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Tumor Necrosis Factor Mediates Lung Antibacterial Host Defense in Murine *Klebsiella* Pneumonia

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Tumor necrosis factor (TNF) is a proinflammatory cytokine which has recently been shown to have beneficial effects in the setting of acquired host immunity. However, the role of TNF in innate immune responses, as in the setting of bacterial pneumonia, has been incompletely characterized. To determine the role of TNF in gram-negative bacterial pneumonia, CBA/J mice were challenged with 10^2 CFU of *Klebsiella pneumoniae* intratracheally, resulting in the time-dependent expression of TNF MRNA and protein within the lung. Passive immunization of animals with a soluble TNF receptor-immunoglobulin (Ig) construct (sTNFR:Fc) intraperitoneally 2 h prior to *K. pneumoniae* inoculation resulted in a significant reduction in bronchoalveolar lavage neutrophils, but not macrophages, at 48 h, as compared with animals receiving control IgG1. Furthermore, treatment with sTNFR:Fc resulted in 19.6- and 13.5-fold increases in *K. pneumoniae* CFU in lung homogenates and plasma, respectively, as compared with animals receiving control IgG1. Finally, treatment of *Klebsiella*-infected mice with sTNFR:Fc markedly decreased both short- and long-term survival of these animals. In conclusion, our studies indicate that endogenous TNF is a critical component of antibacterial host defense in murine *Klebsiella* pneumonia.

Nosocomial bacterial pneumonia is the second most common hospital-acquired infection and is the leading cause of death among all nosocomial infections (20). Despite the development of new broad-spectrum antibiotics, bacterial pneumonia continues to be a major cause of morbidity and mortality in this country (4, 20). The emergence of multidrug-resistant microbes in the immunocompromised host has made the treatment of these infections increasingly difficult (5, 27), highlighting the importance of immune host defense in determining the eventual outcome of severe bacterial infection.

Effective host defense against lung bacterial infection is primarily dependent upon the rapid clearance of the organism from the respiratory tract, mediated by the influx and/or activation of phagocytic cells, including polymorphonuclear neutrophils (PMN) and macrophages (both resident and recruited) (29). The recruitment and activation of leukocytes in the setting of bacterial challenge are complex and dynamic processes which involve the coordinated expression of both pro- and anti-inflammatory cytokines (11, 12, 17). Tumor necrosis factor (TNF) is a 17-kDa cytokine that has been shown to mediate many of the detrimental pathophysiologic events that occur in sepsis and sepsis syndrome (1, 30). However, mounting evidence suggests that TNF is a critical component of effective antibacterial host defense (7, 9, 13, 16). Specifically, TNF is a potent activator of both PMN and macrophages, leading to enhancement of protease release, stimulation of the respiratory burst, and induction of leukocyte and vascular adhesion molecule expression, which are essential for transmigration of these cells into sites of infection (8, 19, 26). Moreover, PMN and macrophage microbicidal activity is augmented by endogenous or exogenous TNF (24, 28). Finally, TNF is expressed in increased amounts in the airspace of humans with bacterial pneumonia (23) and in the lungs of mice challenged with bacterial pathogens (9, 16). Recent studies suggest that neutralization of TNF in vivo can impair neutrophil influx and lung bacterial clearance in response to aerosolized or intratracheally (i.t.) administered *Pseudomonas aeruginosa* (9, 16). While these studies provide compelling evidence in support of TNF as an important mediator in bacterial infection of the lung, the relative importance of TNF in the eventual outcome of bacterial pneumonia has not been defined.

The specific objectives of this study were (i) to determine the time course and cellular sources of endogenously produced TNF in murine *Klebsiella* pneumonia and (ii) to determine the contribution of TNF to lung bacterial clearance and survival in *Klebsiella* pneumonia by in vivo TNF depletion using soluble TNF receptor-immunoglobulin fusion protein (sTNFR:Fc).

MATERIALS AND METHODS

Reagents. Polyclonal antimurine TNF antibodies used in the enzyme-linked immunosorbent assay (ELISA) were produced by immunization of rabbits with murine recombinant TNF (R&D Systems, Minneapolis, Minn.) at multiple intradermal sites with complete Freund's adjuvant. In TNF neutralization experiments, 0.5 ml (100 μg) of the soluble human TNF receptor-immunoglobulin fusion protein (sTNFR:Fc) was administered intraperitoneally (i.p.) 2 h prior to *Klebsiella pneumoniae* administration. The sTNFR:Fc was kindly provided by Michael Widmer (Immunex, Seattle, Wash.) and is composed of soluble dimeric human p80 TNF receptor linked to the Fc region of human immunoglobulin G1 (IgG1), which results in 50 to 1,000 times greater efficacy in neutralizing TNF bioactivity as compared with monomeric soluble TNF receptor (22, 25).

Animals. Specific-pathogen-free CBA/J mice (6- to 12-week-old females; Charles River Breeding Labs, Wilmington, Mass.) were used in all experiments. All mice were housed in specific-pathogen-free conditions within the animal care facility at the University of Michigan (ULAM) until the day of sacrifice.

