Modulation of Bacterial Growth by Tumor Necrosis Factor- α *In Vitro* and *In Vivo*

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Tumor necrosis factor- α (TNF- α) plays an important role in innate immunity. Recent *in vitro* studies have shown that TNF- α may also serve as a growth factor for some bacteria. We examined the physiologic relevance of this phenomenon both *in vitro* and *in vivo*. Recombinant mouse TNF- α increased *in vitro* proliferation of *Escherichia coli* but not *Pseudomonas aeruginosa* in a concentration-dependent manner, and this effect was attenuated by anti-TNF- α antibodies. However, *in vivo*, TNF- α gene-deficient (TNF- $\alpha^{-/-}$) mice showed higher mortality than wild-type (TNF- $\alpha^{+/+}$) mice after inoculation of intranasal bacteria. An impaired bacterial clearance in TNF- $\alpha^{-/-}$ mice was associated with decreased systemic concentrations of chemokine macrophage inflammatory protein-2, reduced pulmonary neutrophil recruitment, and depressed expression of neutrophil CD11b and CD16/CD32, suggesting that the effect of TNF- α on *E. coli* growth was outweighed by the recruited neutrophils. We also demonstrated that neutropenic TNF- $\alpha^{+/+}$ mice had approximately 100-fold higher *E. coli* counts in their lungs than TNF- $\alpha^{-/-}$ mice, although survival rates in both groups were similar. We conclude that TNF- α augments *E. coli* growth *in vitro* and *in vivo*. However, *in vivo*, this effect becomes only apparent in neutropenic animals. The relevance of these findings for immune compromised patients remains to be investigated.

Keywords: cytokines; phagocytosis; neutrophils; lung; transgenic/knockout

Bacterial pneumonia results in the release of proinflammatory cytokines that mediate the antibacterial defense. These cytokines do not usually act directly on the bacteria, but some bacteria appear to have the ability to employ cytokines as growth factors (1–5). Meduri and colleagues (3) showed *in vitro* that clinical isolates of *Staphylococcus aureus*, *Acinetobacter* sp., and *Pseudomonas aeruginosa* were able to use tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-6 for growth enhancement in a concentration-dependent fashion. These investigators also reported increased intracellular growth of *S. aureus*, *Acinetobacter* sp., and *P. aeruginosa* when human monocytes were pretreated with high concentrations of IL-1 β , IL-6, or TNF- α (2).

It is difficult to reconcile these *in vitro* findings with numerous *in vivo* studies that have demonstrated the importance of cytokines such as IL-1 β and TNF- α for the containment of pneumonia (6–8) and increased bacterial growth in their absence (9–11).

Therefore, to compare how cytokines regulate bacterial survival *in vitro* and *in vivo*, it is essential to use the same bacteria under all experimental conditions. In this study, we used the same strain of *Escherichia coli* or *P. aeruginosa* both *in vitro* and *in vivo* to examine the interaction between cytokines and bacteria. The main reason for choosing these two bacteria was their importance in clinical practice: *E. coli* is one of the most common pathogens in patients with abdominal infection and sepsis, which is frequently associated with acute lung injury (12), whereas *P. aeruginosa* is a common pulmonary pathogen that can be fatal in immunocompromised hosts (13).

We chose to study TNF- α because of its key role in many beneficial and detrimental host responses to bacterial infection (2, 3, 14) and its relationship to the outcome of patients with inflammatory lung diseases (4). Among other properties, TNF- α stimulates the release of chemokines and thus is critical for the recruitment of neutrophils from the blood (14, 15). Conversely, TNF- α can also enhance bacterial growth (2, 3).

In the host, TNF- α -mediated inflammatory responses are thought to be mediated through the binding to at least two receptors, TNF- α receptor 1 and TNF- α receptor 2, which are present on most eukaryotic cells. To investigate the effects of TNF- α in the course of bacterial pneumonia, mice deficient in genes for these TNF- α receptors may be used (9–11). However, experiments in TNF- α receptor 1- and TNF- α receptor 2-deficient mice may not give conclusive results as to the role of TNF- α because TNF- α may still bind and activate other related receptors or ligands in these mice. The TNF- α receptor superfamily includes not only TNF- α receptor 1 and TNF- α receptor 2 but also other related membrane-anchored and secreted receptors and ligands such as CD95 (16, 17), CD40 (17), CD27 (17, 18), and TR1 (19). Also, TNF- α has been recently reported to bind to extracellular matrix molecules such as biglycan and decorin (20). Thus, a TNF- α gene knockout (TNF- $\alpha^{-/-}$) mouse model allows one for the study of TNF- α function regardless of the receptor engaged (21).

In this study, we first examined the direct interaction between TNF- α and bacteria with respect to bacterial proliferation *in vitro* and then investigated the host response during the course of *E. coli*- and *P. aeruginosa*-induced pneumonia in TNF- $\alpha^{-/-}$ and TNF- $\alpha^{+/+}$ mice in the presence and absence of circulating neutrophils.

METHODS

Mice

Animal procedures received institutional approval and were conducted according to the *Guide to the Care and Use of Experimental Animals*, published by Canadian Council on Animal Care (2nd ed, 1993). Male B6;129S-Tnf^{tm1Gkl} mice (TNF- $\alpha^{-/-}$) and B6129SF2/J parental control mice (TNF- $\alpha^{+/+}$) (22–28 g of body weight) were obtained from Jackson Laboratories (Bar Harbor, ME).

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Recombinant Protein and Antibodies

Preparation of bacteria. *E. coli* (ATCC25922) and a *P. aeruginosa* (ATCC33358) strains were obtained from American Type Tissue Collection (Rockville, MD). *E. coli* and *P. aeruginosa* were cultured in tryptic soy broth overnight (13–14 hours) at 37°C in a shaking incubator to obtain stationary-phase organisms as previously described (see the online supplement) (22).

