Role of the type 1 TNF receptor in lung inflammation after inhalation of endotoxin or Pseudomonas aeruginosa

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Skerrett, Shawn J., Thomas R. Martin, Emil Y. Chi, Jacques J. Peschon, Kendall M. Mohler, and Christopher B. Wilson. Role of the type 1 TNF receptor in lung inflammation after inhalation of endotoxin or Pseudomonas aeruginosa. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L715–L727, 1999.—To determine the roles of the type 1 tumor necrosis factor (TNF) receptor (TNFR1) in lung inflammation and antibacterial defense, we exposed transgenic mice lacking TNFR1 (TNFR1(−/−)) and wild-type control mice to aerosolized lipopolysaccharide or Pseudomonas aeruginosa. After LPS, bronchoalveolar lavage fluid (BALF) from TNFR1(−/−) mice contained fewer neutrophils and less macrophage inflammatory protein-2 than BALF from control mice. TNF−α, interleukin-1β, and total protein levels in BALF as well as tissue intercellular adhesion molecule-1 expression did not differ between the two groups. In contrast, lung inflammation and bacterial clearance after infection were augmented in TNFR1(−/−) mice. BALF from infected TNFR1(−/−) mice contained more neutrophils and TNF−α and less interleukin-1β and macrophage inflammatory protein-2 than that from control mice, but protein levels were similarly elevated in both groups. Lung inflammation and bacterial clearance were also augmented in mice lacking both TNF receptors. Thus TNFR1 facilitates neutrophil recruitment after inhalation of lipopolysaccharide, in part by augmenting chemokine induction. In contrast, TNFR1 attenuates lung inflammation in response to live bacteria but does not contribute to increased lung permeability and is not required for the elimination of P. aeruginosa.

tumor necrosis factor; pneumonia; lung injury; lipopolysaccharide; cytokines; interleukin-1
rTNF-α but are more susceptible to infection with intracellular bacteria such as Listeria monocytogenes and Mycobacterium tuberculosis (14, 41, 42, 47). TNFR1(−/−) mice also exhibit diminished tissue inflammation in response to systemic or local administration of rTNF-α, a defect associated with diminished upregulation of endothelial adhesion molecules (25, 39). TNFR1(−/−) mice thus provide a focused model in which to test the importance of TNF-α in pulmonary inflammatory responses to different stimuli.

We compared the responses of TNFR1(−/−) and wild-type [TNFR1(+/+) mice to aerosolized LPS and live P. aeruginosa to better define the role of TNF-α in lung inflammation and host defense and to identify the specific roles of TNFR1 in these responses. We hypothesized that TNFR1-deficient animals would have diminished inflammatory responses to LPS and live bacteria and would exhibit defective clearance of P. aeruginosa from the lungs.

MATERIALS AND METHODS

Animals. C57BL/6 TNFR1(−/−) mice and mice lacking both TNFRs [TNFR1(−/−) and TNFR2(−/−)] were bred at the Animal Care Facility of the University of Washington (Seattle, WA) from breeding pairs generated at Immunex Research and Development Corporation (Seattle) (41). Genotypes were confirmed from tail-tip DNA by the polymerase chain reaction with primers specific for the wild-type and disrupted genes for TNFR1 and TNFR2. Specific pathogen-free C57BL/6 wild-type control mice were purchased from Charles River (Wilmington, MA). The animals were housed in sterile microisolator cages in the vivarium of the Seattle Division of the Puget Sound Veterans Affairs Health Care System and permitted free access to sterile food and water. The mice were 7–12 wk of age at the time of study. Male and female animals were used in approximately equal numbers. The experiments were approved by the Animal Studies Committee of the Puget Sound Veterans Affairs Health Care System.

LPS. Escherichia coli O111:B4 LPS was purchased from Sigma (St. Louis, MO), reconstituted to 10 mg/ml in sterile saline for injection, divided into aliquots, and stored at −70°C. The same stock was used for all experiments. For each experiment, an aliquot was thawed, briefly sonicated, and diluted in sterile saline to 10, 33, or 100 µg/ml for injection.

Bacteria. P. aeruginosa PAK was a generous gift from Steve Lory (University of Washington). Several colonies from an agar plate were inoculated into Luria broth (LB) and grown overnight at 37°C in a shaking incubator. The broth was diluted to 30% glycerol, divided into aliquots, and flash-frozen in ethandry-ice before storage at −70°C. For each experiment, an aliquot was thawed, diluted 1:100 in LB, and then incubated for 6 h at 37°C in a shaking incubator. Then 2.5 ml of this suspension was transferred to each of four flasks containing 250 ml of LB, which were incubated for 16 h at 37°C in a shaking incubator. The bacteria then were pelleted, washed twice in phosphate-buffered saline (PBS) containing 10 mM magnesium chloride, and then resuspended in 20 ml of the same buffer.

Exposure to aerosolized LPS or bacteria. Mice were placed in wire mesh cages within a sealed 55-liter Plexiglas chamber connected to a separate aerosol chamber as previously described (53). Airflow through the system was established at 20 l/min by negative pressure. Aerosols were generated by twin jet nebulizers (Marquest Medical Products, Englewood, CO), each containing 8 ml of suspended LPS or bacteria and driven by forced air at 15 psi. Exposures were continued for 30 min.

