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IL-18-deficient mice are resistant to endotoxin-induced liver injury but highly susceptible to endotoxin shock

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Abstract

IL-18 is an IL-1-related cytokine which shares biological functions with IL-12. These include the activation of NK cells, induction of IFN-γ production and T_h1 cell differentiation. In this study we analyzed the effect of IL-18 deficiency on lipopolysaccharide (LPS)-induced liver injury and endotoxin shock in *Propionibacterium acnes*-primed mice. *P. acnes*-primed IL-18-deficient (IL-18KO) mice showed resistance to LPS-induced liver injury. Unexpectedly, *P. acnes*-primed IL-18KO mice were highly susceptible to LPS-induced endotoxin shock. Serum level of tumor necrosis factor (TNF)-α were markedly elevated (~10-fold higher) within 1.5 h after LPS challenge in IL-18KO mice as compared with wild-type mice. Anti-TNF-α antibody administration to IL-18KO mice was significantly protective against endotoxin-induced lethality. *P. acnes*-primed IL-18KO macrophages produced ~6-fold more TNF-α protein than did *P. acnes*-primed wild-type control macrophages. Taken together, these findings demonstrate that IL-18 is responsible for the progression of endotoxin-induced liver injury as well as down-regulation of endotoxin-induced TNF-α production in *P. acnes*-primed mice.

Introduction

IL-18 was originally identified as a cytokine that is secreted from activated macrophages and Kupffer cells, and induces IFN-γ production from T_h1 cells (1–3). In addition, IL-18 enhances NK cell activity and proliferation of activated T cells (2–4). These biological activities are also mediated by IL-12 (5,6). Recent studies using IL-18- and IL-12-deficient mice clearly demonstrated that a collaborative action of IL-18 and IL-12 is essential for NK cell activity and T_h1 cell development (7,8).

In addition to these functions, IL-18 plays an important role in lipopolysaccharide (LPS)-induced liver injury of *Propionibacterium acnes*-primed mice, because *P. acnes*-primed mice were protected from LPS-induced liver injury by administration of anti-IL-18 antibody (3,9). Likewise, anti-IL-12 antibody prevented LPS-induced liver injury in *P. acnes*-primed mice (9,10). During endotoxin shock, several inflammatory mediators such as tumor necrosis factor (TNF)-α, IL-1 and IFN-γ are secreted and evoke serious multiple organ failure (11–13). LPS-induced liver injury is closely coupled to endotoxin shock, a systemic disorder which leads to high mortality (14). Indeed, mice deficient in TNF-α, IFN-γ or the receptors for these cytokines displayed resistance to both LPS-induced endotoxin shock and liver injury (15–18).

IL-1β-converting enzyme (ICE)/caspase-1 has been shown to possess a proteolytic activity for cleaving the inactive IL-18 precursor into the mature form, as in the case of IL-1β (19,20). ICE-deficient mice were defective in the production of IL-1α, IL-1β and IL-18, and resistant to LPS-induced endotoxin shock, suggesting a possible role for IL-1 and IL-18 in the induction of endotoxin shock (19–22). However, mice deficient in IL-1β and the type I IL-1 receptor, the receptor responsible for both IL-1α and IL-1β signaling, displayed no resistance to LPS-induced endotoxin shock (23–25). Therefore, it was expected that IL-18 is a major cytokine that contributes to LPS-induced endotoxin shock.

In the present study we addressed the role of IL-18 in LPS-

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induced endotoxin shock and liver injury, using IL-18-deficient (IL-18KO) mice. IL-18KO mice displayed resistance to LPS-induced liver injury. However, unexpectedly, they were highly sensitive to LPS-induced shock. Serum TNF-α increased to a tremendously high level within 1.5 h after LPS challenge in *P. acnes*-primed IL-18KO mice as compared with *P. acnes*-primed wild-type controls. Intraperitoneal injection of IL-18 to IL-18KO mice before *P. acnes* priming suppressed high serum TNF-α levels after LPS challenge. Reciprocally, anti-IL-18 antibody administration to wild-type controls before *P. acnes* priming increased serum TNF-α levels and resulted in increased lethality. These results indicate that IL-18 plays an important role in the hyporesponsiveness to endotoxin shock as well as in the progression to endotoxin-induced liver injury.

Methods

Mice

IL-18- and IL-12-deficient mice were generated and maintained as previously described (7,8). Age-matched groups of IL-18-deficient, IL-12-deficient and wild-type mice were used for the experiments.

LPS-induced liver injury

Mice (8–12 weeks old) were injected i.p. with 200 μg of heat-killed *P. acnes*, then i.v. with 2 μg of LPS 7 days later. Twenty-four hours after the LPS challenge, mice were bled and sacrificed. Livers were fixed in 10% formalin solution, sectioned at 8 μm thickness, and stained with hematoxylin & eosin. LPS (*Escherichia coli* strain 055:B5) was purchased from Difco (Detroit, MI).

LPS-induced endotoxin shock in P. acnes-primed mice

Mice were injected i.p. with 1 mg of heat-killed *P. acnes*, then i.v. with 1 μg of LPS 7 days later. In some experiments, 100 μl of rabbit polyclonal anti-TNF-α antiserum (Genzyme, Cambridge, MA) or control rabbit Ig (Wako Pure Chemicals, Osaka, Japan) was i.p. injected 30 min before LPS challenge. Survival of mice was checked every hour.

For serum levels of inflammatory mediators during endotoxin shock, mice were primed with 200 μg of heat-killed *P. acnes* and subsequently injected with 2 μg of LPS. Mice were bled 0, 1.5, 3, 6, 12 and 24 h after LPS challenge. Serum concentrations of TNF-α, IL-1β, IL-6, IL-12 and IFN-γ were determined by ELISA (Genzyme, Cambridge, MA). Serum levels of NO were measured using Griess reagent as described previously (26).