Induction of pneumonia. Mice were anesthetized with ketamine hydrochloride (Veterinary Laboratories, Wyeth-Ayerst Canada Inc., Mississauga, ON, Canada) and xylazine (Bayer Inc., Mississauga, ON, Canada). For the induction of acute pneumonia, *E. coli* and *P. aeruginosa* were suspended in sterile saline; a volume of 1.2 μ L/g body weight of the appropriate bacterial suspension was inoculated intranasally. Final concentrations of the inoculated bacteria ranged from $0.5\text{--}1.0 \times 10^{6-9}$ CFU. Each experiment day included at least one pair of TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice so that comparable numbers of inoculated bacteria were applied in the two groups.

Processing of tissue samples after exposure to bacteria. Because only the mice receiving the lowest concentration ($0.5\text{--}1.0 \times 10^6$ CFU) of bacteria survived for 24 hours, these groups of animals were sacrificed by intraperitoneal injection of sodium pentobarbital at 8 or 24 hours after inoculation for comparison of their bacterial clearance and inflammatory responses between TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice. Under aseptic technique, a tracheostomy was performed for intratracheal intubation using a sterile angiocatheter (Angiocath, 20 G; Becton Dickinson Infusion Therapy Systems Inc., Sandy, UT). The surface of the anterior chest wall and abdominal area were sterilized with ethanol. After opening the chest, cardiac puncture was performed for blood sampling for analysis of macrophage inflammatory protein (MIP)-2. The lung lavage was performed with 1 ml of sterile cold saline administered three times and collected for analyses of neutrophil recruitment, cytokine production, and neutrophil opsonin receptor expression. The whole lung was excised and washed with 10 ml of sterile cold saline. Through a midline abdominal incision, the abdominal aorta was dissected, and the spleen was removed and washed with phosphate-buffered saline. The viable bacteria counts of homogenized lung, lung lavage fluid, and spleen tissues were determined after an 18-hour culture at 37°C in TSB-agar plates. Data were expressed as log₁₀ CFUs per milliliter.

A description of the preparation for lung histology, cell differentials, and isolation of neutrophils is provided in the online supplement.

Flow cytometric analysis with CD11b and CD16/CD32. After the neutrophils obtained by lung lavage had been purified in Hanks' balanced salt solution, 100 μ L of this cell suspension were incubated with 0.25 μ g of the fluorescein isothiocyanate-conjugated anti-CD11b or 1 μ g of the fluorescein isothiocyanate-conjugated anti-CD16/CD32 monoclonal antibodies for 30 minutes at 4°C. The cells were fixed in 4% paraformaldehyde for 5 minutes and then analyzed by a FACScan (Becton Dickinson, Palo Alto, CA) using an FL1 detector. Because the mean basal expression levels of CD11b and CD32/CD16 on bone marrow neutrophils were similar in the TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice, final values were expressed as relative fluorescence index by dividing the linear fluorescence of the experimental groups by the values obtained from the resting control cells that were isolated from bone marrow of B6129SF2/J parental control (TNF- $\alpha^{+/+}$) mice.

ELISA assay. MIP-2 levels in lung lavage fluid and plasma were measured by a mouse MIP-2 ELISA kit following the manufacturer's recommended protocol (R&D Systems, Minneapolis, MN).

LiquiChip multiple cytokine assay. Ten separate mouse cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 [p70], granulocyte-macrophage colony-stimulating factor [GM-CSF], interferon- γ , and TNF- α), other than MIP-2, in lung lavage fluid were simultaneously measured using LiquiChip Cytokine Kits (Qiagen Inc., Valencia, CA). Briefly, antibodies that are highly specific for individual cytokines are immobilized on beads with different bead color codes and added to samples. Bead-bound cytokines are detected using a mixture of biotinylated cytokine-specific monoclonal antibodies and Streptavidin-PE. Fluorescence signals from the bead color-coding dyes and the reporter molecules attached to detection reagents are recorded (LiquiChip Workstation; Qiagen Inc.) and digitally processed to translate the signals into quantitative data for each reaction.

Neutropenia by cyclophosphamide. To examine the importance of neutrophils, TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice were given 150 mg/kg of cyclophosphamide intraperitoneally in 0.2 ml of phosphate-buffered saline 4 days and 1 day before the experiment (23). Additional wild-type mice, which served as control subjects, received 0.2 ml of phosphate-buffered saline intraperitoneally at the same time points. The effects of cyclophosphamide treatment on neutrophil numbers were confirmed before the administration of bacteria by phosphate-buffered eosin and thiazine staining (Harleco; EM Science, Gibbstown, NJ) using blood withdrawn from the tail of each mouse. The neutrophil count was more than 97% reduced in cyclophosphamide-treated mice compared with control mice.

Statistical Analysis

An analysis of variance followed by Tukey-Kramer test was used for statistical analysis of the data. Survival analysis was performed using the log rank test, and survival is reported in the form of a Kaplan Meier curve. Differences were considered statistically significant at $p < 0.05$. Data are presented as mean $\pm$ SE.