Processing of tissues after exposure to LPS. In each experiment, 4 and 24 h after exposure to aerosolized LPS, four to six TNFR1(+/+ ) and four to six TNFR1(−/−) mice were anesthetized with intraperitoneal pentobarbital sodium and exanguinated by cardiac puncture. The trachea of each animal was exposed and cannulated with a 22-gauge polyethylene catheter. In some experiments, the lungs were lavaged four times with 1-ml volumes of 0.85% sodium chloride containing 0.6 mM EDTA and prewarmed to 37°C. Bronchoalveolar lavage specimens were centrifuged at 300 g, and the supernatants were aspirated, divided into aliquots, and stored at −70°C. The cell pellets were resuspended in RPMI 1640 medium (BioWhittaker, Walkersville, MD) containing 10% heat-inactivated fetal calf serum (Hydose Laboratories, Logan, UT). Cell counts were performed with a hemocytometer, and differential counts were determined from cytocentrifuged specimens prepared with a modified Wright-Giemsa stain (Diff-Quik, American Scientific Products, McGaw Park, IL). In other experiments, one lung was tied off and the other was inflated to 15 cmH2O pressure with a fixative (10% Formalin in PBS or 4% paraformaldehyde) and then immersed in the same fixative. The fixed tissues were embedded in paraffin, and coronal sections were stained with hematoxylin and eosin.

Processing of tissues after exposure to P. aeruginosa. Immediately after exposure to aerosolized bacteria, four wild-type mice were anesthetized with pentobarbital sodium and exanguinated by cardiac puncture to determine bacterial deposition in each experiment. The left lung and the spleen were harvested, homogenized in PBS-MgC12, serially diluted in the same buffer, and quantitatively cultured by spreading 0.1-ml aliquots on LB agar in duplicate. The colonies were counted after an overnight incubation at 37°C in 5% CO2-humidified air. Bacterial clearance was determined by similarly processing lung and spleen tissues of wild-type and TNFR-deficient mice 4 and 24 h after infection. At the 4- and 24-h time points, the right lung of each mouse was lavaged or fixed in Formalin as described in Processing of tissues after exposure to LPS.

Tissue expression of ICAM-1 by immunohistochemistry. Coronal sections of parafformaldehyde-fixed lung tissue were predigested with 0.01% Pronase in PBS, then blocked with 5% nonfat dry milk in PBS. The sections were incubated with hamster anti-mouse ICAM-1 (Endogen, Woburn, MA) diluted 1:100 in 5% nonfat dry milk in PBS (final concentration of antibody 10.8 µg/ml), hamster IgG (J Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:1,000 in 5% nonfat dry milk in PBS (final concentration of antibody 10 µg/ml), or 5% nonfat dry milk in PBS for 90 min at room temperature. The sections were washed, incubated with biotinylated goat anti-hamster IgG (Jackson ImmunoResearch Laboratories) diluted 1:100 in 5% nonfat dry milk with 1% goat serum for 60 min at room temperature, and then developed with avidin-alkaline phosphatase (Vector Laboratories, Burlingame, CA) before being counterstained with methylene blue.

Measurement of cytokines and total protein in bronchoalveolar lavage fluid supernatants. Immunoreactive TNF-α and interleukin (IL)-1β were measured in sandwich ELISAs, as previously described (54), with mouse-specific antibodies and standards obtained from Genzyme (Cambridge, MA). The C-X-C chemokines macrophage inflammatory protein (MIP)-2 and KC were measured by ELISA with reagents purchased from the manufacturer.

Measurement of total protein in bronchoalveolar lavage fluid supernatants. Protein content was measured in the supernatants obtained from each experiment by the method of Lowry et al. (55). The supernatants were diluted 1:2 in 1% bovine serum albumin in PBS prior to analysis.

Measurement of albumin in bronchoalveolar lavage fluid supernatants. Albumin content was measured in the supernatants obtained from each experiment by the method of Lowry et al. (55). The supernatants were diluted 1:2 in 1% bovine serum albumin in PBS prior to analysis.
from R&D Systems (Minneapolis, MN). TNF-α bioactivity was measured in an L929 cytotoxicity assay as previously described (36). The specificity of the cytopathic effect was confirmed in parallel plates by its abolition in the presence of anti-mouse TNF-α (Genzyme). Total protein concentration was measured with the bicinchoninic acid method (BCA assay, Pierce, Rockford, IL).

Measurement of chemotactic activity. The chemotactic activity for neutrophils that were present in bronchoalveolar lavage fluid (BALF) was measured in microchemotaxis chambers with nitrocellulose filters with 3-µm pores as previously described (34, 36). Peritoneal exudate neutrophils were elicited from wild-type mice by instilling 2 ml of 0.1% oyster glycogen (Sigma) intraperitoneally. Cells were harvested 4 h later by peritoneal lavage with 0.85% saline containing 0.6 mM EDTA, washed twice in Hanks’ balanced salt solution (GIBCO BRL, Grand Island, NY), and suspended to a concentration of 3 × 10⁶/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mM HEPES (BioWhittaker), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (GIBCO BRL). The exudate cells were >95% neutrophils and >90% viable by the exclusion of trypan blue. The bronchoalveolar lavage lavage supernatants were added to the lower wells of 48-well microchemotaxis chambers in triplicate, and the neutrophil suspension was added to the upper well. Control lower wells contained 10% zymosan-activated serum or PBS. Zymosan-activated mouse serum was generated by suspending zymosan particles (Sigma) at 0.5 mg/ml in fresh mouse serum and incubating at 37°C for 1 h, followed by 56°C for 30 min. The particles were pelleted by centrifugation, and the supernatant was used fresh in the chemotaxis assay. The chambers were incubated for 2 h at 37°C in 5% CO₂-humidified air, then the nitrocellulose filters separating the upper and lower wells were removed, stained with Diff-Quik, and mounted on microscope slides. The total number of neutrophils that had migrated through each filter were counted in 10 consecutive high-power fields (×540) with the aid of an eyepiece grid.