In vivo treatment of mice with IL-18 or anti-IL-18 antibodies

IL-18KO mice were i.p. injected with 10 ng of mouse IL-18 or PBS every 2 days from the day before injection of *P. acnes*. After 7 days of *P. acnes* injection, 2 μg of LPS was i.v. injected. Mice were bled 1.5 h after LPS challenge and serum TNF-α concentrations were measured. Wild-type mice were i.p. injected with 200 μg of rabbit anti-mouse IL-18 antibody or control Ig the day before and 3 days after *P. acnes* injection. After 7 days of *P. acnes* injection, LPS was injected and serum concentrations of TNF-α 1.5 h after LPS challenge were measured. Mouse IL-18 and anti-mouse IL-18 antibody were kindly provided by Hayashibara Laboratory (Okayama, Japan).

TNF-α production from peritoneal macrophages

Mice were i.p. injected with 200 μg of heat-killed *P. acnes*. Three days after injection, peritoneal exudate cells were isolated by washing the peritoneal cavity with ice-cold PBS and cultured for 2 h. Non-adherent cells were removed by washing with HBSS and the remaining cell monolayers were used as peritoneal macrophages. Cells were cultured with or without 1 μg/ml LPS. Culture supernatants were analyzed for TNF-α production by ELISA. The culture medium used in this study was RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FCS (Gibco), L-glutamine, 2-mercaptoethanol, penicillin G (100 U/ml) and streptomycin (100 μg/ml)

Northern blot analysis

Mice were injected with heat-killed *P. acnes*. After 3 days of injection, peritoneal macrophages were isolated and stimulated with 1 μg/ml of LPS for 1.5 h. Total RNA was extracted with Trizol reagent (Gibco), electrophoresed, transferred to nylon membrane, and hybridized with ^{32}P-labeled cDNA probes for mouse TNF-α, IL-1β and IL-6. Densitometric analysis was performed with BAS-2000 (Fuji Photo Film, Kanagawa, Japan).

Metabolic labeling and immunoprecipitation

Mice were i.p. injected with 200 μg of *P. acnes*. Three days after injection, peritoneal macrophages were isolated and washed with methionine- and cysteine-free RPMI 1640. Cells (1×10^7) were incubated in 1.5 ml of the same medium containing 2% dialysed FCS with or without 1 μg/ml LPS for 1.5 h. Cells were pulsed with 200 μCi [^{35}S]Methionine (Tran ^{35}S-Label; ICN, Costa Mesa, CA) for the last 1 h. The culture supernatants were harvested and cells were washed with HBSS. Then, cells were solubilized with ice-cold lysis buffer containing 0.5% Nonidet P-40, 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 5 μM leupeptin and 5 μg/ml aprotinin. Cell lysates and supernatants were clarified by centrifugation. Then, 150 μl of 10 times lysis buffer was added to the supernatants. Cell lysates and supernatants were incubated with 30 μl of Protein A–Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C. The Sepharose was removed by centrifugation and 5 μl of polyclonal rabbit anti-mouse TNF-α neutralizing antiserum (Genzyme, Cambridge, MA) was added. After incubation for 12 h at 4°C, 30 μl of Protein A–Sepharose was added and the mixture was incubated for additional 2 h at 4°C. Immunoprecipitates were washed and separated by SDS–PGAE. The gel was fixed in 30% methanol and 10% acetic acid, then treated with EN^{3}HANCE autoradiography enhancer (NEN Research Products, Boston, MA). Dried gel was analyzed after autoradiography and densitometric units were measured by BAS-2000 (Fuji Photo Film, Kanagawa, Japan).

Results

P. acnes-primed IL-18KO mice are resistant to endotoxin-induced liver injury

Treatment of *P. acnes*-primed mice with LPS induces serious liver injury (3,9). IL-18 was originally identified in the liver

extracts of mice treated with *P. acnes* plus LPS (1). Anti-IL-18 antibody blocks the liver injury induced by *P. acnes* plus LPS (3,9). Therefore, we analyzed the effect of IL-18 deficiency on the liver injury induced by *P. acnes* plus LPS. Mice primed with 200 μg of heat-killed *P. acnes* were i.v. challenged with 2 μg of LPS. At 24 h after LPS challenge, mice were bled and sacrificed for histological analyses. In wild-type mice, severe liver injury characterized by focal necrosis was observed (Fig. 1A). In contrast, no focal necrosis was observed in IL-18KO mice, although mild levels of venous congestion existed (Fig. 1B). Serum levels of transaminases such as AST and ALT were significantly elevated in wild-type mice, but serum transaminase levels in IL-18KO mice were almost the same as those in unprimed mice (Fig. 1G). IL-18 is shown to share functions with IL-12, such as activation of NK cells and induction of IFN-γ production. Therefore, we also analyzed the *P. acnes* plus LPS-induced hepatic injury in IL-12KO mice. IL-12KO mice displayed no obvious changes in hepatic tissues (Fig. 1C). Serum transaminase levels were not elevated in IL-12KO mice, supporting that *P. acnes*-primed IL-12KO mice were also resistant to LPS-induced hepatic injury (Fig. 1G). Thus, IL-18KO and IL-12KO mice showed resistance to liver injury induced by *P. acnes* plus LPS.

Injection of wild-type mice with *P. acnes* alone is shown to induce infiltration of mononuclear cells into the liver and subsequent granuloma formation in hepatic lobules within 7 days (Fig. 1D). The formation of granuloma consisting of macrophages and lymphoid cells was also observed in hepatic lobules of *P. acnes*-primed IL-18KO mice despite the resistance to liver injury (Fig. 1E). In contrast, the granuloma was not observed in IL-12KO mice (Fig. 1F). Thus, IL-18KO and IL-12KO mice displayed distinct phenotypes during *P. acnes* priming although both mice were resistant to liver injury.

P. acnes-primed IL-18KO mice are highly susceptible to LPS-induced endotoxin shock

In spite of the resistance to liver injury induced by *P. acnes* (200 μg) and LPS (2 μg), several IL-18KO mice died within 24 h of LPS challenge, although almost all of the wild-type mice survived >24 h. Therefore, we further analyzed the susceptibility to LPS-induced endotoxin shock in IL-18KO mice. For endotoxin shock experiments, mice were i.p. injected with 1 mg of *P. acnes* and 7 days after injection challenged with 1 μg of LPS. Under this condition, about half of the wild-type mice and all of the IL-12KO mice survived for >12 h after LPS challenge. IL-12KO mice displayed no obvious symptoms of endotoxin shock such as a crouched position, shivering and ruffled fur. However, all IL-18KO mice died within 4 h after LPS challenge (Fig. 2). Thus, *P. acnes*-primed IL-18KO mice were highly susceptible to LPS-induced endotoxin shock, whereas *P. acnes*-primed IL-12KO mice were resistant to it.