RESULTS

In Vitro Direct Bacteria-TNF- α Interaction

Recombinant mouse TNF- α enhanced *E. coli* growth in a concentration-dependent manner up to a concentration of 1 ng/ml (Figure 1A). Compared with the control subjects, *E. coli* growth was 13.7- and 15-fold higher at concentrations of TNF- α of 0.5 and 1 ng/ml, respectively. At 1 ng/ml TNF- α , the addition of various concentrations of monoclonal anti-mouse TNF- α antibody gradually attenuated *E. coli* growth, whereas negative control IgG had no

Figure 1. Eight-hour proliferation of *E. coli* (A) and *P. aeruginosa* (C) in antibiotic-free bicarbonate-buffered culture medium in response to different concentrations of recombinant mouse tumor necrosis factor- α (rmTNF- α). A significant increase in *E. coli* growth compared with zero control (without rmTNF- α) was observed with TNF- α at 0.5 ng/ml, and a maximal response was observed at 1 ng/ml. Eight-hour proliferation of *E. coli* (B) and *P. aeruginosa* (D) in response to different concentrations of monoclonal anti-mouse TNF- α antibody in the presence of rmTNF- α (1 ng/ml). Anti-TNF- α antibody at 20 μ g/ml significantly attenuated *E. coli* proliferation, whereas incubation with a negative control (IgG) had no effect (B). There was no difference in *P. aeruginosa* growth under the same concentrations of TNF- α stimulation (C) and the presence of the antibody (D) ($n = 9$ per group; $*p < 0.05$ versus zero control).

effect (Figure 1B). In contrast to *E. coli*, TNF- α had no effect on *P. aeruginosa* growth at any concentration (Figure 1C), nor did the anti-TNF- α antibody (Figure 1D).

In Vivo E. Coli and P. Aeruginosa Survival of TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ Mice

Because we had observed that bacterial proliferation was greater in the presence of TNF- α , we examined the mortality rate in an *in vivo* model of lung infection in both TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice after intranasal inoculation with various concentrations (10^5 – 10^9 CFU) of *E. coli* or *P. aeruginosa*.

After infection with 10^9 CFU *E. coli* ($n = 6$), all TNF- $\alpha^{-/-}$ mice died by 24 hours, whereas 50% of TNF- $\alpha^{+/+}$ mice survived ($p < 0.05$; Figure 2A). Similarly, after an inoculation with 10^8 CFU *E. coli*, the survival rate was 33% in TNF- $\alpha^{-/-}$ mice compared with 100% in TNF- $\alpha^{+/+}$ mice (Figure 2B). All animals survived for 24 hours after inoculation with 10^7 or 10^6 CFU *E. coli*.

After inoculation with 10^9 or 10^8 CFU of *P. aeruginosa*, all TNF- $\alpha^{-/-}$ mice died by 24 hours, whereas 33% and 50% of the TNF- $\alpha^{+/+}$ mice survived, respectively (both $p < 0.05$; Figures 3A and 3B). After inoculation with 10^7 CFU of *P. aeruginosa*, 50% of the TNF- $\alpha^{-/-}$ mice and all of the TNF- $\alpha^{+/+}$ mice survived to 24 hours ($p = 0.055$; Figure 3C). All animals survived for 24 hours after inoculation with 10^6 CFU of *P. aeruginosa*.

In Vivo Bacterial Clearance during Pneumonia

The lower survival rate observed in TNF- $\alpha^{-/-}$ mice was associated with inefficient bacterial clearance by various organs. Bacterial counts in the lung homogenate, lung lavage, and spleen were similar in TNF- $\alpha^{-/-}$ and TNF- $\alpha^{+/+}$ mice 8 hours after inoculation with *E. coli* (Figure 4A); however, it was significantly elevated in TNF- $\alpha^{-/-}$ mice compared with TNF- $\alpha^{+/+}$ mice at 24 hours ($p < 0.05$ both in lung and spleen). The CFU obtained from lung lavage fluid was proportionally less than the CFU obtained from lung homogenate, suggesting that the lavage procedure had no effect on CFU remaining in the lungs.

The bacterial counts of *P. aeruginosa* in the lung homogenate, lung lavage, and spleen were higher in TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice at both 8 and 24 hours after inoculation (Figure 4B).

In Vivo Recruitment of Neutrophils during Pneumonia

We further investigated whether the less efficient bacterial clearance seen in TNF- $\alpha^{-/-}$ mice was associated with the degree of neutrophil recruitment to the lung. Lung lavage fluids were analyzed at 8 and 24 hours after inoculation with bacteria. Neu-

trophil counts were lower in the lungs of TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice after inoculation with the bacteria (Figures 5A and 5B).

Figure 6 illustrates a light microscopic analysis of lung histology (upper panels) and lung lavage cells (lower panels) stained with phosphate-buffered eosin and thiazine from a TNF- $\alpha^{+/+}$ mouse (left panel) and a TNF- $\alpha^{-/-}$ mouse (right panel) at 24 hours after intranasal inoculation with *E. coli*, indicating that depressed neutrophil migration was associated with a greater bacterial load and more severe hemorrhage in TNF- $\alpha^{-/-}$ mice compared with TNF- $\alpha^{+/+}$ mice.

In Vivo Acute MIP-2 Response during Pneumonia

Neutrophil migration into the lung is related to local production of chemotactic factors, including IL-8, a member of the CXC chemokine family. To investigate chemotactic activity in the lungs of TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice, the concentrations of MIP-2, a rodent homologue of human IL-8, were determined. Acute MIP-2 responses in the lung were lower in TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice at 8 hours ($p < 0.05$). The difference became insignificant 24 hours after *E. coli* infection (Figure 7A). Acute MIP-2 responses in plasma were significantly lower in the TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice 8 hours after infection; however, it was higher in TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice 24 hours after infection (both $p < 0.05$; Figure 7B).

Figure 7C shows that the MIP-2 concentration in lung lavage fluid was lower in TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice at 8 hours after *P. aeruginosa* infection ($p < 0.05$) but was significantly higher at 24 hours ($p < 0.05$). Plasma MIP-2 concentrations were somewhat lower in TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice at 8 hours after infection ($p = \text{NS}$) and were significantly higher at 24 hours ($p < 0.05$; Figure 7D).