***K. pneumoniae* inoculation.** We chose to use *K. pneumoniae* 43816, serotype 2 (American Type Culture Collection, Rockville, Md.) in our studies, as this strain has been shown to induce an impressive inflammatory response in mice (2, 11, 12, 21). *K. pneumoniae* was grown in tryptic soy broth (Difco, Detroit, Mich.) for 18 h at 37°C. The concentration of bacteria in broth was determined by measuring the amount of A_{600}. A standard of absorbancies based on known CFU was used to calculate inoculum concentration. Bacteria were pelleted by centrifugation at 10,000 rpm (Jouan centrifuge model MR 1822) for 30 min, washed two times in

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saline, and resuspended at the desired concentration. Animals were anesthetized with approximately 1.8 to 2 mg of pentobarbital per animal i.p. The trachea was exposed, and 30 μl of inoculum or saline was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples.

BAL. Bronchoalveolar lavage (BAL) was performed by standard techniques (11). Briefly, the trachea was exposed and intubated using a 1.7-mm (outside diameter) polyethylene catheter. BAL was performed by instilling phosphate-buffered saline (PBS) containing 5 mM EDTA in 1-ml aliquots. Approximately 5 ml of lavage fluid was retrieved per mouse. Cytospins were then prepared from BAL cells and stained with Diff Quick (Baxter, McGaw Park, Ill.), and differential counts were determined.

Lung harvesting for cytokine analysis and histologic examination. At designated time points, the mice were then anesthetized with inhaled methoxyflurane, blood was collected by orbital bleeding, and the animals were sacrificed. Whole lungs were then harvested for assessment of TNF protein expression. Prior to lung removal, the pulmonary vasculature was perfused with 1 ml of PBS containing 5 mM EDTA, via the right ventricle. After removal, whole lungs were homogenized in 3 ml of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl, and 1 mM MgCl (pH 7.40), using a tissue homogenizer (Biospec Products, Inc.). Homogenates were incubated on ice for 30 min and then centrifuged at 2,500 rpm (Beckman CPR centrifuge) for 10 min. Supernatants were collected, passed through a 0.45-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.), and then stored at −20°C for assessment of cytokine levels. Lungs for histologic examination were excised en bloc without perfusion and inflated with 1 ml of 4% paraformaldehyde in PBS to improve resolution of anatomic relationships.

Determination of lung *K. pneumoniae* CFU. At the time of sacrifice, plasma was collected, the right ventricle was perfused with 1 ml of PBS, and then the lungs were removed aseptically and placed in 3 ml of sterile saline. The tissues were then homogenized with a tissue homogenizer under a vented hood. The lung homogenates were placed on ice, and serial 1:10 dilutions were made. Ten microliters of each dilution was plated on soy base blood agar plates (Difco), the plates were incubated for 18 h at 37°C, and then the colonies were counted.

Murine TNF ELISA. Murine TNF was quantitated using a modification of a double-ligand method as previously described (11). Briefly, flat-bottom 96-well microtiter plates (Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) were coated with 50 μl of rabbit anti-TNF antibody per well (1 μg/ml in 0.6 M NaCl–0.26 M H_3BO_4–0.08 N NaOH [pH 9.6]) for 16 h at 4°C and then washed with PBS (pH 7.5)–0.05% Tween 20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and diluted (neat and 1:10) cell-free supernatants (50 μl) in duplicate were added, followed by incubation for 1 h at 37°C. Plates were washed four times, 50 μl of biotinylated rabbit anti-TNF antibodies was added per well (3.5 μg/ml in PBS (pH 7.5)–0.05% Tween 20–2% fetal calf serum), and the plates were incubated for 30 min at 37°C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) was added, and the plates were incubated for 30 min at 37°C. Plates were washed again four times, and chromogen substrate (Bio-Rad Laboratories) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl of a 3 M H_2SO_4 solution per well. Plates were read at 490 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant murine TNF from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine TNF concentrations above 25 pg/ml. There were no cross-reactions in the ELISA with interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-10, or gamma interferon. In addition, there were no cross-reactions with members of the murine chemokine family, including murine JE/MCP-1, MIP-1α, RANTES, KC, GROα, or ENA-78.

Immunohistochemical localization of antigenic TNF. Paraffin-embedded specimens of whole lung were cut in 3-μm-thick sections and placed on poly-L-lysine-coated slides for immunohistochemical localization of TNF antigen. Paraffin-embedded tissue was processed for immunohistochemical localization of TNF protein using peroxidase staining (Vectastain Elite ABC kit; Vector, Burlingame, Calif.). Briefly, tissue sections were dewaxed with xylene and rehydrated through graded concentrations of ethanol. Tissue nonspecific binding sites were then blocked with blocking solution (Vector). Tissue sections were then washed and incubated with a 1:2,000 dilution of rabbit anti-murine TNF antibodies (1 mg/ml) or equivalent dilutions of purified rabbit IgG for 24 h at 4°C. The tissue sections were washed and then incubated for 30 min with anti-rabbit biotinylated antibodies (Vector) at room temperature. Next, the sections were washed twice in Tris-buffered saline and incubated with Vectastain ABC reagent (Vector). True blue chromogenic substrate (BioGenex) was used for localization of TNF antigen. After optimal color development, tissue sections were rinsed in sterile water, counterstained with fast red, and cover slipped using Permount (Fisher Scientific, Fair Lawn, N.J.) mounting solution.