LPS-induced endotoxin shock is shown to be well correlated with the increased production of several inflammatory mediators such as TNF-α, IL-1, IL-6, IL-12, IFN-γ and NO (11,12,27). Among them, TNF-α appears to play a central role in the pathogenesis of LPS-induced endotoxin shock (16–18). We next measured the serum concentration of several cytokines and mediators that are induced after LPS challenge in *P. acnes*-primed mice. In order to obtain the sera at various time points after LPS challenge, we utilized in this experiment the protocol for endotoxin-induced liver injury in which i.p. injection of 200 μg *P. acnes* is followed by i.v. injection of 2 μg LPS 7 days later. Serum IL-12 levels were almost equally elevated in both wild-type and IL-18KO mice (Fig. 3B). Serum IFN-γ concentrations after LPS challenge in IL-18KO mice were increased but significantly lower than those in wild-type mice, supporting an important role for IL-18 in IFN-γ production (Fig. 3A). Serum concentrations of NO during these periods were almost equally increased in both wild-type and IL-18KO mice (Fig. 3C). The serum concentrations of IL-1β in IL-18KO mice were slightly higher at 3 h after LPS challenge than in wild-type mice and the elevated IL-1β concentrations did not decrease in IL-18KO mice until 6 h after LPS challenge (Fig. 3D). Serum IL-6 concentrations in both wild-type and IL-18KO mice increased to almost the same peak levels until 3 h. However, elevated IL-6 levels were sustained in IL-18KO mice until 24 h, in contrast to the rapid decrease of IL-6 in wild-type mice (Fig. 3E). In the case of TNF-α concentration, significantly increased levels were observed at 1.5 h after LPS challenge in wild-type mice. However, at this time point, a marked increase in TNF-α concentrations (~10-fold increase) was observed in IL-18KO mice when compared with wild-type mice (Fig. 3F). The high serum TNF-α levels rapidly decreased to basal levels within 24 h after LPS challenge in both wild-type and IL-18KO mice. Thus, marked elevation of serum TNF-α level, and sustained levels of serum IL-1β and IL-6 were observed in IL-18KO mice, suggesting an important role for IL-18 as a negative regulator of inflammatory cytokines during endotoxin shock.

Almost all IL-18KO mice died shortly after the period of overproduction of TNF-α during endotoxin shock. To determine the relation between the overproduction of TNF-α and high susceptibility to endotoxin shock, we neutralized the effect of TNF-α during the endotoxin shock by administering anti-TNF-α antibody before LPS challenge. Neutralization of TNF-α markedly improved the survival rate in IL-18KO mice and nearly half of IL-18KO mice survived >24 h (Fig. 4). This result indicates that the high mortality during LPS-induced endotoxin shock in IL-18KO mice was due to the extraordinarily high serum levels of TNF-α.

TNF-α production in response to LPS is also up-regulated in unprimed IL-18KO mice

To determine whether the priming by *P. acnes* is pre-requisite to the hypersensitivity to LPS in TNF-α production, we examined the response to high doses of LPS in unprimed IL-18KO mice (Fig. 5). Unprimed mice were i.p. injected with 200 μg LPS and serum TNF-α levels were measured. In wild-type mice, elevated serum TNF-α levels were observed at 1.5 and 3 h after LPS challenge. Serum TNF-α levels of IL-18KO mice were ~3-fold higher than those of wild-type mice at 1.5 and 3 h after LPS challenge. Thus, unprimed IL-18KO mice also produced more TNF-α in response to LPS than did wild-type mice. However, the difference in serum TNF-α levels between wild-type and IL-18KO mice was more drastic in *P. acnes*-primed mice than in unprimed mice, showing that IL-18KO mice become more hypersensitive to LPS after *P. acnes*-priming (Figs 3F and 5).

