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Neutralization of IL-18 Reduces Neutrophil Tissue Accumulation and Protects Mice Against Lethal *Escherichia coli* and *Salmonella typhimurium* Endotoxemia

Mihai G. Netea,* Giamila Fantuzzi,* Bart Jan Kullberg,† Rogier J. L. Stuyt,* Edward J. Pulido,† Robert C. McIntyre, Jr.,† Leo A. B. Joosten,§ Jos W. M. Van der Meer,† and Charles A. Dinarello²*

In addition to stimulating IFN-γ synthesis, IL-18 also possesses inflammatory effects by inducing synthesis of the proinflammatory cytokines TNF and IL-1β and the chemokines IL-8 and macrophage inflammatory protein-1α. We hypothesized that neutralization of IL-18 would have a beneficial effect in lethal endotoxemia in mice. IL-1β converting enzyme (ICE)-deficient mice, lacking the ability to process mature IL-18 and IL-1β, were completely resistant to lethal endotoxemia induced by LPS derived from either *Escherichia coli* or *Salmonella typhimurium*. In contrast, both wild-type and IL-1β⁺⁻ mice were equally susceptible to the lethal effects of LPS, implicating that absence of mature IL-18 or IFN-γ but not IL-1β in ICE⁻⁻ mice is responsible for this resistance. However, IFN-γ-deficient mice were not resistant to *S. typhimurium* LPS, suggesting an IFN-γ-independent role for IL-18. Anti-IL-18 Abs protected mice against a lethal injection of either LPS. Anti-IL-18 treatment also reduced neutrophil accumulation in liver and lungs. The increased survival was accompanied by decreased levels of IFN-γ and macrophage inflammatory protein-2 in anti-IL-18-treated animals challenged with *E. coli* LPS, whereas IFN-γ and TNF concentrations were decreased in treated mice challenged with *S. typhimurium*. In conclusion, neutralization of IL-18 during lethal endotoxemia protects mice against lethal effects of LPS. This protection is partly mediated through inhibition of IFN-γ production, but mechanisms involving decreased neutrophil-mediated tissue damage due to the reduction of either chemokines (*E. coli* LPS) or TNF (*S. typhimurium* LPS) synthesis by anti-IL-18 treatment may also be involved. The Journal of Immunology, 2000, 164: 2644–2649.

The poor outcome of Gram-negative bacterial sepsis is believed to be due to LPS, and lethal endotoxemia has been extensively used as an experimental model of Gram-negative septic shock (1). Binding of LPS to receptors on leukocytes triggers the production of highly active mediators such as the proinflammatory cytokines TNF, IL-1α, and IL-1β and the chemokines IL-8, macrophage inflammatory protein-1α (MIP-1α),¹ and MIP-2 (2, 3). Stimulation of cytokines and chemokines is of pivotal importance in the pathogenesis of sepsis because they are believed to be the main endogenous mediators of organ injury in endotoxic shock (1, 2). IL-18 (initially described as IFN-γ-inducing factor) is a newly discovered proinflammatory cytokine that triggers the production of highly active mediators such as the proinflammatory cytokines TNF and IL-1β (4). Moreover, IL-18 mediates, through induction of IFN-γ, the activation of NF-kB (5), and MIF-1β (6), and MIP-2 (7), which play a pivotal role in organ injury in endotoxic shock (1, 2).

Mature IL-18 and IL-1β are protected against lethal endotoxemia. The fact that IL-1β⁻⁻ mice are susceptible to lethal endotoxemia (7) suggests that the protection of ICE-deficient animals is mediated by the lack of mature IL-18 or IFN-γ. However, controversial data exist regarding the susceptibility of IFN-γ⁻⁻ mice to LPS (8, 9), suggesting that IFN-γ-independent mechanisms also may be involved in the modulatory activity of IL-18.

