Divergence of Protection Induced by Bacterial Products and Sepsis-Induced Immune Suppression

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Susceptibility to bacterial infections after a primary immune stimulation differs drastically depending on the presensitization of the innate immune system. To determine the conditions that either induce protection or enhanced susceptibility to infection with Salmonella enterica serovar Typhimurium, we pretreated mice either with tumor necrosis factor (TNF), whole killed bacteria, or sublethal cecal ligation and puncture (CLP) as a mouse model for septic peritonitis. Impaired production of the cytokines TNF, interleukin-6 (IL-6), and IL-10 was induced by these pretreatment schedules, with TNF-signaling not being essential for this effect. Injection of TNF or killed bacteria enhanced survival of mice infected subsequently with serovar Typhimurium. In contrast, sepsis such as that induced by CLP only protected from shock induced by 

Local bacterial infections can become systemic and lead to sepsis, which has been defined as the systemic host response to infection and is accompanied by an early release of inflammatory and proinflammatory mediators (1). From extensive experimental work with bacterial lipopolysaccharide (LPS) as a model substance for infection with gram-negative bacteria, it was found that pretreatment with LPS protects mice from endotoxin shock, as well as from subsequent bacterial infections (23). This phenomenon, seemingly preparing the organism for the subsequent infection, became known as “LPS tolerance” (12). Basically, two mechanisms have been proposed to explain this LPS-induced protection. LPS-pretreatment could preactivate antibacterial innate immune responses, e.g., by improving the sensing of molecular patterns typical for infective agents and generation of the required inflammatory responses (16). Pretactivation of monocytes/macrophages for the release of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and IL-12, the production of reactive oxygen intermediates, and enhanced phagocytosis, as well as recruitment and a diminished rate of apoptosis of neutrophils, have been reported to take part in the preparatory mechanisms (10, 23). The crucial requirement for TNF for optimal innate immune defense against bacterial infections has been documented in many models (7, 30). Enhanced resistance to bacterial infection can be induced by pretreatment with TNF, supporting the idea of TNF participating in LPS-induced tolerance (4, 26). Also, TNF seems to initiate the appropriate inflammatory reaction early at the onset of infection (16). Earlier work of our group has shown that LPS-induced resistance to cecal ligation and puncture (CLP)-induced polymicrobial peritonitis was based on the interaction of endogenous TNF with the p75TNFR (6). Besides its beneficial antibacterial effect, TNF, as well as LPS, is also known to induce reduction of subsequent production of inflammatory cytokines. Thus, LPS pretreatment by inducing endogenous TNF seems also to initiate a counter-regulation of the hyper-inflammatory state.

Although well-balanced inflammation is important to control bacterial infection, an excessive systemic inflammatory response can lead to shock and early mortality (5, 8). A large number of clinical and experimental data indicate that septic shock is at least partly due to overshooting the release of inflammatory cytokines (17, 21, 35). Therefore, compensatory anti-inflammatory responses which counter-regulate the immune response are important to avoid detrimental inflammatory responses and achieve immunological homeostasis. Consumption of key inflammatory mediators such as TNF and IL-1, downregulation of their expression, and shedding of their receptors are well-known phenomena. Endogenous inhibitors of Toll-like receptor signaling such as MyD88s and IRAK-M have been reported to limit the activation of inflammatory genes (2, 20). Additional inhibitors of macrophage activation such as SIGIRR, SOCS-1, and ST-2 have recently been described (19, 24, 41). The multitude of ways to limit the inflammatory response underscores the physiological importance of such a tight regulation.

However, the anti-inflammatory counter-regulation can result in transient immune depression leading to insufficient immune reactions for subsequent bacterial infections and to late mortality (21). In patients with prolonged sepsis such a phenomenon has been observed and was termed “immunoparalysis.” Clinically, immunoparalysis was characterized by depressed monocytic HLA-DR antigen expression and decreased TNF secretion capacity (38, 39, 40, 43). Experimental analysis in different models revealed a suppressed innate immune response, e.g., impaired production of inflammatory cytokines, NO, and oxygen radicals (28, 34, 42).