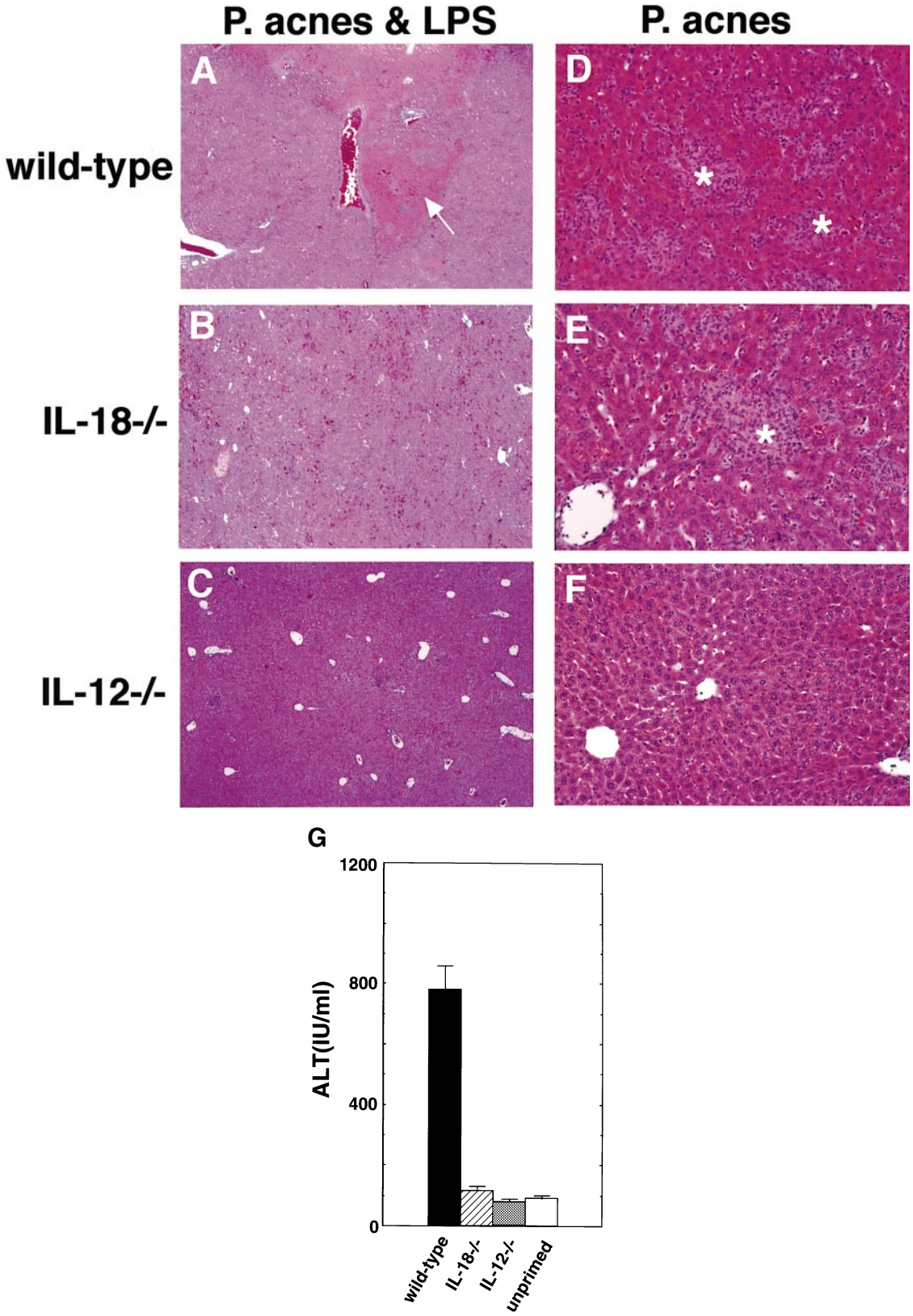

Fig. 1. Liver injuries induced by *P. acnes* and LPS in wild-type, IL-18$^{-/-}$ and IL-12$^{-/-}$ mice. Livers were removed from *P. acnes* (200 μg)-primed mice 24 h after LPS (2 μg) injection [A–C (magnification ×10)] or from mice 7 days after *P. acnes* injection [D–F (magnification ×25)]. Focal necrosis was observed in wild-type mice (A). In contrast, necrotic change was not found in livers of IL-18$^{-/-}$ and IL-12$^{-/-}$ mice (B and C). Granuloma formation was observed in wild-type and IL-18$^{-/-}$ mice (D and E), but not in IL-12$^{-/-}$ mice (F). Asterisks indicate the granulomatous changes. (G) Serum ALT levels at 24 h after LPS challenge in *P. acnes*-primed mice are shown.

Increased TNF-α mRNA and protein synthesis in response to LPS in peritoneal macrophages from P. acnes-primed IL-18KO mice

TNF-α is mainly produced from macrophages in response to LPS (11,28,29). Therefore, we analyzed TNF-α production from peritoneal macrophages (Fig. 6A). Peritoneal macrophages were isolated from *P. acnes*-primed mice and cultured with or without LPS. The culture supernatants were analyzed for TNF-α production by ELISA and the cells were subjected to Northern blot analysis for TNF-α mRNA expression. Although unstimulated macrophages did not secrete any TNF-α, LPS-stimulated wild-type macrophages produced a significant level of TNF-α. IL-18KO macrophages produced ~6-fold more TNF-α in response to LPS stimulation than did wild-type macrophages. Northern blot analysis also demonstrated that the expression of TNF-α mRNA in IL-18KO macrophages was increased ~3-fold when compared with wild-type macrophages. We further examined the TNF-α synthesis in the IL-18KO macrophages. Peritoneal macrophages from *P. acnes*-primed mice were stimulated with LPS for 1.5 h and pulse labeled with [^{35}S]methionine for the last 1 h. The culture supernatants and cell lysates were subjected to immunoprecipitation with anti-TNF-α antibody, and analyzed by SDS–PAGE and autoradiography (Fig. 6B). In culture supernatants, LPS stimulation induced the production of the mature secreted form of TNF-α in both wild-type and IL-18KO macrophages. However, the density of a 17 kDa mature TNF-α band was ~6-fold higher in IL-18KO cells than in wild-type cells. In cell lysates, a 26 kDa TNF-α precursor form was detected after LPS stimulation in both wild-type and IL-18KO macrophages. The density of the TNF-α precursor band was also ~6-fold higher in IL-18KO cells than in wild-type cells, indicating that TNF-α biosynthesis was also enhanced at the translational level in IL-18KO mice. Taken together, these

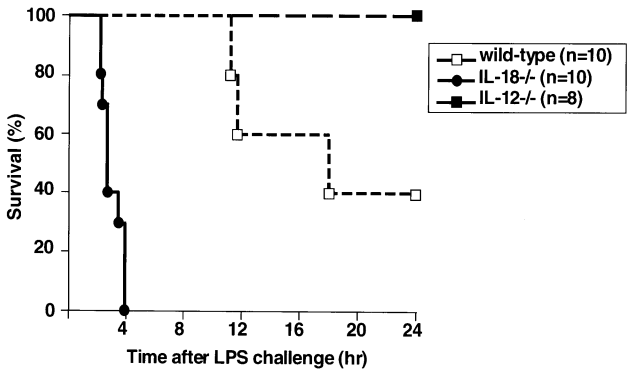

Fig. 2. High susceptibility to LPS in *P. acnes*-primed IL-18$^{-/-}$ mice. *P. acnes* (1 mg)-primed wild-type, IL-18$^{-/-}$ and IL-12$^{-/-}$ mice were i.v. injected with 1 μg LPS. Note that IL-18$^{-/-}$ mice died within 4 h of LPS challenge.