Data analysis. Data are expressed as means ± SE. Comparisons between two groups of animals were made with independent two-tailed t-tests. A P value < 0.05 was considered significant.

RESULTS

Leukocyte recruitment after inhalation of LPS. As shown in Fig. 1A, exposure of mice to aerosolized LPS resulted in a dose-related increase in the number of neutrophils present in bronchoalveolar lavage specimens 24 h later. At each of the three concentrations of LPS that were tested, there were significantly fewer neutrophils in the bronchoalveolar lavage specimens in mice lacking TNFR1 than in wild-type control mice. After exposure to aerosolized LPS at 10, 33, or 100 µg/ml, there were 54, 61, and 38% fewer neutrophils, respectively, in the bronchoalveolar lavage specimens of TNFR1(--/-) mice than in samples from control mice. There also was a dose-related increase in mononuclear cells (mainly macrophages) recovered in bronchoalveolar lavage samples in response to aerosolized LPS (Fig. 1B). The number of mononuclear cells in bronchoalveolar lavage samples did not differ between the two groups of animals.

Figure 2 shows the time course of leukocyte recruitment to the lungs after inhalation of LPS (100 µg/ml). In both groups of animals, the peak number of neutrophils was recovered 4 h after exposure to LPS. In the TNFR1(--/-) mice, there were 24, 36, and 38% fewer neutrophils in BALF 4, 12, and 24 h, respectively, after inhalation of LPS. The differences were significant at the latter two time points. In contrast to neutrophils, the peak number of mononuclear cells was recovered 24 h after exposure to LPS. There were significantly more mononuclear cells in the bronchoalveolar lavage samples from TNFR1(--/-) mice 4 h after inhalation of LPS but no differences at the later time points. Thus neutrophil recruitment to the lungs after inhalation of LPS could occur without normal expression of TNFR1, but sustained neutrophilic inflammation in response to LPS was significantly diminished in mice lacking TNFR1. In contrast, early expansion of the bronchoalveolar mononuclear cell population after inhalation of LPS was augmented in mice lacking TNFR1.

Lung histopathology after inhalation of LPS. Histological changes were more evident 4 h after inhalation of endotoxin than after 24 h. At the 4-h time point in TNFR1(+/+) mice, there were patchy alveolar infiltrates composed of mononuclear and polymorpho-
nuclear leukocytes, red blood cells, and an eosinophilic exudate (Fig. 3A). In other areas, there were scattered interstitial and alveolar neutrophils, with mild interstitial thickening. There was intense submucosal neutrophilic infiltration of bronchi and bronchioles, but margination of leukocytes in the pulmonary vasculature was not evident. In TNFR1(−/−) mice, the same pattern of peribronchial neutrophil infiltration was present, and scattered interstitial and alveolar neutrophils could be found, but there were fewer intra-alveolar cells and no interstitial thickening (Fig. 3C). Inflammation was less evident 24 h after endotoxin exposure in both strains of mice (Fig. 3, B and D). In TNFR1(−/−) mice, the cellularity was homogeneous, with occasional interstitial and alveolar neutrophils; peribronchial inflammation was less intense than at the earlier time point. The histological findings 24 h after endotoxin were very similar in the TNFR1(−/−) mice, but peribronchial neutrophil infiltration was less prominent than in the wild-type animals.

Lung tissue expression of ICAM-1 after inhalation of LPS. In sections of lung tissue from wild-type and TNFR1(−/−) mice that were unexposed to LPS, incubation with anti-ICAM-1 yielded generalized immunoperoxidase staining of alveolar septae, weak and inconsistent staining of venular endothelium, and no staining of bronchial epithelium or arteriolar endothelium. A similar pattern of staining was observed in sections of lung tissue harvested 4 or 24 h after inhalation of LPS; no change in the distribution or intensity of ICAM-1 expression in response to inhaled LPS was evident. There were no differences in lung tissue expression of ICAM-1 between the two strains of mice at either time point after exposure to LPS or among unexposed animals. Sections incubated with control immunoglobulin or nonfat milk-PBS were uniformly negative for any immunoperoxidase staining.

Fig. 2. BAL cells in TNFR1(+/+) and TNFR1(−/−) mice at intervals after inhalation of LPS (100 µg/ml). A: total number of PMN harvested by lavage of both lungs from each animal. B: total number of MN harvested by lavage of both lungs from each animal. Data are means ± SE of combined results from 4 separate experiments; n, no. of mice (nos. in parentheses). *P < 0.05 compared with TNFR1(+/+) mice.