Isolation and reverse transcription-PCR amplification of whole-lung mRNA. Whole lungs were harvested at specific times postinoculation with *K. pneumoniae*, immediately snap frozen in liquid nitrogen, and stored at −70°C. Total cellular RNA from the frozen lungs was isolated by homogenizing the lungs with a tissue homogenizer in a solution containing 25 mM Tris (pH 8.0), 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris (pH 8.0), 10 mM EDTA, and 1.0% sodium dodecyl sulfate (SDS). The mixture was then extracted twice each with phenol-chloroform and chloroform-isoamyl alcohol. The RNA was alcohol precipitated, and the pellet was dissolved in diethylpyrocarbonate-water. Total RNA was determined by spectrometric analysis at a wavelength of 260 nm. One microgram of total RNA was reverse transcribed into cDNA utilizing a reverse transcription kit (Bethesda Research Laboratories) and oligo$(dT)_{12–18}$ primers. The cDNA was then amplified by using the specific primers for TNF, with β-actin primers serving as a control. The TNF primers used had the sequence 5′-CCT-GTA-GCC-CA C-GTC-GTA-GC-3′ and 5′-TTG-ACC-TCA-GCG-CTG-AGT-TG-3′, whereas the murine β-actin sense and antisense primers utilized had the sequences 5′-ATGGATGACGATATCGCTC-3′ and 5′-GATTCCATACCCAGGAAGG-3′, giving amplified products of 380 and 812 bp, respectively. The amplification buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2 mM $MgCl_2$. Specific oligonucleotide primers were added (400 ng per sample) to the buffer, along with 4 μl of the reverse-transcribed cDNA samples. The mixture was first incubated for 5 min at 94°C and then cycled 26 times for TNF and 30 times for β-actin at 93°C for 45 s and 52°C for 45 s and elongated at 72°C for 80 s plus 1 s per cycle. After amplification, the sample was separated on a 2% agarose gel containing 0.3 mg (0.003%) of ethidium bromide per ml, and bands were visualized and photographed by using UV transillumination. Band densities were quantitated by video densitometry using image analysis software from Scion. Values for integrated densities minus background were determined and expressed as percentages of the maximum.

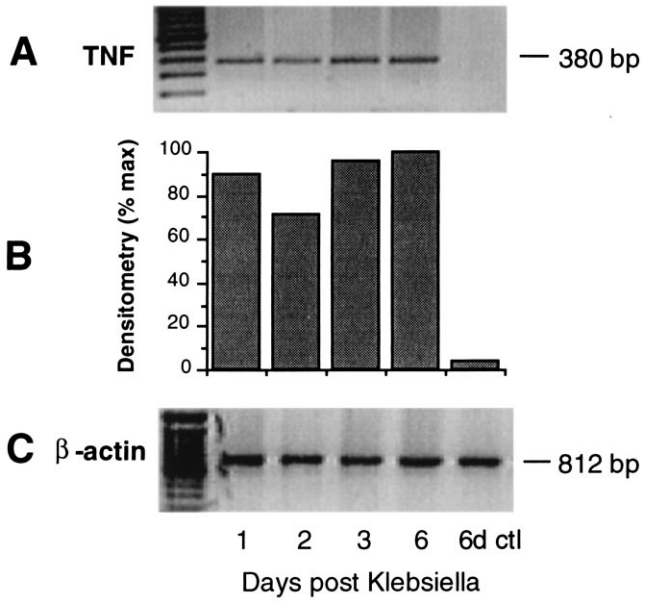

FIG. 1. Time-dependent production of TNF mRNA in lung homogenates after inoculation with *K. pneumoniae* (10^2 CFU). (A) PCR product of TNF (26 cycles); (B) video densitometry of TNF mRNA; (C) PCR product of β-actin (30 cycles). Molecular size markers are found to the left. The lungs from three animals were combined at each specific time point assayed.

Statistical analysis. Data were analyzed with a Macintosh II computer using the Statview II statistical package (Abacus Concepts, Inc., Berkeley, Calif.). The survival data were compared using the chi-square analysis. All other data are expressed as means ± standard errors of the means and were compared using a two-tailed Student's *t* test. Data were considered statistically significant if *P* values were less than 0.05.

RESULTS

Time-dependent production of TNF mRNA within the lung after i.t. inoculation with *K. pneumoniae*. We first performed experiments to determine whether TNF mRNA was expressed within the lung during the evolution of gram-negative bacterial pneumonia. As shown in Fig. 1, TNF mRNA was induced within the lung in response to the i.t. administration of *K. pneumoniae* in a bimodal pattern, with early expression noted on day 1 (89.9% maximum), lesser amounts at day 2 (71.2% maximum), and peak levels at days 3 (96.3% maximum) and 6