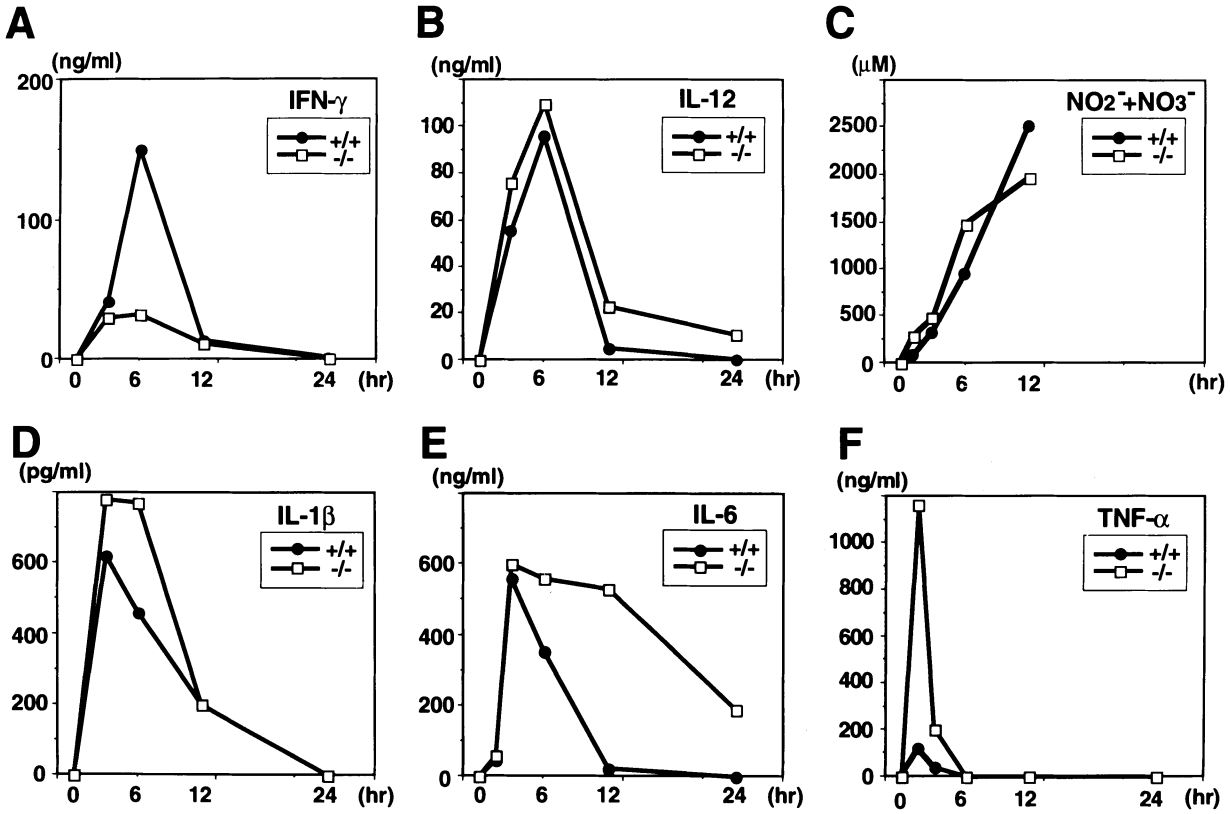

Fig. 3. Serum levels of inflammatory mediators during endotoxin shock. *P. acnes* (200 μg)-primed wild-type and IL-18$^{-/-}$ mice were injected with 2 μg LPS. Sera were taken at the indicated periods after LPS challenge. Each cytokine concentration was measured by ELISA. The level of NO was analyzed using Griess reagent. Results are mean of sera samples from five mice.

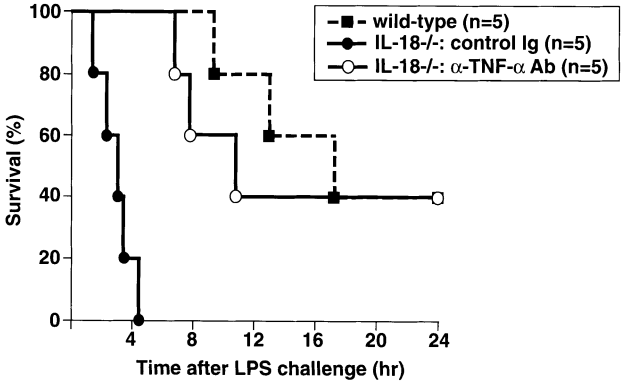

Fig. 4. Treatment with anti-TNF-α antibody improved the mortality in IL-18$^{-/-}$ mice. *P. acnes* (1 mg)-primed mice were i.p. injected with anti-TNF-α antibody or control Ig. After 30 min of treatment, mice were i.v. injected with 1 μg LPS.

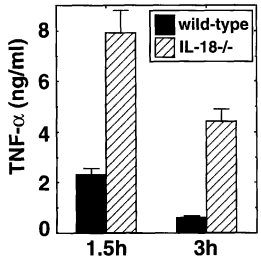

Fig. 5. TNF-α production after LPS treatment in unprimed IL-18$^{-/-}$ mice. Unprimed wild-type and IL-18$^{-/-}$ mice were i.p. injected with 200 μg of LPS. Between 1.5 and 3 h after LPS challenge, mice were bled and serum concentration of TNF-α was measured. Mean ± SD of five mice sera.

results demonstrate that TNF-α production in response to LPS was up-regulated at both mRNA and translational levels in *P. acnes*-primed IL-18KO macrophages.

CD14 is a well known receptor for LPS expressed on monocytes/macrophages (30). Therefore, we examined the expression of CD14 on macrophages of IL-18KO mice. CD14 expression on peritoneal macrophages was not altered between wild-type and IL-18KO mice. In addition, there was no change in CD14 expression on macrophages of *P. acnes*-primed wild-type and IL-18KO mice (data not shown). This indicates that hyper-responsiveness to LPS in IL-18KO macrophages is not due to up-regulation of CD14 expression.

