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Intrapulmonary Delivery of Tumor Necrosis Factor Agonist Peptide Augments Host Defense in Murine Gram-Negative Bacterial Pneumonia

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Tumor necrosis factor alpha (TNF) has been shown to be an essential cytokine mediator of innate immunity in *Klebsiella* pneumonia. Recently, a TNF agonist peptide consisting of the 11-amino-acid TNF binding site (TNF_{70-80}) has been shown to possess many of the leukocyte-activating properties of TNF without the associated toxicity when administered locally or systemically. Given the beneficial effects of TNF in gram-negative pneumonia, we hypothesize that the intratracheal (i.t.) administration of TNF_{70-80} would augment lung innate immunity in mice challenged with intrapulmonary *Klebsiella pneumoniae*. The administration of TNF_{70-80} i.t. to CBA/J mice 7 days prior to, but not concomitantly with, the i.t. delivery of 3×10^3 CFU of *K. pneumoniae* resulted in a marked increase in survival compared to that of animals receiving a control peptide i.t. In addition, pretreatment with TNF_{70-80} resulted in improved bacterial clearance, which occurred in association with enhanced lung myeloperoxidase activity (as a measure of lung polymorphonuclear leukocyte influx), and increased expression of the important activating cytokines TNF, macrophage inflammatory protein-2, interleukin-12, and gamma interferon compared that for animals receiving control peptide. Finally, the administration of TNF_{70-80} intraperitoneally resulted in enhanced rather than decreased lethality of *Klebsiella* pneumonia compared to that for animals receiving either TNF_{70-80} or control peptide i.t. Our studies suggest that the intrapulmonary, but not systemic, administration of the TNF agonist peptide may serve as an important immunoadjuvant in the treatment of murine *Klebsiella* pneumonia.

An effective host defense against lung bacterial infection is dependent primarily upon the rapid clearance of the organism from the respiratory tract, which is mediated by the influx and/or activation of phagocytic cells, including neutrophils (polymorphonuclear leukocytes [PMN]) and macrophages (27). The recruitment and activation of leukocytes in the setting of bacterial challenge is a complex and dynamic process which involves the coordinated expression of both pro- and anti-inflammatory cytokines (10–12, 14, 15).

Tumor necrosis factor alpha (TNF) is a 17-kDa cytokine which is a critical component of an effective antibacterial host defense (2, 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 (7, 19, 24). Moreover, PMN and macrophage microbicidal activity is augmented by endogenous or exogenous TNF (22, 26). Finally, TNF is expressed in increased amounts in the airspaces of humans with bacterial pneumonia (21) and in the lungs of mice challenged with bacterial pathogens (9, 18). The inhibition of TNF has been shown to impair lung bacterial clearance and in some but not all studies to attenuate lung PMN influx in response to aerosolized or intratracheally (i.t.) administered *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (2, 9, 15, 18), resulting in markedly decreased survival in animals with bacterial pneumonia (18). However, TNF can mimic many of the detrimental pathophysiologic events that occur in sepsis and sepsis syndrome (1, 28), making it difficult to obtain the beneficial therapeutic effects of TNF without its cytotoxic consequences.

Recently, a TNF agonist peptide composed of the 11 amino acids that constitute the site of binding of native human TNF to its receptors (referred to as TNF_{70-80}) has been characterized (17, 23). Binding of TNF_{70-80} to TNF receptors (both p55 and p75) has been shown to mediate many leukocyte-activating effects of native TNF. Specifically, this peptide directly stimulates and primes neutrophils for enhanced protease release and respiratory burst, enhances neutrophil phagocytic activity, and augments neutrophil killing of *Plasmodium falciparum* in vitro and clearance of *Plasmodium chabaudi* in mice in vivo (17). In that study, TNF_{70-80} was associated with minimal toxicity when administered systemically, due in part to the fact that this peptide did not alter adhesive properties of the endothelium (17).

The emergence of multidrug-resistant microbes in the immunocompromised host has made the treatment of bacterial infections of the lung increasingly difficult (3, 4, 6, 20, 25), underscoring the importance of the immune host defense in determining the eventual outcome of severe pneumonia. Given these clinical dilemmas, we performed this study to determine if the intrapulmonary and/or systemic administration of TNF_{70-80} was capable of augmenting host innate immunity in the setting of murine gram-negative pneumonia.