Other In Vivo Cytokine Profiles in Responses to Bacterial Pneumonia in TNF- $\alpha^{-/-}$ Mice

To investigate possible compensatory mechanisms elaborating higher levels of other cytokines in response to bacterial inoculation in TNF- $\alpha^{-/-}$ mice, concentrations of 10 additional cytokines, other than MIP-2, were simultaneously measured in lung lavage fluids 24 hours after inoculation with 10^6 CFU of *E. coli* or *P. aeruginosa*.

Figure 8 shows that the concentration of IL-6 was higher in the TNF- $\alpha^{+/+}$ mice and lower in the TNF- $\alpha^{-/-}$ mice. In contrast, the levels of IL-1 β and GM-CSF were significantly lower in the TNF- $\alpha^{+/+}$ mice than in the TNF- $\alpha^{-/-}$ mice, suggesting a

Figure 2. Effect of TNF- α on 24-hour survival of mice after inoculation with *E. coli*. TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice were inoculated intranasally with various concentrations ($n = 6$ per concentration) of *E. coli*. After inoculation with 10^9 CFU of *E. coli*, all of the TNF- $\alpha^{-/-}$ mice died, whereas 50% of the TNF- $\alpha^{+/+}$ mice survived (A). Similarly, after inoculation with 10^8 CFU of *E. coli*, the survival rate was 33% in TNF- $\alpha^{-/-}$ mice, and all TNF- $\alpha^{+/+}$ mice survived for 24 hours after inoculation with 10^7 and 10^6 CFU *E. coli*.

Figure 3. Effect of TNF- α on 24-hour survival of mice after inoculation with *P. aeruginosa*. TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice were inoculated intranasally with various concentrations ($n = 6$ per concentration) of *P. aeruginosa*. After inoculation with 10^9 CFU *P. aeruginosa*, all TNF- $\alpha^{-/-}$ mice died, whereas 33% of TNF- $\alpha^{+/+}$ mice survived (A). After an inoculation with 10^8 CFU of *P. aeruginosa*, none of TNF- $\alpha^{-/-}$ mice survived, whereas 50% of TNF- $\alpha^{+/+}$ mice lived (B). After inoculation with 10^7 CFU of *P. aeruginosa*, 50% of the TNF- $\alpha^{-/-}$ mice and all the TNF- $\alpha^{+/+}$ mice survived (C). All animals survived for 24 hours after inoculation with 10^6 CFU of *P. aeruginosa*.

compensatory mechanism by producing larger amounts of these two cytokines in the TNF- α -deficient mice.

Opsonin Receptor CD11b and CD16/CD32 Expression on Bronchoalveolar Lavage Neutrophils

Because we observed dramatic divergent effects of TNF- α in modulating *E. coli* survival between *in vitro* and *in vivo* conditions where neutrophils appeared to play a crucial role, we examined the expression of opsonin receptors that may have also affected phagocytic capacity in those neutrophils recovered from the bronchoalveolar lavage. Lung lavage neutrophils demonstrated depressed expression of CD11b (Figures 9A and 9B) and CD16/CD32 (Figures 9C and 9D) in TNF- $\alpha^{-/-}$ mice compared with TNF- $\alpha^{+/+}$ mice at 8 and 24 hours after *E. coli* or *P. aeruginosa* infection.

Elimination of Neutrophils Induces Death after *E. coli* and *P. aeruginosa* Inoculation at a Concentration That Was Otherwise Nonlethal in TNF- $\alpha^{-/-}$ and TNF- $\alpha^{+/+}$ Mice

Because the ability of TNF- α to recruit and activate neutrophils appeared to outweigh its ability to stimulate growth of bacteria, we investigated whether the growth-promoting effect observed *in vitro* could be demonstrated in neutropenic animals. To examine this hypothesis, mice were made neutropenic by cyclophosphamide treatment. None of the neutropenic mice survived for 24 hours after intranasal inoculation of 10^6 CFU *E. coli* or

P. aeruginosa in both TNF- $\alpha^{-/-}$ and TNF- $\alpha^{+/+}$ mice (Figures 10A and 10B), whereas all mice without neutrophil depletion survived for 24 hours after the same concentration of bacterial inoculation (data not shown).

Bacterial counts in the lung homogenate, lung lavage, and spleen were obtained from TNF- $\alpha^{-/-}$ and TNF- $\alpha^{+/+}$ mice that were given intranasal inoculation of 10^6 CFU of bacteria and sacrificed 12 hours later. Consistent with *in vitro* and *ex vivo* studies, the number of *E. coli* in the lung homogenate and lung lavage was lower in TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice ($p < 0.05$; Figure 10C), whereas the *P. aeruginosa* counts were similar in lung homogenate, lung lavage, and spleen of TNF- $\alpha^{-/-}$ and TNF- $\alpha^{+/+}$ mice (Figure 10D).

DISCUSSION

Recent studies have suggested that prokaryotic cells may use cytokines as growth factors (1–3, 5, 24). The clinical relevance of these findings, however, is uncertain because in immune competent animals, the ability of TNF- α to recruit phagocytic neutrophils clearly outweighs any growth-promoting effect that might have occurred. In neutropenic animals, the growth-promoting effect of TNF- α could be demonstrated *in vivo* but was not strong enough to affect survival as shown.