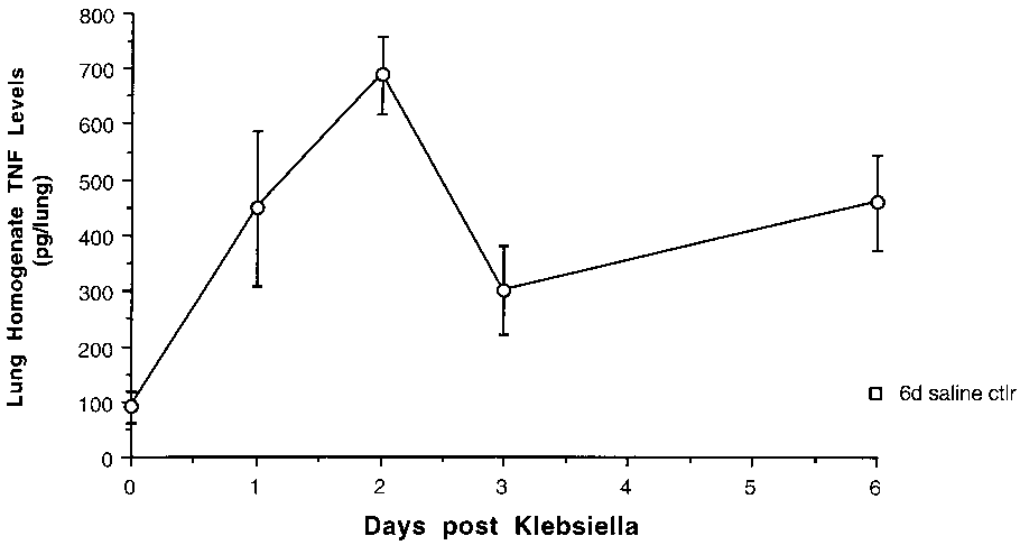

FIG. 2. Time-dependent production of TNF protein in lungs from CBA/J mice after i.t. administration of saline or *K. pneumoniae* (10^2 CFU). $n = 5$ per group.

(100% maximum) postinoculation. Minimal TNF mRNA was detected in saline-challenged lungs at day 6 (4.5% maximum).

Time-dependent expression of TNF protein in lung during the evolution of *Klebsiella* pneumonia. Subsequent experiments were performed to determine whether TNF protein was produced within the lung during the course of murine *Klebsiella* pneumonia. Similar to the time course of TNF mRNA expression, the i.t. administration of *K. pneumoniae* resulted in a bimodal production of TNF protein (Fig. 2). Specifically, early increases in TNF protein production within lung homogenates were noted by day 1, with maximal expression by day 2 postinoculation. Interestingly, a second peak of TNF was detected at 6 days postinoculation, representing an approximate fivefold increase over that observed in lung homogenates prepared from saline-treated control animals at that time ($P < 0.05$). In addition, the increased expression of TNF protein appeared to be compartmentalized to the lung, as there was no significant change in plasma TNF levels detected in animals inoculated with *K. pneumoniae* as compared with animals administered saline i.t. (data not shown).

Immunolocalization of TNF in *Klebsiella* pneumonia. To determine the cellular source(s) of TNF within the lung in *Klebsiella* pneumonia, immunohistochemical studies were performed. At 24 h postinoculation, cell-associated TNF was present within alveolar macrophages (Fig. 3B). At 72 h post-*Klebsiella* administration, alveolar macrophages continue to express substantial quantities of TNF. In addition, staining of some, but not all, PMN was noted at this time (Fig. 3D). The staining for TNF was specific, as no staining was present in sections of lung incubated with purified rabbit IgG from control serum (Fig. 3A and C). Furthermore, no cell-associated TNF was detected in pulmonary epithelial cells, fibroblasts, or endothelial cells.

Effect of passive immunization of animals with sTNFR:Fc on lung histology. To determine the contribution of TNF to inflammatory cell influx in *Klebsiella* pneumonia, mice were administered a single injection of either 100 μg of human IgG1 or sTNFR:Fc i.p. 2 h prior to *K. pneumoniae* inoculation (10^2 CFU); lungs were then harvested at 24 h to assess for early histologic changes. As shown in Fig. 4, a moderate influx of PMN was noted within the airspace of IgG-treated animals challenged with *K. pneumoniae*. Importantly, no intraalveolar bacterial organisms were detected. In contrast, numerous organisms were visualized in the airspace of animals pretreated with sTNFR:Fc, indicating an inability to effectively clear i.t. administered *Klebsiella* organisms early in the course of pneumonia.

Effect of passive immunization of animals with sTNFR:Fc on BAL cell counts and differentials. To assess the role of TNF in mediating lung leukocyte influx in *Klebsiella* pneumonia, animals were pretreated with either IgG1 or sTNFR:Fc i.p. and challenged with saline or *K. pneumoniae* (10^2 CFU) i.t. and then BAL was performed 48 h later. As shown in Table 1, administration of *K. pneumoniae* resulted in a marked increase in the percentage of BAL PMN as compared with the value for saline-treated animals. Interestingly, animals receiving sTNFR:Fc had an approximately 35% reduction in the percentage of BAL PMN, as compared with animals receiving IgG1 ($P < 0.01$). In addition, treatment with sTNFR:Fc resulted in a modest (23%) but significant reduction in total BAL cells ($P < 0.05$) and a 50% reduction in total BAL PMN, without significant changes in total numbers of macrophages (Fig. 5) or lymphocytes (data not shown). Similarly, the administration of sTNFR:Fc reduced total BAL PMN and cells at 24 h post-*Klebsiella* administration by 33 and 30%, respectively. However, these differences did not reach the level of statistical significance.