Although IL-18 exerts some of its proinflammatory effects through induction of IFN-γ, recent data suggest that IL-18 has direct proinflammatory properties. In this respect, IL-18 stimulates activation of NF-κB (10), induces production of proinflammatory cytokines such as TNF and IL-1β and chemokines such as IL-8 and MIP-1α (11), and up-regulates expression of adhesion molecules such as ICAM-1 (12). The hypothesis that these direct inflammatory effects may contribute to disease prompted us to investigate the role of IL-18 in lethal endotoxemia. We have assessed the effect of IL-18 neutralization in endotoxic shock using two methods: the use of ICE⁻⁻ mice known to be deficient in mature active IL-18 (5, 6) and neutralization of IL-18 by treatment with anti-IL-18 Abs. Because the precise effects of *Escherichia coli* and *Salmonella typhimurium* LPS in various types of knockout mice are divergent (8, 9), suggesting differential pathogenic mechanisms involved in mortality induced by these two species of LPS, we have assessed the effect of IL-18 blockade in mice challenged with either *E. coli* or *S. typhimurium* LPS. We have investigated the mechanisms through which anti-IL-18 strategies may beneficially influence the course of lethal endotoxemia.

Materials and Methods

Materials

LPS (*E. coli* serotype O55:B5, catalog no. L4005; and *S. typhimurium*, catalog no. L1519) was obtained from Sigma (St. Louis, MO). The anti-IL-18 antiserum was obtained from a New Zealand rabbit immunized by...
intradermal injection of murine IL-18 (PeproTech, Princeton, NJ) in the presence of Hunter’s Titermax adjuvant. This Ab has been shown to inhibit LPS-induced IFN-γ production in vivo (13).

**Animals**

The in vivo experimental studies were approved by the Animal Use and Care Committee at the University of Colorado Health Sciences Center. The generation and background of ICE−/− and IL-1β−/− mice were previously described (14, 15). The wild-type mice used as controls were of the same genetic background, sex, and age as the knock-out mice. IFN-γ−/− mice and their wild-type littermates were a kind gift from Organon (Oss, The Netherlands). C57BL/6J mice were obtained from Taconic Laboratory (Germantown, NY). For the experiments, 6- to 8-wk-old mice weighing 20–25 g were used. The animals were housed under specific pathogen-free conditions.

**Lethal endotoxemia model**

Knock-out and control mice were injected i.p. with LPS suspended in PBS (40 mg/kg unless otherwise indicated). In separate experiments, C57BL/6J mice were treated i.p. with 200 μl of either normal rabbit serum (NRS) as control or anti-IL-18 antiserum 30 min before the LPS administration. The neutralizing characteristics of the anti-IL-18 antiserum were previously reported for in vitro (13) and in vivo (16) experiments. Ninety minutes after challenge with LPS, five animals from each group were anesthetized with ether and were bled from the retroorbital plexus for measurement of TNF-α and MIP-2 circulating concentrations. Another five animals per group were bled 6 h after LPS challenge for the measurement of IFN-γ circulating concentrations. In addition, lungs and livers from the sacrificed mice were aseptically removed, placed into liquid nitrogen, and stored at −70°C. Half of the tissue material was used for myeloperoxidase (MPO) measurements, and the rest was used for tissue cytokine determinations. For cytokine measurements, the tissue was suspended and homogenized 1:4 (w:v) in sterile PBS containing 0.1% Tween 20 and centrifuged at 20,000 × g for 15 min, and the supernatant was collected and stored at −70°C until assay. In the remaining mice (5–7 animals/group), survival was assessed daily for 7 days.

**Cytokine measurements**

Murine IL-18 was measured by electrochemiluminescence as previously described (45). Murine TNF concentrations were assessed by electrochemiluminescence (17). The detection limits were 160 pg/ml (IL-18) and 62 pg/ml (TNF). IFN-γ measurements were performed with an ELISA kit (Endogen, Woburn, MA) (detection limit 20 pg/ml). MIP-2 concentrations were measured using a commercial ELISA kit (QuantiKine, R&D Systems, Minneapolis, MN) with a detection limit of 7 pg/ml.