The growth-promoting effect of TNF- α on *E. coli* appeared to be highly specific, as it occurred in a concentration-dependent

Figure 4. Bacterial counts in the lung and spleen of TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice. Mice were challenged intranasally with 1×10^6 CFU of *E. coli* (A) or *P. aeruginosa* (B) and then sacrificed after 8 and 24 hours ($n = 6$ each). * $p < 0.05$ versus the same organ between groups at the given time.

Figure 5. Neutrophil transmigration into the lungs of TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice at 8 and 24 hours ($n = 6$ each) after intranasal inoculation of 1×10^6 CFU of *E. coli* (A) and *P. aeruginosa* (B). A portion of the neutrophils recovered from lung lavage fluids was stained with phosphate-buffered eosin and thiazine after cytocentrifuge and was counted under microscopy. * $p < 0.05$ between TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice at given times; † $p < 0.05$ versus 8 hours within groups. BAL = bronchoalveolar lavage; PMN = polymorphonuclear leukocyte.

manner even at subnanomolar TNF- α concentrations and was blocked by anti-TNF- α antibodies. TNF- α appeared to have different effects on *E. coli* growth. At low concentrations of TNF- α , *E. coli* growth was increased. At intermediate levels, it was decreased, and at very high concentrations, growth was

enhanced again. This concept was supported by the quantitative measurements of TNF- α *in vitro* as well as *in vivo* conditions where neutrophils were depleted.

The same *E. coli* growth-promoting effect was not observed with *P. aeruginosa*. These findings raise the possibility that *E. coli*

Figure 6. Light microscopic analysis of lung histology (upper panels, A and B, $\times 40$; C and D, $\times 60$) and lung lavage cells (lower panels, E and F, $\times 100$) stained with phosphate-buffered eosin and thiazine from a TNF- $\alpha^{+/+}$ mouse (left panels) and a TNF- $\alpha^{-/-}$ mouse (right panels) 24 hours after intranasal inoculation with *E. coli*. Depressed neutrophil migration was associated with a greater bacterial load and a more severe hemorrhage in TNF- $\alpha^{-/-}$ mice compared with TNF- $\alpha^{+/+}$ mice. The arrows point to migrated neutrophils.

Figure 7. Macrophage inflammatory protein (MIP)-2 levels in lung lavage fluids and plasma after inoculation of *E. coli* or *P. aeruginosa* in TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice. MIP-2 concentrations were lower in TNF- $\alpha^{-/-}$ mice than in TNF- $\alpha^{+/+}$ mice ($n = 6$ per group; * $p < 0.05$ between TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice at given times).

may express an as yet unidentified receptor for TNF- α . A number of studies have reported the ability of cytokines to bind to bacteria, and this was frequently associated with altered bacterial growth (1–3, 5, 24–27). For example, Luo and colleagues (25) reported that TNF- α can bind to *E. coli*, *Salmonella typhimurium*, and *Shigella flexneri*. Porat and colleagues (5) have shown that IL-1 β , IL-2, and GM-CSF not only bind to *E. coli* but also stimulate its growth. These authors also observed that the growth-promoting effect of TNF- α is lost at higher concentrations (5).

The effect of recombinant TNF- α on *E. coli* growth had previously been studied only *in vitro* under conditions quite different from that *in vivo*. One important difference between *in vitro* and *in vivo* is that the former model cannot recruit leukocytes, which play a critical role in host defense as well as in the development of inflammation. We therefore investigated bacterial growth in live animals. Although TNF- α has effects independent from leukocyte activation such as angiogenesis and cachexia, our findings underscore the critical role of neutrophils for effective bacterial clearance by TNF- α *in vivo* and demonstrate that host response surpasses any effect on bacterial growth that TNF- α exerts. In inflammation, the main role of TNF- α appears to be the orchestration of neutrophils by stimulating all relevant steps in the recruitment and activation of these leukocytes (15), that is, expression of adhesion molecules on endothelial cells and neutrophils, stimulation of chemokine production (e.g., MIP-2), and activation of the neutrophils themselves (e.g., CD11 and CD16/CD32). Although a plethora of evidence exists for each single step, to our knowledge, the critical role of leukocytes for the successful clearance of bacterial infections by TNF- α has not been directly demonstrated with the help of neutropenic animals. In the only similar study known to us, Netea and colleagues (28) showed an increased susceptibility to systemic fungal infection (candidiasis) through impaired

Figure 8. Concentrations of cytokines measured simultaneously from lung lavage fluids 24 hours after intranasal inoculation of *E. coli* (upper panel, $n = 6$) or *P. aeruginosa* (lower panel, $n = 6$). * $p < 0.05$ between TNF- $\alpha^{+/+}$ and TNF- $\alpha^{-/-}$ mice for the given cytokine.

recruitment and activation of neutrophils in TNF- α and lymphotoxin- α double-knockout mice.

Our study demonstrates that insufficient neutrophil accumulation results in higher bacterial loads and mortality in TNF- $\alpha^{-/-}$ compared with TNF- $\alpha^{+/+}$ mice. This is consistent with the observation that the peak time for MIP-2, a major chemokine attracting neutrophils, was delayed in TNF- $\alpha^{-/-}$ mice and that in these mice opsonin receptor expression of CD11b and CD32/CD16 on neutrophils was also depressed. The administration of IL-1 β (10, 29) or GM-CSF (30) has been reported to be associated with an accumulation of neutrophils in the infected lung. Although a higher level of IL-1 β and GM-CSF was observed perhaps as a compensatory mechanism in the TNF- $\alpha^{-/-}$ mice, this increase in endogenous IL-1 β and GM-CSF was unable to restore neutrophil transmigration in the TNF- $\alpha^{-/-}$ mice. Furthermore, elimination of neutrophils resulted in early death in both TNF- $\alpha^{-/-}$ and TNF- $\alpha^{+/+}$ mice. These results indicate that the lack of neutrophil recruitment was the cause for the inability of TNF- $\alpha^{-/-}$ mice to control the bacterial infection.