Effect of passive immunization of animals with sTNFR:Fc on bacterial clearance. To ascertain the biologic relevance of TNF expression in *Klebsiella* pneumonia, we next determined if inhibition of TNF resulted in attenuation of lung bacterial clearance and early dissemination. Mice were administered a single injection of either 100 μg of human IgG1 or sTNFR:Fc i.p. 2 h prior to inoculation with *K. pneumoniae* (10^2 CFU). Lungs were harvested and plasma was collected at 48 h postinoculation. As shown in Fig. 6, animals receiving sTNFR:Fc had a 13.5-fold increase in the number of *K. pneumoniae* CFU isolated from plasma at 48 h postinoculation as compared with animals receiving IgG1 ($P < 0.05$). Even more impressively, animals receiving sTNFR:Fc had a 19.6-fold increase in *K. pneumoniae* CFU in lung homogenates as compared with lungs from control animals ($P < 0.01$). Similarly, we observed greater than 8- and 19-fold increases in lung and plasma bacterial counts, respectively, in animals receiving sTNFR:Fc as

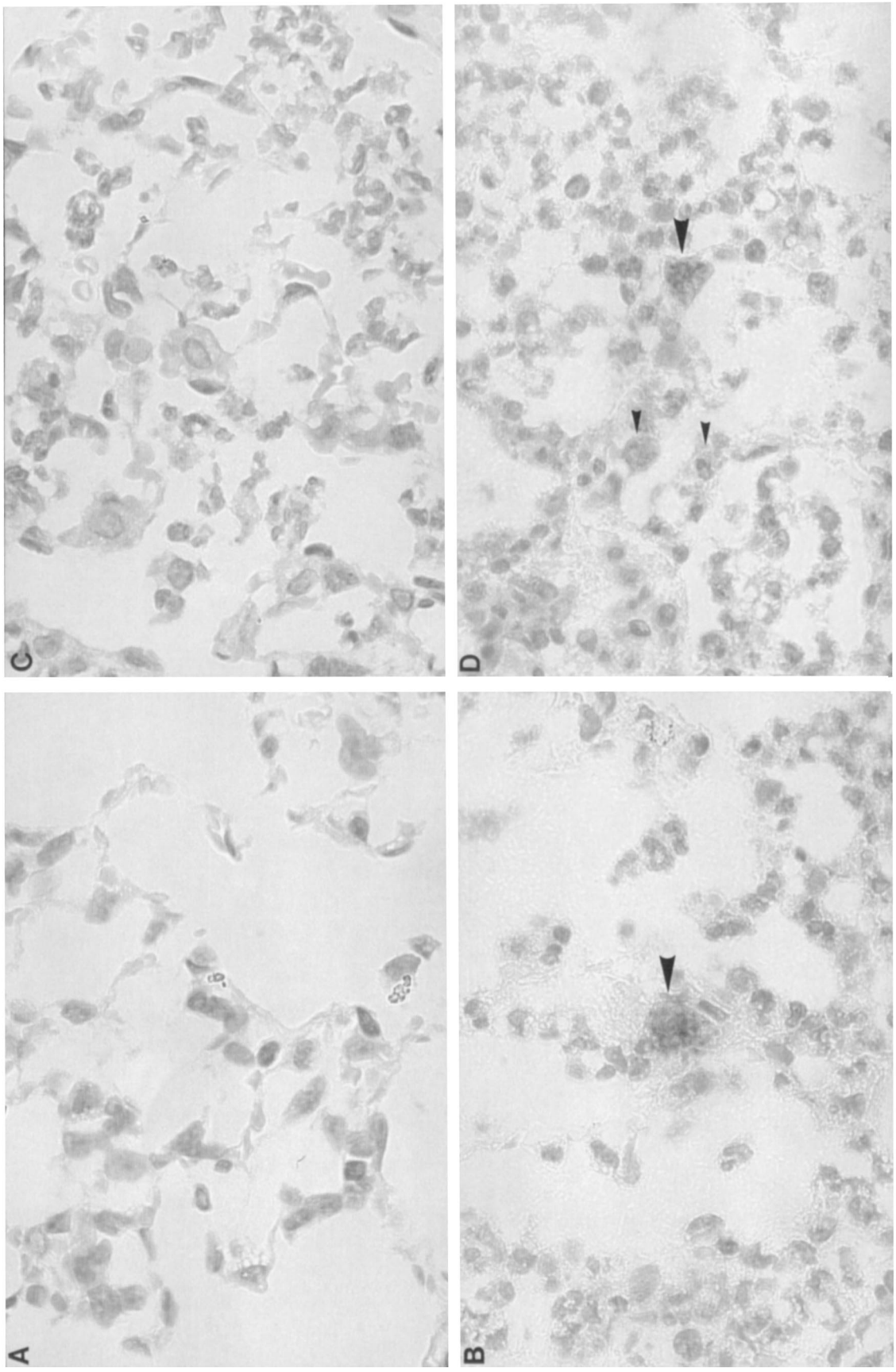

FIG. 3. Expression of cell-associated TNF in *Klebsiella* pneumonia. Representative immunohistochemical staining of murine lung for TNF antigen 24 and 72 h after i.t. inoculation with *K. pneumoniae* (10^2 CFU), showing cell-associated TNF within alveolar macrophages (large arrowheads) at 24 h (B) and both PMN (small arrowheads) and alveolar macrophages at 72 h (D). Control sections showing no staining in lung incubated with purified IgG at 24 (A) and 72 (C) h.