Fig. 3. Light micrographs of hematoxylin and eosin-stained lung tissue after inhalation of LPS (×120). Lung tissue was harvested from TNFR1(+/+) (A and B) and TNFR1(−/−) (C and D) mice 4 (A and C) and 24 h (B and D) after exposure to aerosolized LPS (100 µg/ml).
after inhalation of LPS. The chemotactic activity of PMN in BAL fluid (BALF) supernatants harvested from TNFR1(+/+) and TNFR1(−/−) mice after no treatment (NI BALF) or after exposure to aerosolized LPS (100 µg/ml) was present in the BALFs of TNFR1(+/+) mice but was absent in the BALFs of TNFR1(−/−) mice. Chemotactic activity in positive control (10% zymosan-activated serum) was 288 PMN/10 high-power fields (hpf), and chemotactic activity in negative control (PBS) was 3 PMN/hpf. Data are means ± SE; n, no. of mice (nos. in parentheses). *P < 0.05 compared with TNFR1(+/+) mice.

Chemotactic activity and chemokine levels in BALF after inhalation of LPS. As shown in Fig. 4, no increase in chemotactic activity for neutrophils was detectable in the BALF supernatants of wild-type mice at any time after exposure to aerosolized LPS in comparison with the small amount of activity present in normal BALF. However, a significant increase in chemotactic activity was present in the BALFs of TNFR1(−/−) mice harvested 4 and 12 h after exposure to aerosolized endotoxin. By 24 h, the chemotactic activity had returned to background levels. In contrast to total chemotactic activity, chemokine levels in the BALF after inhalation of LPS were lower in TNFR1(−/−) mice than in wild-type control mice. The concentration of immunoreactive MIP-2 in BALF 4 h after inhalation of LPS was 295.5 ± 60.8 pg/ml in TNFR1(−/−) mice (n = 4), but MIP-2 was detectable in lavage fluid from only one of four TNFR1(+/+) mice (29.0 ± 21.0 pg/ml) if the lower limit of the assay of 8 pg/ml is substituted for in the samples with undetectable MIP-2; P = 0.017). Similarly, KC was detectable in the BALF from two of four TNFR1(+/+) mice 4 h after LPS exposure but was undetectable from all four specimens from TNFR1(−/−) mice. Thus the impaired accumulation of bronchoalveolar neutrophils in TNFR1(−/−) mice after inhalation of LPS was associated with diminished production of C-X-C chemokines but increased total chemotactic activity in BALF.

Cytokines in BALF after inhalation of LPS. The concentrations of immunoreactive TNF-α in BALF 4 h after inhalation of LPS were 2,349.1 ± 603.2 and 2,625.6 ± 499.8 pg/ml in the TNFR1(+/+) and TNFR1(−/−) mice, respectively (n = 12/group; not significant). The concentrations of bioactive TNF-α were 16,368.1 ± 5,303.2 and 8,181 ± 4,898.4 pg/ml in the TNFR1(+/+) and TNFR1(−/−) (n = 11) mice, respectively (not significant). Neither immunoreactive nor bioactive TNF-α was detected in any specimen of BALF harvested 12 or 24 h after inhalation of LPS. Immunoreactive IL-1β at levels of 53–76 pg/ml was detectable in three of eight samples of BALF harvested from TNFR1(−/−) mice 4 h after inhalation of LPS but from none of eight samples from TNFR1(+/+) mice. Thus the transient secretion of TNF-α induced by inhalation of LPS was not affected by the absence of TNFR1, but the levels of LPS-induced IL-1β were more often detectable in the TNFR1(−/−) mice.

Protein concentrations in BALF after inhalation of LPS. As shown in Fig. 5, the protein concentrations in bronchoalveolar lavage supernatants did not change significantly after inhalation of endotoxin in either group of mice. Thus the lung inflammation resulting from the inhalation of endotoxin was not associated with a consistent change in permeability detectable by the measurement of total protein.

Inflammatory cell recruitment after inhalation of P. aeruginosa. As shown in Fig. 6A, the inhalation of virulent P. aeruginosa was followed by a brisk influx of neutrophils to the lungs in both groups of mice. Four hours after infection, there were significantly more neutrophils in bronchoalveolar lavage samples from TNFR1(−/−) mice than in the wild-type control mice; after 24 h, the neutrophil accumulation was nearly identical in the two groups. Similarly, there was a marked increase in the number of mononuclear cells in bronchoalveolar lavage samples from infected animals (Fig. 6B). Significantly more mononuclear cells were recovered from the lungs of TNFR1(−/−) mice than from the TNFR1(+/+) control mice at 24-h time points. Thus the accumulation of inflammatory cells in the lungs in response to the inhalation of P. aeruginosa was augmented in animals lacking TNFR1.

Lung histopathology after inhalation of P. aeruginosa. Four hours after infection, there was diffuse inflammation in both TNFR1(−/−) and wild-type animals (Fig. 7). In the TNFR1(+/+) mice, there was a generalized increase in cellularity, with patchy areas of consolidation (Fig. 7A). Interstitial and alveolar infiltration with neutrophils was evident at high power, and red blood cells were present in many alveoli. Subepithelial consolidation (Fig. 7A) was followed by a brisk influx of neutrophils in both groups of mice.
neutrophilic inflammation of the conducting airways was most apparent in the terminal bronchioles. In the TNFR1(-/-) mice, there was diffuse, nearly confluent inflammation (Fig. 7C). Sheets of neutrophils were present through the interstitium and alveolar spaces, with generalized alveolar hemorrhage. Subepithelial and transepithelial infiltration with neutrophils was evident along conducting airways of all sizes. There were prominent perivascular cuffs composed of mononuclear and polymorphonuclear leukocytes that were not apparent in the wild-type animals. Twenty-four hours after infection, the lungs of TNFR1(+/+) mice exhibited diffuse, predominantly neutrophilic inflammation, with focal areas of dense consolidation (Fig. 7B). Peribronchial infiltration with neutrophils remained evident, and small perivasculares cuffs of mixed cellularity could be discerned. In the TNFR1(-/-) mice, the pattern was very similar to that in the wild-type animals, but perivascular cuffs of mononuclear and polymorphonuclear leukocytes were more prominent (Fig. 7D).