Our results obtained from the TNF- $\alpha^{-/-}$ mice were consistent with those reported using an anti-TNF- α monoclonal antibody during pneumococcal pneumonia in mice (7). The previous study demonstrated that treatment with an anti-TNF- α mAb 2 hours before inoculation resulted in a fourfold increase in *Streptococcus pneumoniae* CFU isolated from lungs. Anti-TNF- α -treated mice died significantly earlier from pneumococcal pneumonia than control mice, suggesting that endogenously produced TNF- α is important for host defense during pneumonia. A recent study demonstrated that lymphocyte proliferation in response to blood monocytes and alveolar macrophages infected with *Histoplasma capsulatum* was inhibited by treatment with the anti-TNF- α antibody infliximab, whereas control antibody had no effect. This study suggests that patients receiving anti-TNF- α therapy maybe at risk for developing disseminated histoplasmosis because of immunosuppressive effects (31).

However, the results of using soluble inhibitors against TNF- α are not always clear from prior studies, suggesting that different inhibitors may provide different results (32–37). Several studies reported that soluble inhibitors of TNF- α do not affect neutrophil

Figure 9. Mean fluorescence intensity of the neutrophil opsonin receptors CD11b and CD16/CD32 in $TNF-\alpha^{+/+}$ mice and $TNF-\alpha^{-/-}$ mice at 8 and 24 hours ($n = 6$ each) after intranasal inoculation of *E. coli*. (A and C) and *P. aeruginosa* (B and D). Neutrophils were recovered from lung lavage fluid, incubated with relevant monoclonal antibodies, and fixed. Fluorescence intensity was determined by a flow cytometer. * $p < 0.05$ between $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ mice at the given time.

accumulation during 5 hours of pneumonia induced by instillation of IL-1 α (32), during 4 hours of IgG immune complex pneumonia (33), or during 48 hours of *Klebsiella pneumoniae* pneumonia (34). Others showed that soluble inhibitors compromise emigration during 3 or 6 hours of LPS pneumonia (35, 36), and the effects on neutrophil emigration observed at 6 hours were not observed at 2, 4, or 12 hours (35). In contrast to the $TNF-\alpha$ gene-deficient mice, TNF receptor-deficient mice lacking both p55 and p75 receptors for $TNF-\alpha$ might show different responses to *E. coli* challenge. Mizgerd and colleagues reported that the acute neutrophil emigration was not compromised and was actually increased in TNF receptor-deficient mice compared with wild-type mice at 6 hours after *E. coli* pneumonia (9). The enhanced neutrophil sequestration may reflect a decreased loss of transmigrated neutrophils due to apoptosis as $TNF-\alpha$ receptors p55 and p75 are required in provoking the progress of a subset of neutrophils to apoptosis (38). However, the TNF receptor-deficient mice also showed high mortality and impaired bacterial clearance.

The reduced expression of opsonin receptors on $TNF-\alpha^{-/-}$ neutrophils indicates impaired bacterial phagocytosis, as was directly demonstrated for *Candida albicans* in $TNF-\alpha$ and lymphotoxin- α double-knockout mice (28). Fc receptors such as CD16/CD32 act as a bridge between humoral and cellular responses in the immune system and are one of the pivotal elements for maintaining peripheral tolerance. Fc receptors mediate various effects on immune cells, including proliferation of B cells and phagocytosis by phagocytes (39, 40). Engagement of activated CD16/CD32 triggers phagocytosis and simultaneously downregulates inflammatory responses (39, 40). Hostoffer and colleagues (41) demonstrated that exposure of neutrophils to $TNF-\alpha$ resulted in a twofold to threefold increase in surface expression of Fc α R (CD89), a fivefold elevation of superoxide production, and a threefold to fourfold enhancement of phagocytosis in response to aggre-

Figure 10. Effect of neutropenia on 24-hour survival of mice after intranasal inoculation of bacteria (A and B). (Upper panel) $TNF-\alpha^{+/+}$ ($n = 6$) and $TNF-\alpha^{-/-}$ ($n = 6$) mice were inoculated with 10^6 CFU *E. coli*, or *P. aeruginosa*. None of the mice survived to 24 hours. Note that all $TNF-\alpha^{+/+}$ ($n = 6$) and $TNF-\alpha^{-/-}$ ($n = 6$) mice without neutrophil depletion survived for 24 hours after inoculation with the same concentration of the bacteria (as mentioned in the Results). (Lower panel) Bacterial counts in the lung homogenate, lavage fluid, and spleen of $TNF-\alpha^{+/+}$ mice ($n = 3$) and $TNF-\alpha^{-/-}$ ($n = 3$) mice (C and D). Mice were challenged intranasally with 10^6 CFU *E. coli*. (C) or *P. aeruginosa* (D) and then sacrificed at 12 hours. * $p < 0.05$ between $TNF-\alpha^{+/+}$ and $TNF-\alpha^{-/-}$ mice for lung bacterial count.

gated IgA. Thus, an imbalance between stimulatory and inhibitory Fc receptors might have taken place in $TNF-\alpha^{-/-}$ mice in response to the bacterial invasion, resulting in severe lung injury and systemic bacteremia.