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MATERIALS AND METHODS

Reagents. The polyclonal antimurine TNF, macrophage inflammatory protein-2 (MIP-2), interleukin-12 (IL-12), and gamma interferon (IFN-γ) antibodies

used in the enzyme-linked immunosorbent assays (ELISAs) were produced by immunization of rabbits with murine recombinant cytokines in multiple intradermal sites with complete Freund's adjuvant (5, 10). Carrier-free murine recombinant TNF, MIP-2, and IFN-γ were purchased from R&D Systems, Minneapolis, Minn., whereas carrier-free murine IL-12 (p75 heterodimer) was a generous gift from the Genetics Institute (Cambridge, Mass.). TNF_{70-80} and control peptides were synthesized at the University of Michigan Protein and Carbohydrate Structure Facility and were composed of the amino acid sequences H-Pro-Ser-Thr-His-Val-Leu-Ile-Thr-His-Thr-Ile-OH and H-Gly-Gly-Asp-Pro-Gly-Ile-Val-Thr-His-Ser-OH, respectively (17). Both peptides were purified by high-pressure liquid chromatography and mass spectrometry and contained no detectable lipopolysaccharide as determined by the *Limulus* lysate assay (ICN Biomedicals, Costa Mesa, Calif.).

Animals. Specific-pathogen-free CBA/J mice (8- 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 until the day of sacrifice.

BAL. Bronchoalveolar lavage (BAL) was performed to obtain alveolar cells. The trachea was exposed and intubated by using a 1.7-mm-outer-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.

***K. pneumoniae* inoculation.** We chose to use *K. pneumoniae* 43816, serotype 2 (American Type Culture Collection, Rockville, Md.), in our studies, as *K. pneumoniae* is a common respiratory tract pathogen clinically and this particular strain has been shown to induce an impressive lobar pneumonia in mice (10–12, 18). *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 absorbance at 600 nm. A standard of absorbencies based on known CFU was used to calculate the inoculum concentration. Bacteria were pelleted by centrifugation at 3,000 rpm in a Beckman GS-6R centrifuge for 15 min, washed twice in saline, and resuspended at the desired concentration. Animals were anesthetized with approximately 1.8 to 2 mg of pentobarbital per animal intraperitoneally (i.p.). The trachea was exposed, and 30 μl of inoculum or saline was administered via a sterile 26-gauge needle. A *K. pneumoniae* dose of 3×10^3 CFU was used in all experiments. The skin incision was closed with surgical staples.

Lung harvesting for cytokine analysis. 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 cytokine 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 2 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) by using a tissue homogenizer (Biospec Products, Inc.). Homogenates were incubated on ice for 30 min and then centrifuged at 2,500 rpm in a Beckman GS-6R 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.

Determination of lung and plasma *K. pneumoniae* CFU. At the time of sacrifice, plasma was collected, the right ventricle was perfused with 1 ml of PBS, and then lungs were removed aseptically and placed in 3 ml of sterile saline. The lungs 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) and incubated for 18 h at 37°C, and then colonies were counted.

Murine cytokine ELISAs. Murine TNF, MIP-2, IL-12, and IFN-γ were quantitated by using a modification of a double-ligand method as previously described (10–12, 18). Briefly, flat-bottomed 96-well microtiter plates (Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) were coated with 50 μl of rabbit anticytokine antibodies (1 mg/ml in 0.6 M NaCl–0.26 M H_3BO_4–0.08 N NaOH, pH 9.6) per well 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, followed by the addition of 50 μl of biotinylated rabbit anticytokine antibodies (3.5 mg/ml in PBS [pH 7.5]–0.05% Tween 20–2% fetal calf serum) per well, and plates were incubated for 30 min at 37°C. The 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 3 M H_2SO_4 solution per well. Plates were read at 490 nm in an ELISA reader. Standards were 1/2-log-unit dilutions of recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine TNF and MIP-2 concentrations of above 25 pg/ml and murine IL-12 and IFN-γ concentrations of above 100 pg/ml. The ELISA did not cross-react with IL-1, IL-2, IL-4, IL-6, or IL-10. In addition, the ELISA did not cross react with other members of the murine chemokine family, including murine JE/MCP-1, MIP-1α, RANTES, KC, GROα, and ENA-78. Importantly, TNF_{70-80} did not cross-react with native murine TNF in the TNF ELISA, nor did TNF_{70-80} alter the ability to detect native TNF.