Chemotactic activity and chemokine levels in BALF after inhalation of P. aeruginosa. Chemotactic activity for neutrophils was detected in BALF harvested 4 and 24 h after inhalation of P. aeruginosa in both TNFR1(+/+) and TNFR1(-/-) mice (Fig. 8). There was a trend toward more chemotactic activity in the BALF harvested from wild-type mice 4 h after infection, but there were no significant differences between the two groups of animals at either time point. Paralleling the trend in total chemotactic activity, the levels of MIP-2 and KC in BALF 4 h after exposure to P. aeruginosa were approximately twofold higher in TNFR1(+/+) mice than in TNFR1(-/-) animals (Fig. 9), although only the difference in MIP-2 concentration reached significance. No chemokines were detected in the lavage fluid from uninfected animals. Thus the greater number of bronchoalveolar neutrophils in TNFR1-deficient mice 4 h after infection did not result from an increased production of chemotactic factors.

Cytokines in BALF after inhalation of P. aeruginosa. Levels of both TNF-α and IL-1β in BALF were highest...
4 h after infection but remained detectable at 24 h in both groups of mice (Fig. 10). Immunoreactive TNF-α concentrations were significantly higher in TNFR1(−/−) than in wild-type animals 4 h after infection. A similar trend was observed in levels of bioactive TNF-α, which did not reach significance (P = 0.085). In contrast, bronchoalveolar levels of IL-1β were significantly lower in TNFR1(−/−) mice at both time points.

Total protein concentration in BALF after inhalation of P. aeruginosa. As shown in Fig. 11, there was a marked increase in the total protein concentration in BALFs harvested from both groups of animals at both time points after infection with P. aeruginosa. There were no differences between the TNFR1(+/+) and TNFR1(−/−) mice. Thus TNFR1 did not contribute to the protein leak in this model.

Clearance of aerosolized P. aeruginosa from the lungs. Inhalation of P. aeruginosa was followed by a gradual net bacterial clearance by 24 h after infection in both TNFR1(+/+) and TNFR1(−/−) mice (Fig. 12). At the 4-h time point, there were significantly fewer bacteria remaining in the lungs of TNFR1(−/−) mice than in the wild-type control mice. However, by 24 h after infection, both groups had achieved equivalent net clearance.

Thus the early clearance of P. aeruginosa from the lungs was augmented in the absence of TNFR1.

Spleen cultures. There were no differences between TNFR1(−/−) and TNFR1(+/+) mice in the systemic dissemination of infection after inhalation of P. aeruginosa. Four hours after infection, 5 of 12 spleen cultures were positive in TNFR1(−/−) mice vs. 3 of 9 spleen cultures in TNFR1(+/+) mice. After 24 h, 3 of 12 spleen cultures were positive in each group.

Inflammatory cell recruitment, chemokine levels, and bacterial clearance in TNFR1(−/−) and TNFR2(+/−) mice. The unexpected finding of increased lung inflammation and accelerated bacterial clearance in the

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**Fig. 8.** Chemotactic activity for PMN in BALF supernatants harvested from TNFR1(+/+) and TNFR1(−/−) mice after no treatment or after inhalation of live P. aeruginosa (PA). Chemotactic activity was measured in microchemotaxis chambers with peritoneal exudate PMN harvested from normal mice. Chemotactic activity in positive control (10% zymosan-activated serum) was 181.3 PMN/10 hpf, and chemotactic activity in negative control (PBS) was 3.3 PMN/hpf. Data are means ± SE; n = 5 normal control mice and 10/group of infected mice.

**Fig. 9.** Chemokine levels in BALF 4 h after inhalation of P. aeruginosa. Macrophage inflammatory protein (MIP)-2 and KC were measured by ELISA. Data are means ± SE; n = 10 mice for MIP-2 and 5 mice for KC. *P < 0.05 compared with TNFR1(+/+) mice.