**MPO assay**

The content of MPO in the tissues was measured as previously described (18). Briefly, organs were weighed and then either both lungs or a segment of the liver (150–200 mg of tissue) was homogenized by a Virtishear homogenizer (Germantown, NY). The samples were sonicated for 90 s at full power with an Ultrasonic homogenizer (Cole-Parmer Instrument, Chicago, IL), incubated in a 60°C water bath for 2 h, and centrifuged for 10 min at 20,000 × g. The supernatant (25 μl) was added to 725 μl of 50 mM phosphate buffer (pH 6.0) containing 0.5 g/dl cetrimonium bromide. The samples were sonicated for 90 s at full power with an Ultrasonic homogenizer (Cole-Parmer Instrument, Chicago, IL), incubated in a 60°C water bath for 2 h, and centrifuged for 10 min at 20,000 × g. The supernatant (25 μl) was added to 725 μl of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine (Sigma) and 5 × 10−4% hydrogen peroxide. Absorbance of 460 nm visible light was measured between 1 and 3 min with a Beckman DU7 spectrophotometer (Beckman Instruments, Palo Alto, CA). The pellet was resuspended in 4 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 g/dl cetrimonium bromide. The samples were sonicated for 90 s at full power with an Ultrasonic homogenizer (Cole-Parmer Instrument, Chicago, IL), incubated in a 60°C water bath for 2 h, and centrifuged for 10 min at 20,000 × g. The supernatant (25 μl) was added to 725 μl of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine (Sigma) and 5 × 10−4% hydrogen peroxide.

**Statistical analysis**

The differences between groups were analyzed by Mann-Whitney U test and, if appropriate, by Kruskal-Wallis ANOVA test. Survival curves were analyzed by the Kaplan-Meier log-rank test. Experiments were performed twice on two separate occasions, and the data are presented as cumulative results of both experiments.

**Results**

**Survival during endotoxemia**

All wild-type and IL-1β−/− mice injected with 40 mg/kg LPS died within 5 days after the endotoxin challenge (Fig. 1, A and B). In contrast, ICE−/− mice were completely protected against the lethal effects of LPS derived either from E. coli or from S. typhimurium LPS (Fig. 1, A and B). None of the wild-type or knockout mice died when they were injected with 20 mg/kg LPS (n = 4 wild-type and 4 knockout mice).
mice and n = 5 each for IL-1β−/− and ICE−/− mice). These data suggest that the protection of ICE−/− mice to the lethal effects of LPS is mediated through the lack of mature IL-18, presumably resulting in the reduced production of IFN-γ. However, the hypothesis that the ICE−/− mice are protected due to the lack of IFN-γ is not supported by the observation that IFN-γ−/− mice were only partially protected against lethal endotoxemia induced by E. coli LPS (Fig. 1C) and were totally susceptible to S. typhimurium LPS (Fig. 1D).

The above data suggest an important role for IL-18 during endotoxic shock, independent of IFN-γ production. C57BL/6J mice were injected i.p. with NRS or anti-IL-18 antiserum 30 min before E. coli (A) or S. typhimurium (B) LPS challenge. Experiments involved 10–12 mice/group and were performed twice on two different occasions. Cumulative data are presented in the figure. ∗, p < 0.05.

FIGURE 2. Anti-IL-18 Ab treatment protects against lethal endotoxemia. Mice were injected i.p. with NRS or anti-IL-18 antiserum 30 min before E. coli (A) or S. typhimurium (B) LPS challenge. Experiments involved 10–12 mice/group and were performed twice on two different occasions. Cumulative data are presented in the figure. ∗, p < 0.05.