We attempted to clarify the apparent discrepancy in sensitivity to bacterial infections after a primary immune stimulation by determining the conditions that lead either to protection from bacterial infection due to “LPS tolerance” or
enhanced susceptibility due to “immunoparalysis.” As the infecting agent, _Salmonella enterica _serovar Typhimurium (Stm) was chosen as a well-characterized mouse pathogenic bacterial infection model. The role of TNF was analyzed in particular since it is a central mediator of the inflammatory immune response, as well as a cytokine with important antibacterial activity. After CLP, the mouse model for septic peritonitis was used in the present study, an immunoparalysis-like state in terms of the reduced cytokine production capacity became evident 2 days after surgery (7). In addition, such CLP-treated mice are highly susceptible to secondary infections with different gram-negative and gram-positive bacteria (3, 7, 36). Although the reduction of cytokine production was TNF independent, the generation of enhanced susceptibility to bacterial infections was TNF dependent and was only achieved by CLP-induced sepsis.

**MATERIALS AND METHODS**

Mice. Female or male NMRI mice (20 to 25 g) were purchased from Charles River, Sulzfeld, or Harlan-Winkelmann, Borchten, Germany. TNF-deficient (TNF-/-) and control (TNF+/-) mice on a mixed genetic background 129/SV x C57BL/6 (32) were provided by L. Hüttner, GSF-Forschungszentrum, Munich, Germany. Mice deficient for the p55TNFR (33) or p75TNFR (9) were kept and bred in the animal facility of the University of Regensburg. All infection experiments were performed in compliance with the federal guidelines for animal experimentation.

CLP. Mice were anesthetized by intraperitoneal (i.p.) injection of 75 mg of Ketanest (Parke, Davis, & Company, Munich, Germany) and 16 mg of Rompun (Bayer AG, Leverkusen, Germany)/kg. The cecum was exteriorized, and the diameter was ligated and punctured once with a needle (0.4 mm in diameter) to achieve a sublethal CLP as described previously (5). Mice were observed for 2 weeks. Kaplan-Meier survival curves were compared by using the log-rank test.

Bacterial infections. Stm (ATCC 14028s) was grown in LB medium, Gibco-BRL, Karlsruhe, Germany, and counted on XLD-Agar (Schleicher & Schuell, Dassel, Germany). The numbers of CFU injected were estimated from the optical density at 560 nm and checked by plating aliquots of the inocula. Mice were injected i.p. with Stm in 500 μl as indicated in the figure legends. _Pseudomonas aeruginosa _103 strain were grown in LB medium, Gibco-BRL, Karlsruhe, Germany, and counted on Cetrimid agar plates (Schleicher & Schuell, Dassel, Germany).

d-Galactosamine/LPS-induced hepatotoxicity. d-Galactosamine HCl (d-GaIN; Sigma Aldrich, Steinheim, Germany) at 15 mg/mouse was administered i.p. (22), together with LPS from _S. enterica _serovar Abortus-Equi (15) at 100 ng/mouse, and survival was monitored.

Reagents. Recombinant human TNF (rhTNF; BASF Bioresearch, Ludwigshafen, Germany) has a specific bioactivity of 6.6 × 10^6 U/mg in the cytotoxicity assay on L929 cells without actinomycin D. Lipoteichoic acid was provided by E. Hartung, University of Konstanz (27). CpG-ODN 1668 (Invitrogen GmbH, Technologiepark Karlsruhe, Germany) was commercially obtained.

Measurements of circulating cytokines. Serum samples were taken 90 min after intravenous challenge with lipopolysaccharide (LPS) from _S. enterica _serovar Abortus-Equi (15) at 1 μg/200 μl/mouse. Quantification of TNF, IL-6, and IL-10 serum titers was performed by enzyme-linked immunosorbent assay (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s recommendations. LPS binding protein (LBP) levels were determined as described previously (18).

Statistics. All infection experiments were performed at least three times with similar results, and one representative experiment is shown. The log-rank test was used to compare Kaplan-Meier survival curves. The unpaired, two-tailed Mann-Whitney test was used for the determination of the P values for all other data.

**RESULTS**

Reduced cytokine production capacity after pretreatment with rhTNF, dead Stm, or CLP. As seen in Fig. 1A, a single dose of rhTNF, which exclusively interacts with the mouse p55TNFR given 48 h prior to in vivo stimulation with LPS inhibited TNF, IL-6, and IL-10 production. This effect of pretreatment with rhTNF was dose dependent (Fig. 1A, inset). A similar reduction in cytokine production capacity was also observed when mice were pretreated with dead Stm (Fig. 1B).