**Fig. 10.** Cytokine levels in BALF at intervals after inhalation of P. aeruginosa. Immunoreactive (Immuno) TNF-α (A) and interleukin (IL)-1β (C) were measured by ELISA. Bioactive TNF-α (B) was measured as lysis of L929 cells. Data are means ± SE; n = 5 normal control mice and 10/group of infected mice. *P < 0.05 compared with TNFR1(+/+) mice.
TNFR1(−/−) mice, in contrast to the impaired neutrophil recruitment to inhaled LPS observed in these animals, raised the possibility that the inflammatory response to *P. aeruginosa* might be mediated, in part, through TNFR2. To investigate this possibility, we conducted a single experiment with mice lacking both TNFRs. As shown in Fig. 13, there were significantly fewer bacteria in the lungs of TNFR1(−/−) and TNFR2(−/−) mice 4 and 24 h after infection than in wild-type control mice. The augmented bacterial clearance was associated with a trend toward increased early neutrophil accumulation in the TNFR-deficient mice. Four hours after infection, there were 1.7 ± 0.2 × 10⁶ and 6.9 ± 3.6 × 10⁵ bronchoalveolar neutrophils/lung in wild-type and TNFR1(−/−) and TNFR2(−/−) mice, respectively (n = 3/group). After 24 h, there were 7.4 ± 1.1 × 10⁶ and 7.7 ± 1.5 × 10⁶ neutrophils/lung in the control and TNFR1(−/−) and TNFR2(−/−) mice, respectively (n = 4/group). Despite the robust inflammation, chemokine levels were diminished in the TNFR-deficient mice. The concentrations of MIP-2 in BALF 4 h after infection were 10.2 ± 1.8 and 3.1 ± 1.8 ng/ml in the wild-type and TNFR1(−/−) and TNFR2(−/−) mice, respectively (P = 0.03; n = 4). The levels of KC were 7.4 ± 1.5 and 1.6 ± 0.6 ng/ml in the wild-type and knockout mice, respectively (P = 0.01; n = 4). No chemokines were detected in the lavage fluid from uninfected animals. Thus mice lacking both TNF receptors responded to aerosolized *P. aeruginosa* with the same pattern of accelerated bacterial clearance and increased neutrophil accumulation in the lung despite a blunted chemokine response that was observed in mice lacking only TNFR1.

**DISCUSSION**

The major finding of this study is that the role of TNFR1 in regulating pulmonary inflammation differed with the inciting stimulus. After exposure to aerosolized LPS, there was a persistent reduction in neutrophil recruitment to the air spaces of the lungs in TNFR1-deficient mice, in comparison with wild-type animals, that was associated with depressed chemokine levels in BALF. In contrast, early neutrophil influx and bacterial clearance after exposure to aerosolized *P. aeruginosa* were augmented in mice lacking TNFR1, as well as in mice deficient in both TNFRs, despite diminished bronchoalveolar concentrations of chemokines. These data suggest that TNFR1 facilitates neutrophil lung inflammation in response to inhaled LPS but serves to downregulate acute inflammatory responses to virulent *P. aeruginosa*.

Our observations support a role for TNF-α in promoting lung inflammation in response to inhaled LPS and suggest that this effect is mediated, at least in part, through TNFR1. Several investigators (9, 73) have reported that neutralization of TNF-α reduces lung inflammation after systemic challenge with endotoxin, and there is evidence that neutrophilic infiltration of the lungs in response to systemic TNF-α requires TNFR1 (39). However, previous efforts to define the role of TNF-α in mediating neutrophil recruitment to the lung in response to airway challenge with LPS have been inconclusive. Ulich et al. (66) found that coadministration of human soluble TNFRI reduced by 50–60% the number of bronchoalveolar neutrophils recovered 6 h after intratracheal injection of LPS in rats but did not affect the number of neutrophils 4 or 12 h after LPS challenge. Ulich et al. (65) also reported that coadminis-
provides a more stable defect in TNF-a recruitment. The use of transgenic mice lacking TNFR1 neutralized bronchoalveolar TNF-a. (58) found that coadministration of anti-TNF-a BALF 3 h after intratracheal injection of LPS. Tang et al. bioactivity and reduced the number of neutrophils in linked to mouse IgG heavy chain neutralized TNF-a vector encoding for the extracellular domain of TNFR1 intratracheal injection of mice with an adenoviral in BALF (65). Kolls et al. (24) reported that systemic or blunted TNF-a is known to stimulate its own synthesis in TNFR1-deficient animals, indicating that signaling through TNFR1 is not re-

TNF-a/2 impaired in TNFR1(-/-) mice. It is possible that the administration of soluble receptors or antibodies, which may have incomplete or transient effects. Pe-

schon et al. (41) found that TNFR1-deficient mice exhibited a marked defect in neutrophil recruitment to the lungs after intranasal deposition of Micropolyspora faeni but did not observe any differences from control mice in bronchoalveolar lavage cell populations 2 and 24 h after nasal instillation of LPS. In contrast, we identi-

fied a defect in neutrophil recruitment in TNFR1-deficient animals that was sustained over 24 h and was evident at three different concentrations of aerosolized LPS.

Although we observed a consistent reduction in bronchoalveolar neutrophils after inhalation of LPS in TNFR1-deficient animals, histological sections demonstrated an inflammatory response in these animals, indicating that signaling through TNFR1 is not required for neutrophil emigration into the air spaces of the lungs. TNF-a is known to stimulate its own synthesis and release in vitro, (43), raising the possibility of a blunted TNF-a response to LPS in mice lacking TNFR1. However, we found no deficiency in bronchoalveolar TNF-a induction in TNFR1(-/-) mice, indicating that positive feedback through TNFR1 is not required for maximal TNF-a release in response to inhaled LPS. Similarly, other investigators (41, 47) found that serum TNF-a responses to systemic LPS challenge were not impaired in TNFR1(-/-) mice. It is possible that TNF-a contributes to the inflammatory response in TNFR1(-/-) mice via TNFR2. TNFR2 can mediate nuclear factor-kB activation in some cell lines in the absence of TNFR1 (48) and thus may potentially stimulate nuclear factor-kB-dependent proinflammatory gene transcription (1). Indeed, transgenic mice that overex-