To investigate further the role of neutrophils in the interaction between $TNF-\alpha$ and bacteria in *in vivo* situations, we investigated bacterial growth in leukopenic animals. In line with our expectations, *E. coli* numbers were approximately 100-fold higher in the lungs of $TNF-\alpha^{+/+}$ mice as compared with $TNF-\alpha^{-/-}$ mice. These findings could be relevant with respect to the development of pneumonia in neutropenic patients, for example, after chemotherapy. However, the higher bacterial burden was not reflected by an increased mortality. This discrepancy might be explained by a number of factors, including similar bacterial load in other organs such as the spleen, the severity of the model, or other leukocyte-independent actions of $TNF-\alpha$. In any case, our findings indicate that bacteria can use $TNF-\alpha$ as a growth factor also *in vivo*.

The fact that bacteria have acquired the ability to respond to $TNF-\alpha$ suggests that this must bear some fitness advantage for the bacteria. The fitness of a pathogen can be defined as its ability to multiply within a host and disseminate from that host, translocate to a new host, and colonize and/or cause infection. Being able to adapt to a cytokine rich milieu may give the organism, in this case *E. coli*, a fitness advantage over its competitors. Strains of *E. coli* are known to be able to enter a transient hypermutable state that allows them to acquire new genetic variations at times critical for survival and colonization of new hosts (42). Therefore,

one may speculate that this is an adaptation to the cytokine milieu found in some organ systems such as the gut where the concentration of TNF- α was higher than that in systemic circulation in patients undergoing abdominal surgery (43, 44).

In conclusion, *E. coli* can use TNF- α as a growth factor *in vitro* and *in vivo*. However, the effect of TNF- α on *E. coli* growth is surpassed early in the course of lung infection by neutrophil recruitment and activation. Further studies are required to determine the clinical relevance of the growth-promoting effect of TNF- α on *E. coli* especially in relationship to neutropenic patients.