A similarly reduced capacity for production of pro- and anti-inflammatory cytokines upon LPS stimulation 2 days after septic peritonitis induced by CLP was also observed (Fig. 1C). Intravenous challenge with purified lipoteichoic acid (60 μg per mouse, given i.p.) or CpG (40 μg per mouse, given i.p.) 48 h after CLP treatment also exhibited reduced cytokine release upon stimulation, although cytokine levels were lower than after LPS challenge, indicating that the effect was not restricted to challenge with LPS as a model for gram-negative bacteria (data not shown). Such CLP-induced reduction of cytokine production was evident in wild-type C57BL/6 mice, as well as in p55TNFR-deficient mice concerning TNF, IL-6, and IL-10 production capacity (Fig. 1C). The impaired cytokine production capacity 2 days after CLP was similar in p55TNFR-deficient and p75TNFR-deficient mice, however, with noticeably higher TNF serum levels in the p75TNFR-deficient mice. LPS-induced IL-12(p40) serum levels, however, 2 days after CLP were higher in wild-type and p55TNFR-deficient mice than in mice that had not experienced CLP previously (data not shown).

Since the reduction in cytokine production capacity was not restricted to TNF production but was also seen with IL-6 and IL-10, TNF-deficient mice could also be analyzed for the influence of CLP on cytokine production. Two days after CLP, TNF-deficient mice reacted like wild-type mice with reduced LPS-induced production of IL-6 and IL-10 (Fig. 1D), demonstrating that in terms of cytokine production capacity wild-type mice and mice deficient for any of the two TNF receptors, as well as TNF-deficient mice, behaved in a comparable manner downregulating cytokine production upon CLP. Thus, even though TNF can induce a reduction of cytokine production (Fig. 1A) similar to pretreatment with dead bacteria (Fig. 1B), CLP-induced reduction of cytokine production also occurs independently of TNF action.

Improved survival after injection of d-GaIN/LPS or a high dose of bacteria after CLP. Two days after sublethal CLP improved survival was observed in mice treated with d-GaIN and LPS. Without previous CLP, all mice died quickly within 6 h due to the hepatotoxic effect of d-GaIN/LPS (P = 0.022) (Fig. 2A). Also, when a high dose of Stm was injected, most mice died very quickly within the next 24 h. Mice which had undergone sublethal CLP 2 days previously, however, survived such a high dose of living Stm significantly longer (P = 0.0004) (Fig. 2B). Similarly, when mice received a very high dose of living _P. aeruginosa_, which by itself killed most mice within 24 h, previous subjection to sublethal CLP saved all animals (P = 0.0016) (Fig. 2C). Thus, the impaired production capacity for inflammatory cytokines after sublethal CLP can be beneficial in cases of life-threatening hyperinflammation and shock.

Improved survival of Stm infection after pretreatment with rhTNF or dead Stm. Improved survival of Stm infection was achieved by pretreatment of mice either with rhTNF (P < 0.0001) (Fig. 3A) or with phenol-inactivated Stm (P < 0.0001) (Fig. 3B). Obviously, pretreatment with rhTNF or dead Stm prepared the organism for the Stm infection and protected the
FIG. 1. Cytokine production capacity after pretreatment with rhTNF, dead STm, or CLP. Cytokine serum levels were determined 90 min after stimulation with LPS (1 μg/mouse, i.p.). (A) Mice received rhTNF (100 μg, i.p.) or phosphate-buffered saline (PBS), and 48 h later LPS-induced serum levels of TNF, IL-6, and IL-10 were measured (n = 9 for each group). ***, P < 0.0001. The inset shows the dose response after pretreatment with PBS (○) or 1, 10, or 100 μg of rhTNF (■) 48 h previous to stimulation. (B) Mice had received phenol-inactivated STm (2.5 × 10^9 CFU, i.p.) or PBS 48 h later LPS-induced serum levels of TNF, IL-6, and IL-10 were measured (n = 6 for each group). ***, P < 0.004; **, P < 0.008. (C) Mice were subjected to sublethal CLP (C57BL/6 wild type, n = 7 without CLP, n = 5 with CLP; p55TNFR^-/-, n = 8 for each group; p75TNFR^-/-, n = 6 for each group), and 48 h later LPS-induced serum levels of TNF, IL-6, and IL-10 were measured. *, P < 0.04; **, P < 0.004. (D) TNF-deficient mice (n = 3 for each group) were subjected to sublethal CLP, and 48 h later LPS-induced serum levels of IL-6 and IL-10 were measured in pooled sera. wt, wild type; S.tm, STm.
mice even though their cytokine production capacity was impaired by the pretreatment.

**CLP-induced enhanced susceptibility to a secondary STm infection.** In order to investigate the mechanisms by which TNF affected the susceptibility to a secondary bacterial infection, mice were infected with live STm i.p. with a dose of bacteria depending on the sensitivity of the respective genetic background of the mouse strain under investigation. Although most mice of the mixed background SvJ129 × C57BL/6 infected with 1.25 × 10^5 CFU of STm survived the infection for more than 2 weeks, all TNF-deficient mice infected with the same number of STm died within 5 days (P < 0.002) (Fig. 4A), supporting the well-known importance of TNF for survival of bacterial infections.