IL-18 expression during lethal endotoxemia

In healthy mice, circulating concentrations of IL-18 were under the detection limit of the assay (160 pg/ml). In contrast, constitutive expression of IL-18 was measured in the lungs (6020 ± 456 pg/g) and liver (9928 ± 1204 pg/g) of healthy mice. LPS challenge induced circulating IL-18 in the bloodstream of septic mice 90 min after endotoxin administration (273 ± 41 pg/ml after E. coli LPS and 279 ± 54 pg/ml after S. typhimurium LPS). At all other time points (30 min–24h), circulating IL-18 concentrations were below the detection limit of the assay. Ninety minutes after Salmonella LPS, there was an increase in the IL-18 content of the lung and liver (14,326 ± 1,737 and 13,111 ± 993 pg/g, respectively; p < 0.05), whereas E. coli LPS stimulation led to an increase in IL-18 levels only in the lungs (9,757 ± 1,028 pg/g; p = 0.06) and led to a significant decrease in IL-18 levels in the liver (5,027 ± 260 pg/g; p < 0.05).

The effect of anti-IL-18 treatment on the in vivo proinflammatory cytokine production

C57BL/6J mice were injected i.p. with 40 mg/kg LPS, and blood was drawn 90 min and 6 h later for cytokine measurements. Administration of the anti-IL-18 Abs did not influence TNF serum concentrations measured 90 min after E. coli LPS and 279 ± 54 pg/ml after S. typhimurium LPS). At all other time points (30 min–24h), circulating IL-18 concentrations were below the detection limit of the assay. Ninety minutes after Salmonella LPS, there was an increase in the IL-18 content of the lung and liver (14,326 ± 1,737 and 13,111 ± 993 pg/g, respectively; p < 0.05), whereas E. coli LPS stimulation led to an increase in IL-18 levels only in the lungs (9,757 ± 1,028 pg/g; p = 0.06) and led to a significant decrease in IL-18 levels in the liver (5,027 ± 260 pg/g; p < 0.05).

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reducing IFN-γ activation after *E. coli* LPS (92% inhibition; \( p < 0.01 \)) than it was after *S. typhimurium* LPS (48% inhibition; \( p < 0.05 \)).

MPO levels in lungs and liver

To assess the effect of anti-IL-18 treatment on LPS-induced lung and liver neutrophil accumulation, we assessed the MPO content in these two organs 6 h after LPS challenge. As shown in Fig. 4, LPS injection dramatically increased MPO content in the lungs and liver. However, whereas *E. coli* LPS was more effective than *S. typhimurium* LPS in increasing MPO content of the lung, the latter induced more MPO in the liver. Compared with NRS, treatment of mice with anti-IL-18 Abs significantly reduced the MPO levels in the lungs of mice challenged with *E. coli* but not of those challenged with *S. typhimurium* LPS (Fig. 4A). In contrast, anti-IL-18 significantly reduced hepatic MPO after *E. coli* LPS (79%; \( p < 0.05 \)) and *S. typhimurium* LPS (61%; \( p < 0.05 \)) (Fig. 4B).

Modulation of chemokine expression by anti-IL-18 treatment

Because neutrophil accumulation in the organs is largely dependent on chemokines such as MIP-2, we investigated the content of this chemokine in the lungs and liver of mice treated with anti-IL-18 and challenged with LPS. In mice injected with *E. coli* LPS, anti-IL-18 treatment reduced MIP-2 expression in the lungs (52% decrease; \( p < 0.05 \)) and liver (49% decrease; \( p < 0.05 \)) (Fig. 5). In contrast, no effects of anti-IL-18 Ab on MIP-2 synthesis after *S. typhimurium* LPS injection were apparent (Fig. 5).