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References

1. Denis M, Campbell D, Gregg EO. Interleukin-2 and granulocyte-macrophage colony-stimulating factor stimulate growth of a virulent strain of *Escherichia coli*. *Infect Immun* 1991;59:1853-1856.
2. Kanangat S, Meduri GU, Tolley EA, Patterson DR, Meduri CU, Pak C, Griffin JP, Bronze MS, Schaberg DR. Effects of cytokines and endotoxin on the intracellular growth of bacteria. *Infect Immun* 1999; 67:2834-2840.
3. Meduri GU, Kanangat S, Stefan J, Tolley E, Schaberg D. Cytokines IL-1 β , IL-6, and TNF- α enhance *in vitro* growth of bacteria. *Am J Respir Crit Care Med* 1999;160:961-967.
4. Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. Inflammatory cytokines in the BAL of patients with ARDS: persistent elevation over time predicts poor outcome. *Chest* 1995;108:1303-1314.
5. Porat R, Clark BD, Wolff SM, Dinarello CA. Enhancement of growth of virulent strains of *Escherichia coli* by interleukin-1. *Science* 1991;254: 430-432.
6. Takashima K, Tateda K, Matsumoto T, Iizawa Y, Nakao M, Yamaguchi K. Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice. *Infect Immun* 1997;65:257-260.
7. Van der Poll T, Keogh CV, Buurman WA, Lowry SF. Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice. *Am J Respir Crit Care Med* 1997; 155:603-608.
8. Laichalk LL, Kunkel SL, Strieter RM, Danforth JM, Bailie MB, Standiford TJ. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella pneumoniae*. *Infect Immun* 1996;64:5211-5218.
9. Mizgerd JP, Peschon JJ, Doerschuk CM. Roles of tumor necrosis factor receptor signaling during murine *Escherichia coli* pneumonia. *Am J Respir Cell Mol Biol* 2000;22:85-91.
10. Mizgerd JP, Spieker MR, Doerschuk CM. Early response cytokines and innate immunity: essential roles for TNF receptor 1 and type I IL-1 receptor during *Escherichia coli* pneumonia in mice. *J Immunol* 2001; 166:4042-4048.
11. Skerrett SJ, Martin TR, Chi EY, Peschon JJ, Mohler KM, Wilson CB. Role of the type 1 TNF receptor in lung inflammation after inhalation of endotoxin or *Pseudomonas aeruginosa*. *Am J Physiol* 1999;276: L715-L727.
12. Matthay MA, Uchida T, Fang X. Clinical acute lung injury and acute respiratory distress syndrome. *Curr Treat Options Cardiovasc Med* 2002; 4:139-149.
13. Hoiby N, Krogh Johansen H, Moser C, Song Z, Ciofu O, Kharazmi A. *Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth. *Microbes Infect* 2001;3:23-35.
14. Strieter RM, Kunkel SL. Acute lung injury: the role of cytokines in the elicitation of neutrophils. *J Invest Med* 1994;42:640-651.
15. Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. *J Immunol* 2002;168:4620-4627.
16. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 1991;66: 233-243.
17. Armitage RJ. Tumor necrosis factor receptor superfamily members and their ligands. *Curr Opin Immunol* 1994;6:407-413.
18. Camerini D, Walz G, Loenen WA, Borst J, Seed B. The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. *J Immunol* 1991;147:3165-3169.
19. Kwon BS, Wang S, Udagawa N, Haridas V, Lee ZH, Kim KK, Oh KO, Greene J, Li Y, Su J, et al. TR1, a new member of the tumor necrosis factor receptor superfamily, induces fibroblast proliferation and inhibits osteoclastogenesis and bone resorption. *FASEB J* 1998;12:845-854.
20. Tufvesson E, Westergren-Thorsson G. Tumour necrosis factor-alpha interacts with biglycan and decorin. *FEBS Lett* 2002;23:124-128.
21. Marino MW, Dunn A, Grahl D, Inglesc M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, et al. Characterization of tumor necrosis factor-deficient mice. *Proc Natl Acad Sci USA* 1997; 94:8093-8098.
22. Redford P, Welch RA. Extraintestinal *Escherichia coli* as a model system for the study of pathogenicity islands. *Curr Top Microbiol Immunol* 2002;264:15-30.
23. Abraham E, Carmody A, Shenkar R, Arcaroli J. Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1137-L1145.
24. Kanangat S, Bronze MS, Meduri GU, Postlethwaite A, Stentz F, Tolley E, Schaberg D. Enhanced extracellular growth of *Staphylococcus aureus* in the presence of selected linear peptide fragments of human interleukin(IL)-1 β and IL-1 receptor antagonist. *J Infect Dis* 2001; 183:65-69.
25. Luo G, Niesel DW, Shaban RA, Grimm EA, Klimpel GR. Tumor necrosis factor alpha binding to bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties. *Infect Immun* 1993;61: 830-835.
26. Bermudez LE, Petrofsky M, Shelton K. Epidermal growth factor-binding protein in mycobacterium avium and mycobacterium tuberculosis: a possible role in the mechanism of infection. *Infect Immun* 1996;64: 2917-2922.
27. Hirsch CS, Yoneda T, Averill L, Ellner JJ, Toossi Z. Enhancement of intracellular growth of mycobacterium tuberculosis in human monocytes by transforming growth factor-beta 1. *J Infect Dis* 1994;170:1229-1237.
28. Netea MG, Van Tits LJ, Curfs JH, Amiot F, Meis JF, Van der Meer JW, Kullberg BJ. Increased susceptibility of TNF- α lymphotoxin- α double knockout mice to systemic candidiasis through impaired recruitment of neutrophils and phagocytosis of *Candida albicans*. *J Immunol* 1999;163:1498-1505.
29. Hybertson BM, Jepson EK, Clarke JH, Spelts RJ, Repine JB. Interleukin-1 stimulates rapid release of cytokine-induced neutrophil chemoattractant (CINC) in rat lungs. *Inflammation* 1996;20:471-483.
30. Wollin L, Uhlig S, Nusing R, Wendel A. Granulocyte-macrophage colony-stimulating factor amplifies lipopolysaccharide-induced bronchoconstriction by a neutrophil- and cyclooxygenase 2-dependent mechanism. *Am J Respir Crit Care Med* 2001;163:443-450.
31. Wood KL, Hage CA, Knox KS, Kleiman MB, Sannuti A, Day RB, Wheat LJ, Twigg HL III. Histoplasmosis after treatment with anti-tumor necrosis factor-alpha therapy. *Am J Respir Crit Care Med* 2003;167: 1279-1282.
32. Hybertson BM, Jepson EK, Cho OJ, Clarke JH, Lee YM, Repine JE. TNF mediates lung leak, but not neutrophil accumulation, in lungs of rats given IL-1 intratracheally. *Am J Respir Crit Care Med* 1997;155: 1972-1976.
33. Mulligan MS, Ward PA. Immune complex-induced lung and dermal vascular injury: differing requirements for tumor necrosis factor-alpha and IL-1. *J Immunol* 1992;149:331-339.
34. Laichalk LL, Kunkel SL, Strieter RM, Danforth JM, Bailie MB, Standiford TJ. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella pneumoniae*. *Infect Immun* 1996;12:5211-5218.
35. Ulich TR, Yin S, Remick DG, Russell D, Eisenberg SP, Kohno T. Intratracheal administration of endotoxin and cytokines. IV: the soluble tumor necrosis factor receptor type I inhibits acute inflammation. *Am J Pathol* 1993;142:1335-1338.
36. Kolls JK, Lei D, Nelson S, Summer WR, Greenberg S, Beutler B. Adenovirus-mediated blockade of tumor necrosis factor in mice protects against endotoxic shock yet impairs pulmonary host defense. *J Infect Dis* 1995;171:570-575.
37. Ulich TR, Yi ES, Yin S, Smith C, Remick D. Intratracheal administration of endotoxin and cytokines. VII: the soluble interleukin-1 receptor and the soluble tumor necrosis factor receptor II (p80) inhibit acute inflammation. *Clin Immunol Immunopathol* 1994;72:137-140.
38. Murray J, Barbara JA, Dunkley SA, Lopez AF, Van Ostade X, Condliffe

- AM, Dransfield I, Haslett C, Chilvers ER. Regulation of neutrophil apoptosis by tumor necrosis factor- α : requirement for TNFR55 and TNFR75 for induction of apoptosis *in vitro*. *Blood* 1997;90:2772-2783.
39. Takai T. Roles of Fc receptors in autoimmunity. *Nat Rev Immunol* 2002; 2:580-592.
40. Amigorena S, Bonnerot C. Fc receptor signaling and trafficking: a connection for antigen processing. *Immunol Rev* 1999;172:279-284.
41. Hostoffer RW, Krukavets I, Berger M. Enhancement by tumor necrosis factor- α of Fc α receptor expression and IgA-mediated superoxide generation and killing of *Pseudomonas aeruginosa* by polymorphonuclear leukocytes. *J Infect Dis* 1994;170:82-87.
42. LeClerc JE, Li B, Payne WL, Cebula TA. High mutation frequencies among *Escherichia coli* and *Salmonella* pathogens. *Science* 1996;274: 1208-1211.
43. Carlet J, Tamion F, Cabie A. Intestinal cytokines. Rombeau JL, Takala J, editors. In: Gut dysfunction in critical illness. Berlin Heidelberg, Germany: Springer-Verlag; 1996. p. 177-88.
44. Cabie A, Farkas JC, Fitting C, Laurian C, Cormier JM, Carlet J, Cavaillon JM. High levels of portal TNF- α during abdominal aortic surgery in man. *Cytokine* 1993;5:448-453.