Since mice of the C57BL/6 strain are extremely sensitive to infection with STm due to a mutation of the *nramp1* allele (11), only very few bacteria are sufficient to lethally infect these mice. Almost 50% of the wild-type C57BL/6 mice died within 8 days when 10 CFU of STm were injected i.p. (Fig. 4B). Mice with the same genetic background but also deficient for the p55TNFR were even more sensitive, and most of these mice succumbed to such an infection within 1 week, also supporting the known importance of TNF interaction with the p55TNFR in bacterial infection. The susceptibility to STm infection of

**FIG. 2.** Survival after CLP and D-GalN/LPS or high-dose bacterial challenge. (A) Mice without pretreatment (n = 8) (●) or with sublethal CLP 48 h previously (n = 15) (▼) were challenged i.p. with 15 mg of D-GalN in combination with 0.1 μg of LPS. Survival was monitored, and the difference in mortality was determined. P = 0.022. (B) Mice without pretreatment (n = 7) (●) or with sublethal CLP 48 h previously (n = 7) (▼) were challenged with live STm (10^6 CFU, i.p.). Survival was monitored, and the difference in mortality was determined. P = 0.0004. (C) Mice without pretreatment (n = 6) (●) or with CLP 48 h previously (n = 5) (▼) were challenged with live *P. aeruginosa* (10^7 CFU, i.p.). Survival was monitored, and the difference in mortality was determined. P = 0.0016. Ctr., control; P.a., *P. aeruginosa*.

**FIG. 3.** Survival of infection with STm after pretreatment with rhTNF or dead STm Mice were pretreated either with rhTNF (100 μg, i.p) (n = 10) (▼) or PBS (n = 10) (●) and infected with STm (9 × 10^4 CFU, i.p.) 48 h later (A) or with phenol-inactivated STm (3 × 10^9 CFU, i.p.) (n = 6) (▼) or PBS (n = 5) (●) and infected with STm (2 × 10^5 CFU, i.p.) 48 h later (B). Survival was monitored, and the difference in mortality was determined. P < 0.001. S.tm, STm.
mice deficient for the p75TNFR, however, was comparable to that of wild-type mice of the same genetic background (data not shown), which is also in agreement with the literature.

Requirement of TNF function for CLP-induced enhanced susceptibility to a secondary STm infection. Although CLP protected mice from a shock-inducing challenge with d-GalN/LPS or high numbers of STm or \textit{P. aeruginosa} (Fig. 2A to C), it rather sensitized mice to a subsequent infective bacterial challenge. To investigate the contribution of TNF to the development of such immune suppression, mice deficient for TNF were superinfected with STm on day 2 after CLP surgery. In contrast to wild-type mice (Fig. 5A), TNF-deficient mice were not hypersensitive to STm superinfection after CLP but rather protected from the secondary infection with STm, as demonstrated in a prolonged survival period ($P < 0.001$) (Fig. 5B).

To find out whether TNF interaction with the p55TNFR or with the p75TNFR is required to exert the immune suppressive effect induced by CLP, mice deficient for either the p55TNFR or the p75TNFR were subjected to CLP and superinfected 2 days later with STm. p55TNFR-deficient mice were protected in the superinfection model in a way similar to that of TNF-deficient mice ($P = 0.047$) (Fig. 5C and D). Mice deficient for the p75TNFR did not exhibit enhanced sensitivity to the secondary STm infection as were the wild-type mice nor were they protected (data not shown). Thus, TNF-specific activation of the p55TNFR seems to be responsible for the generation of hypersensitivity to bacterial superinfection after CLP-induced sepsis.

**DISCUSSION**

Two opposite effects concerning the immune status can be observed in mice within 48 h after treatment with rhTNF, dead bacteria, or CLP. (i) Protective equipment of the host for a subsequent bacterial infection is generated due to initiation of the innate antibacterial inflammatory reaction. TNF is not essential for the initiation of this pathway; however, TNF can serve as the initiating molecule and can mediate this effect, as shown in LPS-mediated protection in the CLP model where activation of the p75TNFR was required (6). (ii) In order to achieve homeostasis, this inflammatory reaction limits itself. Downregulation of inflammatory cytokine production constitutes part of this counter-regulatory mechanism, thus reducing the risk of overinflammation and shock. This effect is TNF independent, as shown by CLP-induced reduction of cytokine production capacity in TNF-deficient and TNF receptor-deficient mice. The high concentration of soluble TNF in p75TNFR-deficient mice is mainly due to the lower abundance of soluble TNF receptors, as described earlier (29, 31, 37).