We next analyzed mRNA expression of other inflammatory cytokines in peritoneal macrophages. Peritoneal macrophages from *P. acnes*-primed mice were stimulated with LPS *in vitro* and subjected to Northern blot analysis (Fig. 6C). Levels of TNF-α mRNA in IL-18KO macrophages after 1.5 h stimulation with LPS were ~3-fold higher than those in wild-type macrophages as shown above. However, levels of IL-1β and IL-6 mRNA were not altered between IL-18KO and wild-type macrophages, indicating that TNF-α mRNA expression was specifically increased in LPS-stimulated IL-18KO macrophages.

Down-regulation of endotoxin-induced TNF-α production is induced by IL-18 during the early stage of P. acnes priming

We examined the critical time point for the action of IL-18 in the regulation of reactivity to LPS by administration of recombinant IL-18 to IL-18KO mice, or administration of anti-IL-18 antibody to wild-type mice. First, *P. acnes*-primed IL-18KO mice were administered with IL-18 30 min before LPS challenge. This treatment did not have any effect on TNF-α production or the mortality rate (data not shown). However, administration of 10 ng IL-18 to IL-18KO mice on the day prior to *P. acnes* priming and every second day thereafter for 6 days resulted in marked reduction of TNF-α production and improved mortality after LPS challenge (Fig. 7A). We next treated wild-type mice with anti-IL-18 neutralizing antibody. Administration of anti-IL-18 antibody to *P. acnes*-primed wild-type mice 30 min before LPS challenge did not have any effect on TNF-α production (data not shown). However, treatment with anti-IL-18 antibody on the day prior to *P. acnes* priming resulted in a significant increase in TNF-α production as compared with mice treated with control Ig (Fig. 7A). In addition, all of these mice died within 4 h after LPS challenge (data not shown). Taken together, these results demonstrate that IL-18 renders mice hyporesponsive to LPS for TNF-α production, especially during the early stage of *P. acnes* priming.

Consistent with these *in vivo* results, TNF-α production from macrophages in response to LPS was not reduced when peritoneal macrophages from *P. acnes*-primed IL-18KO mice were cultured with IL-18 for 18 h before LPS stimulation (data not shown). However, when peritoneal macrophages from IL-18KO mice, that were pretreated with i.p. injection of recombinant IL-18 before *P. acnes* priming, were stimulated with LPS *in vitro*, TNF-α production was markedly reduced compared with macrophages from PBS-pretreated IL-18KO mice (Fig. 7B). Reciprocally, macrophages from anti-IL-18 antibody pre-treated *P. acnes*-primed wild-type mice produced significantly elevated levels of TNF-α in response to LPS as compared with macrophages from control Ig pre-treated wild-type mice (Fig. 7B). Thus, IL-18 rendered macrophages hyporesponsive to LPS-stimulated TNF-α production during the early stage of *P. acnes* priming.

Discussion

Resistance to endotoxin-induced liver injury in P. acnes-primed IL-18KO mice

Infection of Gram-negative bacteria and/or the release of its products can induce several sequential events resulting in septic shock. Septic shock is accompanied by multiple organ failure including liver injury and leads to high mortality. LPS, an endotoxin derived from Gram-negative bacteria, has been shown to play an important role in the pathogenesis of septic shock (14). LPS triggers the production of several inflammatory mediators including TNF-α, IL-1, IL-6, IL-12, IFN-γ and NO. Among these mediators, TNF-α has been shown to be one of the factors most responsible for the pathogenesis of septic shock (11,12,31,32). It is now hypothesized that TNF-α, which is produced and released in the first 2 h of endotoxin shock, induces several mediators such as IL-1β, IFN-γ and NO, which