Discussion

The results of the present study demonstrate that a reduction of IL-18, either in ICE \(^{−/−} \) mice or by administration of neutralizing anti-IL-18 Abs to wild-type mice, protects against the lethal effects of LPS derived from *E. coli* or *S. typhimurium*. Neutralizing IL-18 was accompanied by a decrease in the MPO content in the lung and liver, reflecting a reduction in neutrophil accumulation. It is well established that infiltration of neutrophils into vital organs plays an essential role in endotoxin lethality (19). There are at least five mechanisms by which anti-IL-18 affords protection in these studies: 1) anti-IL-18 treatment reduces IFN-γ production, particularly after *E. coli* LPS (13), which is consistent with IFN-γ \(^{−/−} \) mice being partially resistant to lethal *E. coli* LPS, 2) inhibition of MIP-2 production in tissues of mice challenged with *E. coli* LPS probably resulted in decreased neutrophil infiltration, 3) the beneficial effects of anti-IL-18 administration during *S. typhimurium* endotoxemia may be mediated through decreased production of TNF, leading to protection against liver injury, 4) anti-IL-18 treatment likely reduces ICAM-1 expression (12), and 5) because IL-18 induces Fas ligand expression which plays an important role in hepatic toxicity (20), inhibition of IL-18 may decrease liver toxicity through inhibition of Fas ligand expression.
IL-18 in IFN-γ production induced by LPS has been confirmed in IL-18-deficient mice (23).

The importance of IL-18 as a proinflammatory cytokine is suggested by experiments in which pretreatment with an anti-IL-18 Ab protected against LPS-induced liver injury (21). A similar protection was reported in IL-18−/− mice (24). Although ICE−/− mice lacking mature forms of IL-18 and IL-1β are completely resistant to lethal endotoxemia as shown in the present study and by others (5, 6), IL-1β−/− mice are not (7). Therefore, it has been proposed that the absence of mature, active IL-18 determines the resistance of ICE−/− mice via reduced IFN-γ production. However, in the case of S. typhimurium LPS, we have found that IFN-γ−/− mice are not protected against endotoxemia in that others have reported that absence of IFN-γ-R does not afford protection (9). One disadvantage of experiments performed in knock-out mice may be the modified homeostasis due to the absence of some genes, which may lead to multiple abnormalities. Indeed, ICE−/− and IFN-γ−/− mice lack not only mature IL-1β, but also lack mature IL-18 and IFN-γ; in addition, production of other cytokines such as γ, IL-1α, TNF, and IL-6 is also impaired compared with their wild-type counterparts (14, 25). In contrast, this is not the case for the IL-1β−/− mice (26). Therefore, our experiments with anti-IL-18 Abs are crucial, and these experiments suggest that our conclusions regarding the role of IL-18 in lethal endotoxemia are valid. Indeed, administration of an anti-IL-18 polyclonal Ab protected mice against the deleterious effects of both LPS species tested, supporting the concept that IL-18 has an important pathogenic role in both species of lethal endotoxemia.

The beneficial effect of the anti-IL-18 Ab is consistent with the study of Xu et al. (27), showing protection against lethality induced by Salmonella LPS using an anti-IL-18 receptor Ab. Interestingly, this contrasts with the observation of Sakao et al. (24), who reported an increased mortality of IL-18−/− mice in the Propionibacterium acnes-sensitization LPS model, despite protection of animals against liver injury. However, this model is different from the model of high-dose LPS used by Xu et al. (27) and by us in the present study. The use of IL-18 knock-out mice adds a new variable to the experimental outcome because deficient animals probably up-regulate other cytokines and cytokine receptors, with subsequent hyperreactivity to inflammatory stimuli as has been shown for other proinflammatory cytokine knock-out mouse strains (28).

In contrast to E. coli LPS, it is unlikely that IFN-γ plays an important role in the protection afforded by anti-IL-18 Abs after S. typhimurium LPS. After S. typhimurium, we observed only a moderate inhibition of IFN-γ synthesis by the anti-IL-18 and a lack of resistance in IFN-γ−/− mice. Other studies support our observation because mice deficient in IFN-γ-R are also susceptible to lethal S. typhimurium LPS injection (9). Whereas anti-IL-18 had no effect on circulating TNF levels induced by E. coli LPS, the Ab significantly decreased levels of TNF (55%; p < 0.02) after challenge of mice with S. typhimurium LPS. This suggests that the effect of anti-IL-18 during S. typhimurium endotoxemia may be at least in part due to inhibition of TNF.