Another part of the counter-regulatory mechanism, however, seems to be dependent on TNF action. In the case of an ongoing sepsis such as after CLP, where TNF acts as an important effector molecule of the innate antibacterial inflammatory reaction, manifestation of immune suppression is observed. The TNF-dependent protective mechanisms seem to be either used up or efficiently inhibited by the bacterial infection. In the absence of TNF (even though reduced cytokine production develops), protective measures of the innate immune reaction remain stable. This was also the case in the absence of the p55TNFR. To a lesser extent it was also seen in the absence of the p75TNFR, where immune suppression was not observed after CLP but previous CLP also did not protect from infection.

Since impairment of cytokine expression can develop independently of TNF and can be observed after challenge with LPS, lipoteichoic acid, or CpG, this phenomenon is not restricted to gram-negative bacterial infections. If no sepsis was ongoing, such reduced cytokine production correlated with protection for secondary bacterial infections. Even after sepsis, such impaired cytokine production protected mice from death due to overshooting inflammation, as seen in the models of d-GalN/LPS hepatotoxic shock and septic shock with high numbers of STm or \textit{P. aeruginosa} 2 days after CLP.
However, if sepsis was ongoing after CLP the impaired cytokine production capacity did not lead to protection but, in contrast, to enhanced susceptibility to secondary bacterial infections. In this case, the reduced TNF production capacity after CLP seemed to contribute to the enhanced susceptibility to bacterial challenge since mice could be at least partly protected by rhTNF injection before the superinfection challenge (7). A lack of LBP after CLP implicating impaired sensing of bacterial infection was excluded since LBP levels were elevated after CLP in all mouse lines (data not shown). Several other mechanisms have been suggested for the induction of hypersensitivity to bacterial superinfections during sepsis or sepsis-like conditions. It has clearly been shown that mice infected with Mycobacterium bovis or pretreated with killed Propionibacterium acnes became extremely sensitive to subsequent endotoxin shock and that this hypersensitivity was due to IFN-γ and IL-12 action (13, 14, 44). In our study the IL-12(p40) serum levels were more than twofold higher after CLP compared to untreated mice. However, this was the case in wild-type, as well as in p55TNFR-deficient mice, indicating that the involvement of IL-12 in immune suppression after CLP needs to be further investigated. The molecular basis responsible for the difference of immune stimulation by defined agents activating the Toll-like receptor signaling pathways and leading to protection versus bacterial infection leading to immune suppression is still not clear.

TNF-deficient mice have been reported to be unable to limit inflammation in a model of experimental allergic encephalomyelitis (25, 30) and die from unlimited inflammation after injection of killed Propionibacterium acnes (30), suggesting that TNF action not only initiates but also downregulates inflammation. Details of such TNF-dependent downregulation of inflammation are still not entirely clear. These findings correlate with our observations that TNF-deficient or TNFR-deficient mice fail to develop immune suppression after CLP. Since the immune suppression observed in wild-type mice after CLP also affects immune responses of T and B cells (unpublished data), it seems as if sustained inflammation during bacterial infection downregulates the responses of the adaptive immune system, as well as innate immune responses. Experiments to clarify this observation, including a possible participation of regulatory cell populations, are currently under way.

FIG. 5. Survival after CLP and superinfection with STm. Mice were subjected to sublethal CLP. SvJ129 × C57BL/6 wild-type (n = 5 for each group) (A) and TNF−/− mice (n = 5 for CLP treated, n = 7 for STm infected, and n = 6 for CLP treated and STm infected) (B) were infected with 1.25 × 10^3 CFU STm 48 h later. C57BL/6 wild-type mice (n = 7 for each group) (C) and p55TNFR−/− mice (n = 6 for each group) (D) were infected with 10 CFU of STm 48 h later (○, CLP only; ●, STm only; ▽, CLP + STm). The difference in mortality between STm and CLP+STm in each experiment was significant (P = 0.018 [A], P = 0.001 [B], P = 0.002 [C], P = 0.047 [D]).
Taken together, these data indicate that during sepsis TNF and activation of the TNF receptors, mainly the p55TNFR, are crucially involved in generating a suppressed immune status in the host leading to reduced immune defenses for subsequent secondary bacterial infections. Thus, TNF is not only required for the initiation of an optimal antibacterial innate immune defense but also causes the ensuing “immunoparalysis.”

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