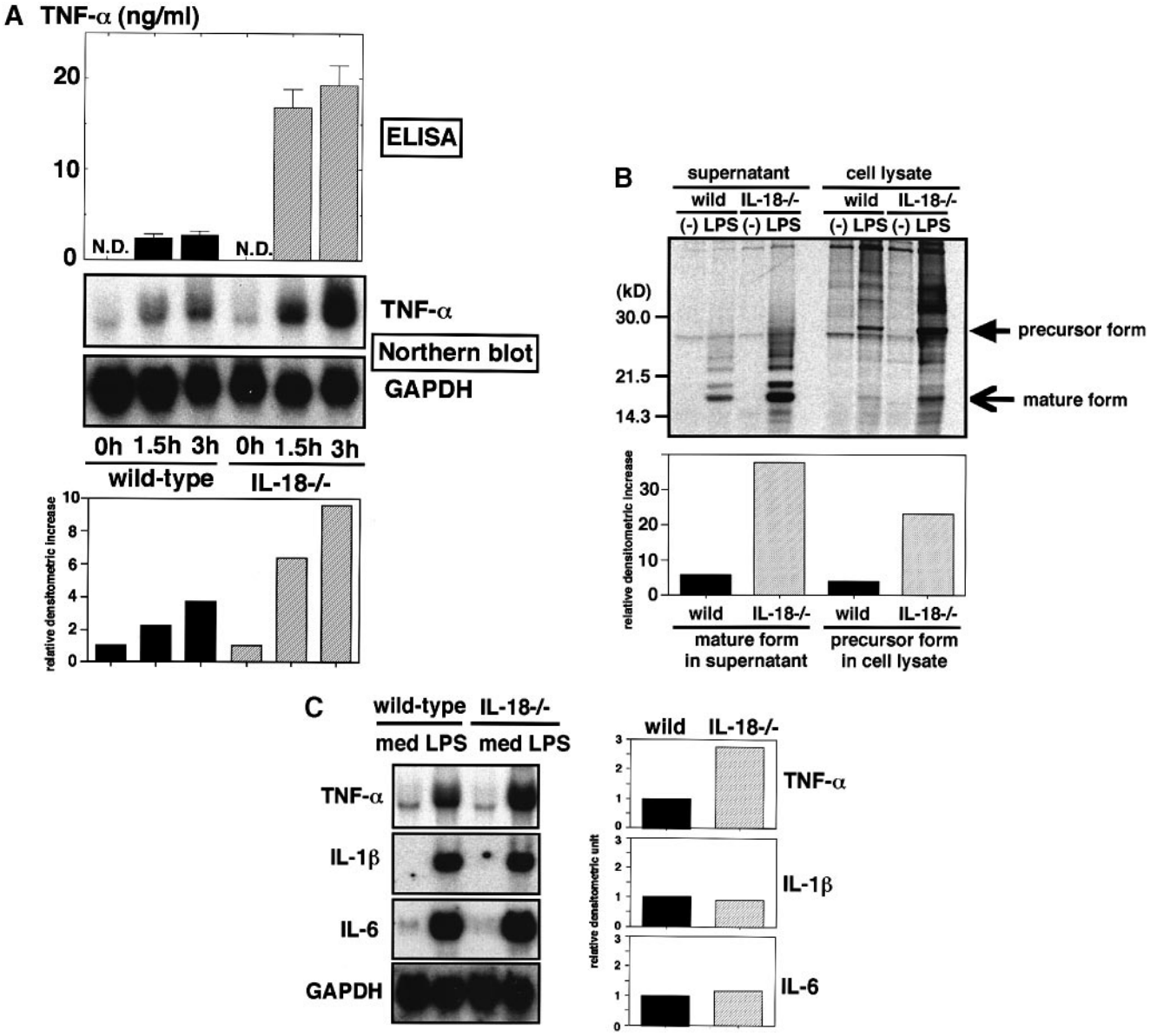

Fig. 6. TNF-α production from peritoneal macrophages of *P. acnes*-primed IL-18$^{-/-}$ mice. (A) Mice were i.p. injected with *P. acnes*. Three days after injection, peritoneal macrophages were stimulated with 1 μg/ml LPS for 1.5 or 3 h. The culture supernatants were analyzed for TNF-α production by ELISA. Total RNA was extracted from cells and subjected to Northern blot analysis for TNF-α expression. Results of densitometric analysis are shown. The relative level of TNF-α mRNA expression was normalized to the respective amount of GAPDH in each lane. The figure is representative of three independent experiments. ND, not detected. (B) Peritoneal macrophages from four *P. acnes*-primed mice were incubated with or without 1 μg/ml LPS for 1.5 h and pulse labeled with [^{35}S]methionine for the last 1 h. The culture supernatants and cell lysates were immunoprecipitated with anti-TNF-α antibody, subjected to SDS–PAGE and analyzed by autoradiography. Results of densitometric analysis are shown. Representative of three independent experiments. (C) Peritoneal macrophages were isolated 3 days after *P. acnes* priming and incubated with or without 1 μg/ml LPS for 1.5 h. Total RNA was extracted from cells and analyzed for expression of mRNA of TNF-α, IL-1β and IL-6 by Northern blot analysis. Results of densitometric analysis are shown. The relative level of mRNA expression was normalized to the respective amount of GAPDH in each lane.

leads to serious systemic disorders (13,33). The importance of TNF-α was clearly demonstrated in studies using mice deficient in the 55 kDa subunit of the TNF receptor (TNF-R p55) or TNF-α (16–18). These mice, when sensitized with D-galactosamine, exhibited resistance to LPS-induced liver injury and endotoxin shock. In addition, a study using anti-IL-18 antibody has revealed that IL-18 is also involved in the induction of liver injury (3,9). *P. acnes*-primed IL-18KO mice were resistant to LPS-induced liver injury, confirming an important role for IL-18 in the induction of liver injury. In addition, *P. acnes*-primed IL-12KO mice displayed resistance to LPS-induced liver injury. This is consistent with a study using monoclonal IL-12 antibody, which blocked *P. acnes* plus LPS-induced liver injury in normal mice (9,10).

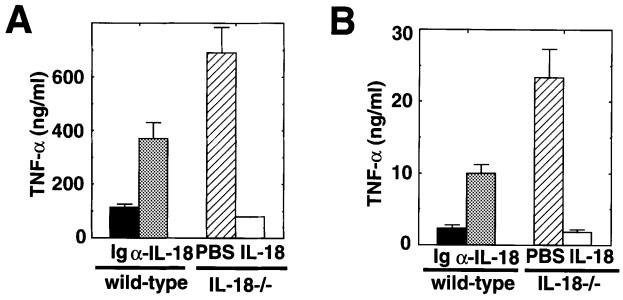

Fig. 7. IL-18 is required for LPS hyporesponsiveness during *P. acnes* priming. (A) IL-18$^{-/-}$ mice were administered with 10 ng of IL-18 or PBS every 2 days from the day before *P. acnes* priming. Wild-type mice were administered with 200 μg of anti-IL-18 antibody or control rabbit Ig the day before and 3 days after *P. acnes* priming. After 7 days of *P. acnes* priming, mice were injected with 2 μg LPS and serum concentration of TNF-α 1.5 h after LPS challenge was analyzed. Mean ± SD of four mice sera. (B) IL-18$^{-/-}$ mice were administered with 10 ng IL-18 the day before *P. acnes* priming. Wild-type mice were administered with anti-IL-18 antibody the day before *P. acnes* priming. Peritoneal macrophages were isolated 3 days after *P. acnes* priming and stimulated with 1 μg/ml LPS for 1.5 h. Culture supernatants were analyzed for TNF-α production by ELISA. Mean ± SD of triplicate samples of one experiments. The figure is representative of the results obtained from three independent experiments.

The pathogenic process of liver injury caused by *P. acnes* plus LPS is shown to consist of two phases; an early priming phase induced by injection of *P. acnes* and a late excitation phase elicited by LPS (9,34–36). At the priming phase, *P. acnes* activates the immune system leading to the formation of granuloma consisting of infiltrated mononuclear cells in hepatic lobules (34). In TNF-R p55-deficient mice, no granuloma formation was observed after *P. acnes* priming and the animals were resistant to LPS-induced liver injury (36). In the present study, we demonstrated that *P. acnes*-primed IL-12KO mice showed no granuloma formation in the liver. This indicates that IL-12 as well as TNF-α is important in the priming phase during the pathogenesis of *P. acnes* plus LPS-induced liver injury. On the other hand, *P. acnes*-primed IL-18KO mice displayed granuloma formation. Although it has been shown that expression of IL-18 mRNA was induced within 3 days of *P. acnes* injection in peritoneal macrophages (8) and that Kupffer cells of mice during *P. acnes* priming secreted the mature IL-18 (35), our present results demonstrate that IL-18 is not responsible for the granuloma formation during *P. acnes* priming.