Lung and liver injury during endotoxemia is largely mediated through neutrophil infiltration (29, 30), which can be assessed by the MPO content in the respective tissues (19). Interestingly, E. coli LPS induced more neutrophil infiltration in the lungs compared with S. typhimurium LPS, whereas the latter was more effective in promoting neutrophil accumulation in the liver. Treatment of mice with anti-IL-18 Abs before challenge with E. coli LPS was accompanied by a significant decrease in the lung and liver MPO content compared with that of mice challenged with LPS that did not receive anti-IL-18. The anti-IL-18-associated decrease in MPO content may have been mediated through diminished chemokine production in these animals. Indeed, the reduction in the neutrophil infiltration of lung and liver in the anti-IL-18-treated mice after E. coli LPS challenge was accompanied by a decrease in MIP-2 levels in the organs of IL-18-treated animals compared with those of controls. Therefore, it is conceivable that part of the effects of anti-IL-18 treatment during E. coli endotoxemia is IFN-γ-independent and mediated through decreased MIP-2 expression. In contrast, we suggest that the beneficial effect of anti-IL-18 treatment in S. typhimurium endotoxemia is mediated by the reduction in circulating TNF concentrations, which is supported by the observed effect of anti-IL-18 on MPO. Although anti-IL-18 treatment significantly decreased the MPO content of the liver after S. typhimurium challenge, it had no influence on the MPO content of the lungs. This pattern is consistent with the decreased TNF concentration after anti-IL-18 Ab treatment during S. typhimurium endotoxemia in that TNF has been shown to be involved in LPS-mediated liver damage (31) but not lung injury (32).

The present observations of differential responses to these two LPS species may have important theoretical consequences. The two species differ not only at the level of the polysaccharide chains but also at the level of lipid A. Compared with E. coli lipid A, lipid A from Salmonella contains an additional fatty acid (33) and different phosphate groups (34). Because lipid A binding to LPS binding protein (LBP) and CD14 results in cytokine production (35), it is not surprising that differences in the structure of lipid A may stimulate a different combination of cytokines. An additional argument is provided by studies performed in LBP-deficient (LBP−/−) mice. These mice responded with reduced cytokine production after stimulation with S. abortus equi LPS (36) compared with that after E. coli LPS (37). This differential cytokine response of LBP−/− mice to E. coli or Salmonella LPS raises the interesting possibility that these two LPS species may interact differently with the various Toll-like receptors (TLRs) known to be involved in the intracellular signaling induced by LPS (38, 39). In support of this hypothesis are the data of Yang et al. (38), which show that Salmonella-derived LPS is much more potent in inducing cytokines through TLR2 compared with E. coli LPS, whereas the two types of LPS are equally potent in inducing cytokines through TLR4 (40). Alternatively, differences between the two LPS preparations may be due to quantitative and/or qualitative differences in the “endotoxin-associated proteins.” In our experiments, we have used commercial LPS chromatographically purified by gel filtration with a protein content less than 1%. However, even very small amounts of endotoxin-associated proteins may influence cytokine induction by LPS, as previously suggested by some (41) although not all (42) authors. Despite the fact that we used E. coli and S. typhimurium LPS isolated and purified by identical methods, suggesting that protein contamination is similar for both preparations, we cannot exclude a role of these endotoxin-associated proteins in the biological activities of the various LPS used. However, the importance of our data remains valid regardless of the exact source of differences between the two LPS used (differences in either the lipid A structure or the endotoxin-associated proteins) because these preparations have been used in all models of lethal endotoxemia reported in the literature. Therefore, our data on the differences in the cytokine network and pathogenic mechanisms between these two types of LPS are likely to explain some of the contradictory data in the literature such as resistance of TNF−/− mice to E. coli but not to Salmonella LPS (43, 44). The capacity of anti-IL-18 treatment to induce resistance against both of these two species of LPS underscores the importance of IL-18 as a regulator of pathological mechanisms in lethal endotoxemia.
References