TABLE 1. Effect of TNF_{70-80} on BAL cell differentials at 24 h, 48 h, and 7 days postadministration[a]

Time	Treatment	Total cells (10^5)	% BAL:		
			Macrophages	PMN	Lymphocytes
24 h	Control	21.4 ± 2.0	96.0 ± 2.0	2.0 ± 0.6	2.0 ± 1.1
	TNF_{70-80}	26.7 ± 8.3	90.0 ± 6.5	6.3 ± 3.8	3.7 ± 0.9
48 h	Control	19.3 ± 7.2	97.0 ± 2.0	1.6 ± 0.4	1.3 ± 1.9
	TNF_{70-80}	25.1 ± 6.6	90.0 ± 2.5	5.7 ± 2.2	4.0 ± 2.5
7 days	Control	18.0 ± 3.8	99.0 ± 1.0	0.4 ± 0.4	0.7 ± 0.2
	TNF_{70-80}	19.9 ± 7.9	98.0 ± 2.0	2.0 ± 1.3	0 ± 0

[a] Animals were treated with 10 μg of TNF_{70-80} or control peptide i.t. Results are means and SEM (n = 5 or 6 per time point).

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

RESULTS

Effect of i.t. administration of TNF_{70-80} on BAL cell differentials. To assess the effects of intrapulmonary administration of TNF_{70-80} alone without bacterial challenge on the development of airspace inflammation, CBA/J mice were administered 10 μg of either control peptide or TNF_{70-80}, and BAL was performed at 24 h, 48 h, and 7 days posttreatment. Although administration of TNF_{70-80} resulted in a modest increase in BAL PMN at 24 and 48 h, this difference was not statistically significant compared to results for animals receiving control peptide i.t. (Table 1). In addition, no differences in total BAL cells or percent BAL macrophages or lymphocytes were observed at any of the time points examined.

Effect of i.t. administration of TNF_{70-80} on survival in *Klebsiella* pneumonia. To determine if the i.t. treatment with TNF_{70-80} influenced survival of animals with *Klebsiella* pneumonia, mice were administered control peptide or 100 ng, 1 μg, or 10 μg of TNF_{70-80} concomitantly with *K. pneumoniae* inoculation; or 10 μg of TNF_{70-80} 7 days prior to *K. pneumoniae* inoculation. Animals were inoculated i.t. with 3×10^3 CFU of *K. pneumoniae*, as this inoculum represents the dose at which approximately 90 to 100% of control animals died. In those animals administered 10 μg of TNF_{70-80} i.t. concomitantly with *K. pneumoniae* inoculation, a modest but statistically insignificant increase in survival was noted compared to animals receiving control peptide i.t. concomitantly with *K. pneumoniae* (Fig. 1). In contrast, animals that received 10 μg of TNF_{70-80} i.t. 7 days prior to *K. pneumoniae* inoculation had significantly improved survival, with 75% of animals surviving long term (>10 days [$P < 0.01$]), compared to 6% of animals pretreated with control peptide.

Effect of i.t. TNF_{70-80} administration on bacterial clearance. To determine if the survival advantages observed in animals pretreated with TNF_{70-80} were attributable to enhanced lung and blood bacterial clearance, mice were administered 10 μg of either control peptide or TNF_{70-80} i.t. 7 days prior to *K. pneumoniae* inoculation (3×10^3 CFU), and then plasma and lungs were harvested 48 h after *K. pneumoniae* administration. As shown in Fig. 2, animals pretreated with TNF_{70-80} had approximately 15-fold fewer *K. pneumoniae* CFU isolated from lung homogenates than did animals treated with the control peptide