press TNFR2 are more susceptible to lethal challenge with LPS or rTNF-a and exhibit chronic tissue inflammation that is independent of TNF-a or TNFR1 (12). However, TNF-a-mediated upregulation of endothelial

and epithelial adhesion molecule expression appears to be controlled exclusively by TNFR1 (25, 39), and neutrophilic lung inflammation in response to M. faeni is accentuated in TNFR2-deficient mice (41). It is likely that other inflammatory mediators with overlapping functions can partially fulfill the proinflammatory activities of TNF-a in TNFR1-deficient mice. IL-1B has many of the same effects as TNF-a, including stimulation of chemokine release and upregulation of adhesion molecules (10), and we found that IL-1B levels were more readily detectable in TNFR1-deficient mice than in wild-type control mice after LPS challenge.

One mechanism by which TNF-a amplifies lung inflammation is by augmenting chemokine production. TNF-a stimulates chemokine release by macrophages and by cells that do not respond directly to LPS in vitro, such as epithelial cells and fibroblasts (56). TNF-a also induces bronchoalveolar chemokine release in vivo (23). Chemokines are major components of the chemotactic activity of BALF in endotoxin-exposed animals (16), and chemokine depletion impairs neutrophil recruit-

ment in response to LPS or gram-negative infec-

tion (2, 16, 19, 49, 59, 63). We found that levels of MIP-2 and KC in BALF harvested 4 h after inhalation of LPS were lower in TNFR1-deficient mice than in wild-type control mice, suggesting that TNFR1 is required for optimal chemokine induction in vivo in response to inhaled LPS. However, we also found that BALF from LPS-exposed TNFR1-deficient mice contained more chemotactic activity for neutrophils than lavage fluid from LPS-exposed wild-type animals. It is possible that the lavage fluid from TNFR1(-/-) mice contained additional, unmeasured chemotactic factors or lacked inhibitors of chemotaxis in comparison with lavage fluid from control animals. Alternatively, the peritoneal exudate neutrophils used in the chemotaxis assay may have responded to the net chemotactic activity differently from circulating neutrophils in vivo. Neverthe-

less, it seems likely that the diminished bronchoalveo-

lar chemokine response of TNFR1(-/-) mice contributed to the impaired neutrophil recruitment observed in these animals in response to aerosolized LPS.

Another mechanism by which TNF-a promotes acute lung inflammation is by upregulating the expression of adhesion molecules on endothelial cells and circulating leukocytes (56). Both E. coli LPS and live P. aeruginosa elicit neutrophil emigration to the alveolar spaces by mechanisms dependent on ICAM-1 and CD18 in normal mammals (11, 27, 28, 44). It has been reported that pulmonary endothelial ICAM-1 expression is upregulated in response to gram-negative pneumonia or airway deposition of LPS (4, 44, 59) and that TNF-a is an important mediator of pulmonary vascular ICAM-1 expression in vivo (31, 37). Furthermore, TNF-a-induced endothelial adhesion molecule expression is mediated by TNFR1 (39). Thus it is attractive to hypothesize that diminished endothelial ICAM-1 expression may have contributed to the impaired neutrophil recruitment in TNFR1(-/-) mice exposed to aerosolized LPS. However, we were unable to detect any
change in ICAM-1 expression after inhalation of LPS because of high baseline expression of ICAM-1 in the alveolar interstitium. Other investigators (4, 39) also have found it difficult to detect changes in pulmonary ICAM-1 expression by immunohistochemistry because of high constitutive expression. Furthermore, there are ICAM-1- and CD18-independent mechanisms of neutrophil recruitment into the alveolar spaces in response to bacterial stimuli (11, 27, 28, 44) that may be more developed in TNFR-1-deficient transgenic animals.

A key finding in our studies is that inflammatory cell recruitment to the lungs of TNFR1-deficient mice was augmented after inhalation of live P. aeruginosa, in contrast to the impaired response to aerosolized LPS. Bronchoalveolar levels of chemokines were depressed in TNFR1(/−/) mice in response to both stimuli. One potential explanation for these divergent effects is that live bacteria elicit neutrophils directly as well as indirectly. Whereas inflammatory responses to LPS are dependent on host-derived mediators such as chemokines and complement fragments, neutrophil recruitment in response to gram-negative infection involves bacterial-derived chemotactic factors such as formylated peptides, as well as endogenous mediators stimulated by LPS and other bacterial products (32, 51). As a result, neutrophil recruitment to the lung in gram-negative pneumonia is only partly dependent on endogenous cytokines (19, 20) and is not reduced in the absence of C5a receptors (21). Moreover, some strains of P. aeruginosa are directly toxic to the alveolar epithelium and induce a bidirectional protein leak across the epithelial and endothelial barriers (26, 46, 72), in contrast to the compartmentalized response to airway challenge with LPS (38, 72). The exudation of serum proteins such as LPS binding protein and soluble CD14 into the air spaces amplifies cytokine responses to bacterial products (33, 35, 72). Furthermore, contamination of the bloodstream with live bacteria and bacterial products leads to endothelial and circulating leukocyte activation that primes the host for greater responsiveness to tissue chemotactic signals (60, 72).