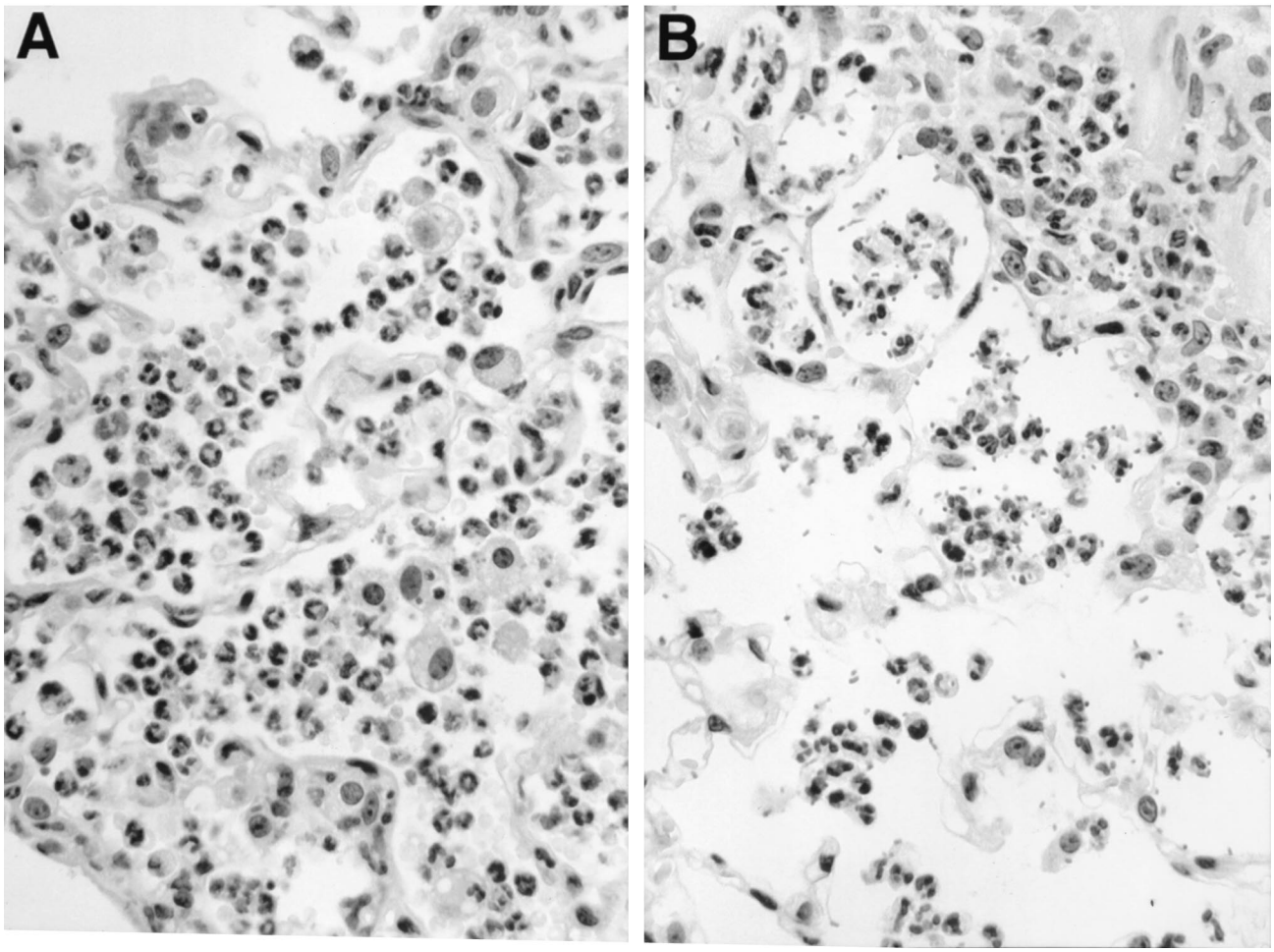

FIG. 4. Effect of sTNFR:Fc on lung histology in *Klebsiella* pneumonia. CBA/J mice were passively immunized with sTNFR:Fc or human IgG1 and then inoculated with 10^2 CFU of *K. pneumoniae*, and lungs were harvested 24 h postinoculation. Representative of three experiments.

compared with controls when animals were challenged with a 90% lethal dose of *K. pneumoniae* (10^3 CFU; data not shown).

Effect of passive immunization of animals with sTNFR:Fc on survival in *Klebsiella* pneumonia. To determine the contribution of TNF to the ultimate survival of animals with *Klebsiella* pneumonia, mice were administered either 100 μg of sTNFR:Fc or human IgG1 i.p. 2 h prior to *K. pneumoniae* inoculation, with repeat administration of 50 μg of sTNFR:Fc or IgG1 i.p. every 48 h. Animals were inoculated with 10^2 CFU of *K. pneumoniae*, as this inoculum represents the dose at which approximately 80 to 90% of control animals survived long-term (>14 days). In those animals passively immunized with IgG1, minimal mortality was observed, with 80% of animals surviving long-term (Fig. 7). In contrast, animals that received sTNFR:Fc had significantly increased mortality at all time points after 4 days, with only 10% of animals surviving past 6 days ($P < 0.001$).

TABLE 1. Effect of sTNFR:Fc on BAL cell differentials 48 h after *K. pneumoniae* administration[a]

Treatment	% Mϕ	% PMN
Saline + IgG1	99.0 ± 0.57	0.7 ± 0.7
Klebsiellae + IgG1	26.4 ± 4.6†	73.6 ± 4.6†
Klebsiellae + sTNFR:Fc	51.4 ± 4.3*†	48.2 ± 4.5*†

[a] Animals were pretreated with either 100 μg of sTNFR:Fc or human IgG1 i.p. 2 h prior to i.t. inoculation. *, $P < 0.01$ as compared with animals receiving IgG1; †, $P < 0.01$ as compared with saline-challenged animals. % Mϕ, percent macrophages.

