/CD3⁺ T cells via IL-12 production by Kupffer cells, resulting in the hepatotoxicity observed in septic shock (27). Moreover, de-

Table I. Sensitivity or Resistance of Genetically Deficient Mice in Endotoxic Shock Models

Mouse strain	High-dose LPS	Low-dose LPS
C57BL/6 control	Sensitive*	Sensitive
ICAM-1-deficient	Resistant [†]	Sensitive
ICE-deficient	Resistant	Sensitive
TNF-RII-deficient	Partially resistant	Sensitive
IL-1 β -deficient	Sensitive	Sensitive
TNF-RI-deficient	Sensitive	Resistant
IFN- γ R-deficient	Sensitive	Resistant
CD7-deficient	Partially resistant	Resistant

*Sensitive means animals developed shock and succumbed to LPS injection.

[†]Resistance means animals are resistant to the effects of LPS and survive LPS or LPS plus D-gal injections.

pletion of these cells significantly decreased mortality in the IL-12-primed LPS-induced shock syndrome (24).

We have found a significant reduction in the percentage and absolute number of NK1.1⁺/CD3⁺ cells in livers from untreated CD7-deficient mice versus age-matched control mice (Fig. 5). These data suggested that the CD7 molecule may play a critical role in development, function, and/or migration of liver NK1.1⁺/CD3⁺ T cells. The ontogeny of NK1.1⁺/CD3⁺ T cells is currently unknown; however, it is clear that they are a functionally and phenotypically distinct subset of cells (23). That CD7-deficient animals have selectively diminished numbers of CD3⁺/NK1.1⁺ T cells with normal numbers of CD3⁻/NK1.1⁺ NK cells and CD3⁺/NK1.1⁻ T cells, also suggests that the CD3⁺/NK1.1⁺ T cell subset may be a distinct cell lineage. We hypothesize that CD7, which is expressed early in T and NK cell ontogeny (7), may play a role in development and/or migration of NK1.1⁺ T cells in the liver. Decreased numbers of these T cells in the livers of CD7-deficient mice may result in blunted cytokine production and be sufficient to protect mice from both high- and low-dose LPS-induced shock.

Thus, CD7-deficient mice are unique in their resistance to both high-dose and low-dose LPS-induced shock models. Our data suggest that CD7 is an essential molecule that is involved in the lethal cellular and molecular events leading to low-dose LPS-induced hepatocyte necrosis/apoptosis, and to ischemia/reperfusion injury in high-dose LPS-induced shock. Understanding the role of CD7 in LPS shock syndromes and in NK1.1⁺ T cell maturation and function may spur the development of new treatments for endotoxic shock syndrome in humans.

We acknowledge the expert technical assistance of Mr. Jonathan L. Baron, helpful discussions with Dr. Charles Dinarello, and expert secretarial assistance from Ms. Kim R. McClammy.

This study was supported by National Institutes of Health grant CA28936.

Received for publication 18 December 1998 and in revised form 27 January 1999.

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