Our observations argue strongly that the dominant role of TNFα in response to inhaled P. aeruginosa is to attenuate lung inflammation. One mechanism by which this might occur is suggested by the evidence that soluble TNFαR1 shed from the surface of cells can bind free TNFα and thereby inhibit peak TNFα activity (67). Thus if any signaling occurs through TNFαR2 in TNFR1(/−/) mice, then the absence of soluble TNFαR1 could result in increased stimulation from free TNFα by way of TNFαR2. However, it is unlikely that this is the explanation for the augmented inflammatory response to infection in TNFR1(/−/) mice for two reasons. First, the ratios of bioactive to immunoreactive TNFα in postinfection BALF were ~5:1 in both TNFR1(/−/) and TNFR1(+/+) mice (Fig. 10), rendering improbable any differences in soluble inhibitors of TNFα. Second, we observed an increased inflammatory response to P. aeruginosa in mice lacking both TNFR1 and TNFR2, indicating that signaling through TNFR2 did not account for the exaggerated response in TNFR1-deficient mice. Another pathway by which TNFR1 may contribute to the downmodulation of inflammatory responses is by signaling the induction of anti-inflammatory mediators. For example, TNFα stimulates the secretion of IL-10 (6), which inhibits pulmonary inflammation (5, 50). It is possible that an altered balance of pro- and anti-inflammatory mediators led to the more robust early neutrophil response after inhalation of P. aeruginosa in mice lacking TNFR1.

Although the increased numbers of neutrophils in the lungs of TNFR1-deficient mice with early P. aeruginosa infection may reflect increased cellular recruitment, an alternative explanation is that elicited neutrophils survive longer in the air spaces of animals lacking TNFR1. Neutrophils are short-lived cells and ordinarily undergo apoptosis within hours of their entry into tissues (55), after which they undergo endocytosis and are cleared by macrophages. Exposure to TNFα and ingestion of gram-negative bacteria accelerate neutrophil apoptosis (57, 69). Because TNFR1 can mediate apoptosis (7), it is possible that in the absence of TNFR1, apoptosis of neutrophils was delayed, leading to greater accumulation of these cells in the lungs. Prolonged neutrophil survival might improve microbial killing, perhaps accounting for the accelerated clearance of P. aeruginosa in TNFR1(/−/) mice.

We found no evidence that TNFR1 plays a role in increasing lung permeability in response to LPS or P. aeruginosa. The protein concentration in BALF from LPS-exposed animals did not differ from that in control animals, indicating that inhalation of LPS resulted in little, if any, alteration in epithelial permeability despite the influx of neutrophils. Other investigators (22, 72) have made similar observations, but some (30, 40, 71) have reported that intratracheal injection of LPS or exposure to high concentrations of aerosolized LPS in rats causes an increase in epithelial permeability. In contrast to the results with aerosolized LPS, we found that the BALF protein concentration was markedly elevated in infected mice whether or not TNFR1 was present. These observations indicate that the mechanism of increased epithelial permeability in these animals is not dependent on TNFR1. Similarly, Rezaiguia et al. (46) observed that TNFα depletion did not influence protein epithelial permeability in rats with P. aeruginosa pneumonia but did reduce the increase in alveolar fluid clearance in infected animals. It is likely that bacterial-derived factors and host mediators other than TNFα are important in altering epithelial permeability (26, 72).

We were surprised to find that pulmonary clearance of P. aeruginosa was augmented in TNFR1(/−/) and TNFR1(+/−) and TNFR2(/−/) mice because most investigations have supported an important role for TNFα in host resistance to gram-negative bacteria. Buret et al. (3) reported that intratracheal injection of rTNFα improved pulmonary clearance of P. aeruginosa in rats, apparently by stimulating the phagocytosis of bacteria by bronchoalveolar neutrophils. Kolls et al. (24) found that systemic administration of an adenoviral vector encoding a soluble inhibitor of TNFα re-
sulted in impaired clearance of aerosolized P. aeruginosa in BALB/c mice. Similarly, Laichalk et al. (29) reported that systemic administration of human soluble TNF-R2 linked to the Fc fragment of human IgG resulted in impaired neutrophil recruitment and bacterial clearance after intratracheal challenge of CBA/J mice with K. pneumoniae. However, Rezaigiu et al. (46) reported that antibody-mediated depletion of TNF-α in rats did not affect pulmonary neutrophil recruitment or clearance of P. aeruginosa (46). The study by Gosselin et al. (18), using a model of chronic P. aeruginosa airway infection in mice, suggested that the role of TNF-α is host dependent: anti-TNF-α impaired pulmonary bacterial clearance in BALB/c mice without influencing neutrophil recruitment but had no effect in DBA/2 mice. Our finding of accelerated bacterial clearance in TNFR-deficient mice may be related to the greater early accumulation of neutrophils in these animals. These data suggest that TNF-α is not required for resistance to P. aeruginosa in C57BL/6 mice.

In summary, we found that TNFR1-deficient mice exhibited impaired pulmonary inflammatory responses to inhaled LPS but augmented responses to live P. aeruginosa despite impaired chemokine responses to both stimuli. The absence of TNF1 did not affect the protein leak associated with gram-negative pneumonia nor did it impair bacterial clearance from the lungs. These findings support a complex role for TNFR1 in regulating pulmonary inflammation that varies with the nature of the inciting stimulus. Elucidation of the mechanisms underlying augmented neutrophil recruitment and accelerated bacterial clearance in TNFR1-deficient mice challenged with P. aeruginosa will require further investigation.

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