DISCUSSION

Bacterial infection of the lung continues to be a difficult and costly medical problem (10, 20). Therefore, a more complete understanding of the cytokine mediators that constitute innate immunity against bacterial pathogens is essential to allow for the development of effective new therapies. In this study, we demonstrated that inhibiting TNF bioactivity in vivo resulted in significant impairment in lung PMN influx, bacterial clearance, and survival after challenge with *K. pneumoniae*. Comparatively, TNF appears to play a major role in host defense against *K. pneumoniae*, as inhibition of other cytokines believed to contribute to antibacterial host defense, specifically MIP-2 and gamma interferon, has modest to no measurable effect on the eventual outcome in murine *Klebsiella* pneumonia (12 and unpublished observations). In contrast, inhibition of TNF dramatically altered survival in these animals.

TNF likely mediates lung antibacterial host defense through

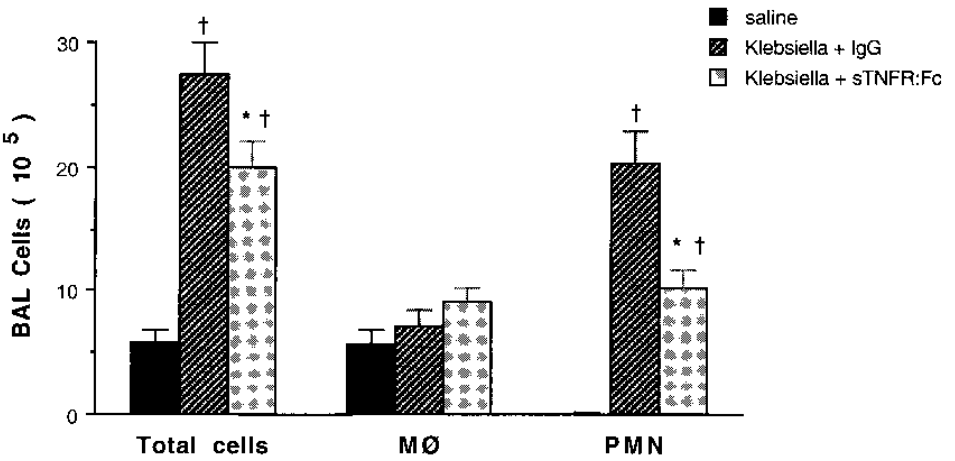

FIG. 5. Effect of sTNFR:Fc on BAL cell numbers in *Klebsiella* pneumonia. CBA/J mice were passively immunized with sTNFR:Fc or human IgG1 and then inoculated with 10^2 CFU of *K. pneumoniae* ($n = 5$ per group). *, $P < 0.05$ as compared with preimmune-treated animals. Mϕ, macrophages.

several related but distinct pathways. PMN have clearly been shown to be important effector cells in the phagocytosis and killing of bacterial pathogens, and our studies indicate that the inhibition of TNF resulted in some attenuation of PMN influx. This observation is consistent with previous studies with rats which have shown that the PMN influx into the airspace in response to i.t. administered endotoxin is inhibited by neutralization of TNF (32). Since TNF is not directly chemotactic for PMN, it is probable that TNF mediates PMN influx in vivo through regulation of leukocyte and vascular adhesion molecule expression (8, 19) or, alternatively, through the induction of distal chemotactic peptides or lipid mediators (31). However, the effects of sTNFR:Fc on PMN influx were not profound, especially at early time points (24 h), suggesting that the leukocyte-activating effects of TNF are relevant in vivo. Specific TNF effects include activation of PMN and macrophage phagocytic and microbicidal activity in vitro (14, 24, 28). Furthermore, we have observed that TNF can augment the killing of ingested *Escherichia coli* by murine alveolar macrophages (data not shown). Enhancement of leukocyte bactericidal activity by TNF can occur by several mechanisms, including direct stimulation of the respiratory burst, release of proteolytic enzymes, and induction of nitric oxide synthesis (9, 19). In support of the premise that the activating effects of TNF may predominate over effects on inflammatory cell recruitment, Gosselin and colleagues found that inhibition of TNF using polyclonal anti-TNF antibodies can attenuate clearance of *Pseudomonas aeruginosa* without influencing lung PMN in flux (9).

The cellular sources of TNF in vivo have not been well characterized. Previous studies have demonstrated that mac-