High susceptibility to endotoxin shock in IL-18KO mice

In general, endotoxin-induced productions of several inflammatory cytokines and factors are involved in both endotoxin shock and endotoxin-induced liver injury. These two events are closely coupled (13,33). Indeed, TNF-R p55- and TNF-α-deficient mice, both of which displayed resistance to liver injury, were also resistant to endotoxin shock (16–18). IFN-γ receptor-deficient mice were also resistant to LPS-induced endotoxin shock and liver injury (15,37). Furthermore, *P. acnes*-primed IL-12KO mice were resistant to LPS-induced shock and liver injury (Figs 1 and 2). However, surprisingly, IL-18KO mice displayed high susceptibility to LPS-induced endotoxin shock when primed with *P. acnes*. Thus, IL-18KO mice were resistant to endotoxin-induced liver injury but highly sensitive to endotoxin shock. These results indicate that septic shock and liver injury occur via distinct mechanisms although these two events are mediated by several common cytokines. This may be in agreement with the findings in which athymic nude mice are resistant to *P. acnes* plus LPS-induced shock but not to liver injury (3). It is shown that, in nude mice, the absence of T cells accounts for the resistance to endotoxin shock, but NK cells are responsible for endotoxin-induced liver injury (H. Tsutsui, unpublished data).

IL-18 is involved in down-regulation of LPS-induced TNF-α production

P. acnes-primed IL-18KO mice showed greater susceptibility to LPS-induced septic shock than did wild-type mice. In IFN-γ receptor-deficient mice that display resistance to endotoxin shock induced by D-galactosamine plus low dose LPS, the production of TNF-α was profoundly reduced during septic shock due to the impaired capacity of macrophages to recognize LPS (15). These observations, together with the results from studies using TNF-R p55- and TNF-α-deficient mice, support that TNF-α plays a central role in the pathogenesis of septic shock. In IL-18KO mice, the production of several inflammatory cytokines during septic shock was significantly altered. The high serum levels of cytokines IL-1β and IL-6, both of which are responsible for endotoxin shock, were sustained in IL-18KO mice. Furthermore, most strikingly, tremendously high serum TNF-α levels were observed at the early stage of endotoxin shock in IL-18KO mice. The overproduction of TNF-α is proceeded a few hours later by the death of IL-18KO mice. In addition, treatment with anti-TNF-α neutralizing antibody just before LPS challenge improved mortality during endotoxin shock in IL-18KO mice. Taken together, we conclude that the high sensitivity to LPS-induced endotoxin shock in IL-18KO mice is due to a marked elevation in the serum TNF-α level. Thus, several inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, were overproduced during endotoxin shock in IL-18KO mice, indicating that IL-18 is a negative regulator for these cytokines during sepsis, especially for TNF-α.

Although unprimed IL-18KO mice displayed a lot of TNF-α secretion in response to high dose LPS when compared with wild-type mice, the amount of TNF-α produced in unprimed IL-18KO mice was much less than that in *P. acnes*-primed IL-18KO mice. In addition, administration of exogenous IL-18 to IL-18KO mice after *P. acnes* priming did not affect TNF-α production, but administration before *P. acnes* priming markedly reduced TNF-α production and improved the mortality in IL-18KO mice. Conversely, anti-IL-18 antibody treatment of wild-type mice before *P. acnes* priming resulted in increased TNF-α production and high mortality. These results indicate that IL-18 is responsible for down-regulation of LPS-induced TNF-α production during *P. acnes* priming. TNF-α is secreted from macrophages and T cells in response to a variety of stimuli. Among these, LPS is the most potent inducer of TNF-α from macrophages (38,39). Consistent with this, macrophages of *P. acnes*-primed IL-18KO mice were highly sensitive to LPS for the production of TNF-α. *In vivo* treatment of IL-18KO mice with recombinant IL-18 or of wild-type mice

with anti-IL-18 antibody significantly altered TNF-α production from LPS-stimulated macrophages. We tried to sensitize resident IL-18KO macrophages with *P. acnes in vitro* in the presence of IL-18. However, the presence of IL-18 during *P. acnes* priming *in vitro* resulted in no significant reduction in TNF-α production. In addition, there was no significant increase in TNF-α production from LPS-stimulated IL-18KO macrophages after *in vitro P. acnes* priming relative to wild-type macrophages (our unpublished data). These *in vitro* results suggest that IL-18 secreted from activated macrophages does not affect macrophages in an autocrine manner during *P. acnes* priming, but acts on cells other than macrophages to induce cytokine(s) or molecule(s), which in turn renders macrophages hyporesponsive to LPS-induced TNF-α production.

LPS is shown to induce a tolerance to its own effects in macrophages *in vitro*. Pre-exposure of macrophages to low dose LPS induces a desensitization for secondary TNF-α responses to LPS (40,41). Pre-exposure to components from Gram-positive bacteria and to a synthetic lipid structurally related to lipid A induces similar effects (42). The supernatants of macrophages toleralized *in vitro* to LPS have been shown to contain a factor capable of rendering naive macrophages refractory to LPS stimulation (43). We examined whether LPS-induced tolerance is dependent on IL-18 or not. IL-18KO mice pre-treated with low-dose LPS displayed reduced TNF-α production after LPS challenge as in the case of wild-type mice (our unpublished data). Therefore, it is unlikely that IL-18 is the sole factor responsible for LPS-induced endotoxin tolerance, although it is possible that IL-18 is involved.

In conclusion, the present study demonstrates that IL-18 suppresses TNF-α production from monocytes/macrophages in response to LPS during *P. acnes* priming, and that IL-18 plays an important role in the pathogenesis of sepsis, in addition to TNF-α, IL-1, IL-6, IL-8, IFN-γ and IL-10. Further study will reveal the complicated cytokine network during sepsis and provide a good indication as to how to treat these disorders.

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Abbreviations

ICE	IL-1β-converting enzyme
KO	knockout
LPS	lipopolysaccharide
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor receptor

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