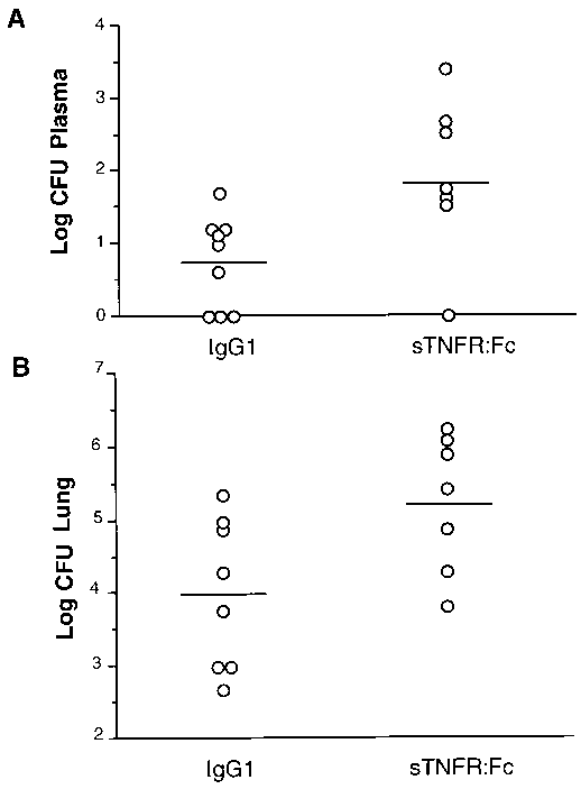

FIG. 6. Effect of sTNFR:Fc on *K. pneumoniae* CFU in plasma (A) and lungs (B) at 48 h postinoculation. Animals were pretreated with either 100 μg of sTNFR:Fc or human IgG1 i.p. 2 h prior to i.t. inoculation with 10^2 CFU of *K. pneumoniae*. Each dot represents the log CFU for each individual animal studied. Lines represent mean values for each group.

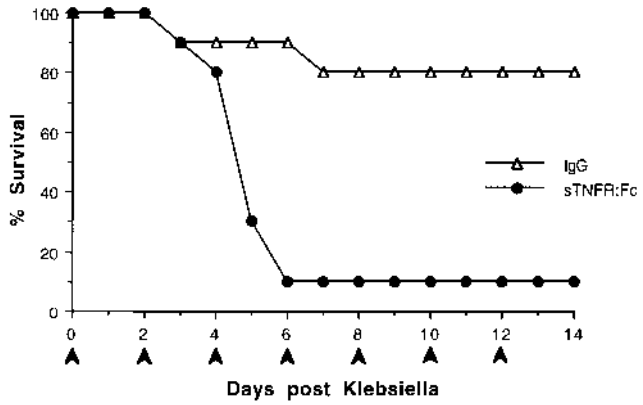

FIG. 7. Effect of sTNFR:Fc on survival in *Klebsiella* pneumonia. CBA/J mice were passively immunized with sTNFR:Fc or human IgG1 and then inoculated with 10^2 CFU of *K. pneumoniae* ($n = 10$ per group). Arrowheads indicate times of sTNFR:Fc administration.

rophages represent major cellular sources of TNF in vitro and in vivo (19, 23). In addition, PMN have been shown to synthesize and secrete TNF (6). Utilizing immunohistochemical techniques, we have demonstrated temporal changes in the cellular sources of TNF in the lung after i.t. challenge with *K. pneumoniae*. Early after *Klebsiella* administration, TNF localized primarily to alveolar macrophages. At later time points, however, recruited PMN appear to represent important cellular sources of TNF. Although PMN have a limited capacity to synthesize and secrete specific cytokines in vitro (6 and personal observations), the fact that large numbers of PMN are present within the airspace at the later time points examined raise the possibility that these cells may account for the second peak of TNF mRNA and protein production noted between 3 and 6 days post-*K. pneumoniae* administration. A similar bimodal cascade of cytokine production has been observed with the expression of IL-8 mRNA within the canine airway in response to *P. aeruginosa*, whereby the production of IL-8 by airway epithelium and ductal cells results in the recruitment of PMN, which then elaborate additional IL-8 (15). The production of TNF by alveolar macrophages may serve to localize the inflammatory response, whereas the expression of this cytokine by PMN may serve as an important mechanism whereby the inflammatory response can be amplified, resulting in more effective clearance of the invading organism.

The current studies have clearly identified TNF as an important cytokine mediator in antibacterial host defense. Recent advances in our understanding of cytokine biology have made possible the implementation of novel strategies to deliver specific cytokines to desired sites of action. However, immunotherapy is often complicated by significant toxicity, especially when specific cytokines are given systemically. This is particularly true for TNF, as the systemic administration of this cytokine has been complicated by fever, hemodynamic instability, end-organ injury, and even death (30). These side effects have dramatically limited the clinical use of TNF and other cytokines given systemically. Compartmentalized delivery of cytokines or cytokine receptors to the lungs has been achieved by direct i.t. administration (31, 32). More recently, we and others have successfully employed i.t. gene therapy using recombinant human adenoviruses to overexpress specific cytokines and cytokine antagonists in the lung, including murine IL-12 (3, 10a, 16). An alternative approach is the systemic delivery of synthetic TNF agonist peptides, which have been shown to inhibit the growth of *Plasmodium chabaudi* in mice without the associated systemic toxicity of native TNF (18). We are currently investigating both the effect of i.t. TNF gene therapy and the systemic administration of synthetic TNF peptides in the treatment of mice with lethal murine *Klebsiella* and *Pseudomonas* pneumonia.

In summary, our studies demonstrate that the administration of *K. pneumoniae* to mice i.t. results in the compartmentalized expression of the important regulatory cytokine TNF. Furthermore, endogenous TNF appears to be an important mediator of lung bacterial clearance and ultimate survival in *Klebsiella* pneumonia. As the treatment of infections becomes increasingly complicated by aggressive immunosuppressive regimens and the emergence of resistant strains of bacteria, including *K. pneumoniae* (5), manipulation of the immune system with cytokine-anticytokine strategies may play an important adjuvant role in the management of patients with severe gram-negative bacterial infection. Moreover, the current wave of enthusiasm regarding the treatment of patients with anti-TNF antibodies or soluble receptors must be tempered by the awareness of potential infectious complications that may occur as a result of this specific therapy.

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