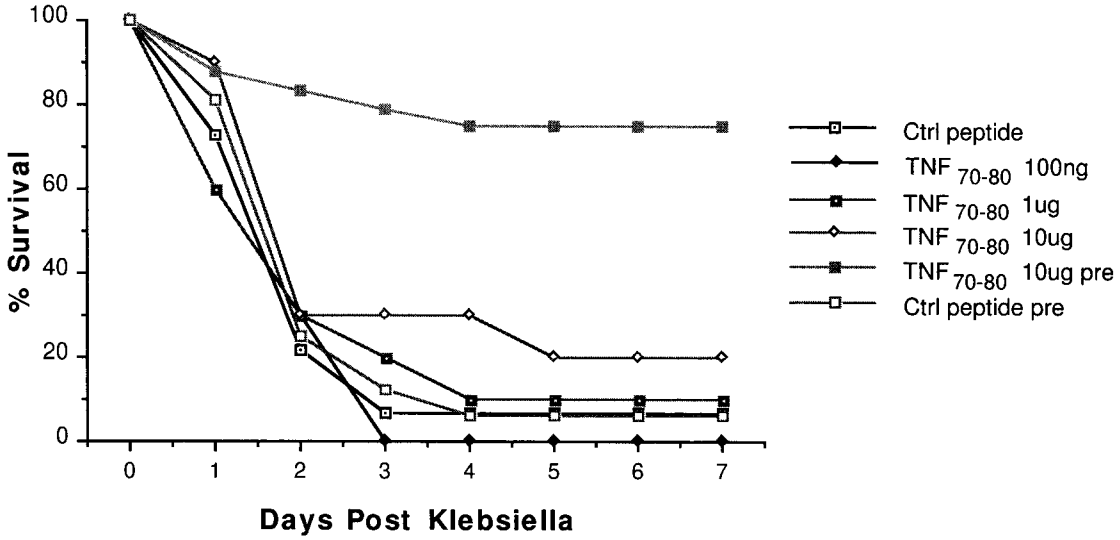

FIG. 1. Effect of TNF_{70-80} on survival in *Klebsiella* pneumonia. CBA/J mice were treated with control peptide (Ctrl) (10 μg) or 100 ng, 1 μg, or 10 μg of TNF_{70-80} concomitantly with *K. pneumoniae* inoculation or with 10 μg of control peptide or TNF_{70-80} 7 days prior to *K. pneumoniae* inoculation (pre). Animals were inoculated i.t. with 3×10^3 CFU of *K. pneumoniae* (n = 10 to 24 per group).

($P < 0.05$). More impressively, plasma from animals treated with the TNF peptide contained >800-fold-fewer bacteria per ml than did that from animals treated with the control peptide ($P \leq 0.01$). Furthermore, 88% of control peptide-treated animals had *K. pneumoniae* isolated from plasma, whereas only 38% of animals given TNF_{70-80} were bacteremic at 48 h postinoculation (data not shown). Importantly, plasma and lung *K. pneumoniae* CFU in animals receiving TNF_{70-80} concomitantly with bacterial administration were not different from those observed in animals receiving control peptide.

Effect of i.t. TNF_{70-80} administration on lung PMN recruitment. To determine whether the enhanced bacterial clearance observed in mice treated with TNF_{70-80} was the result of a more vigorous influx of PMN, animals were treated with either control peptide or TNF_{70-80} 7 days prior to the i.t. inoculation of *K. pneumoniae*. Lungs were harvested 48 h following inoculation and assayed for lung myeloperoxidase (MPO) activity as a measure of lung PMN influx (8). The 48-h time point was examined, as maximal influx of PMN is observed at 48 to 72 h after bacterial administration. Importantly, the i.t. inoculation with *K. pneumoniae* in animals pretreated with TNF_{70-80} resulted in an approximately 1.8-fold increase in lung MPO activity at 48 h compared to that in animals receiving control peptide i.t. (Fig. 3) ($P < 0.05$).

Effect of TNF_{70-80} administration on the production of proinflammatory cytokines within the lung during the evolution of *Klebsiella* pneumonia. Subsequent experiments were performed to determine if the beneficial effects of TNF_{70-80} administration were partially attributable to augmented production of important proinflammatory cytokines. Previous studies in our laboratory and others have demonstrated that several cytokines, particularly TNF, chemokines, and the T1-phenotype cytokines IL-12 and IFN-γ, are necessary components of the cytokine-mediated lung antibacterial host defense in gram-negative infections (2, 9, 10–12, 14, 16, 18). We observed minimal production of TNF, MIP-2, IL-12, and IFN-γ protein in uninfected animals, and these cytokine levels were not altered by i.t. pretreatment with either control peptide or TNF_{70-80} (data not shown). However, in animals challenged with *K. pneumoniae*, the i.t. administration of TNF_{70-80} 7 days prior to *K. pneumoniae* resulted in 1.5-, 1.5-, 1.7-, and 1.5-fold increases in the production of TNF, MIP-2, IL-12, and IFN-γ protein, respectively, within the lung at 48 h compared to those in animals receiving control peptide i.t. (Fig. 4).

Effect of i.p. versus i.t. administration of TNF_{70-80} on survival in *Klebsiella* pneumonia. To determine if the beneficial effects of TNF_{70-80} in bacterial pneumonia required direct intrapulmonary, rather than systemic, delivery, animals were treated with either 10 μg of either TNF_{70-80} i.t., TNF_{70-80} i.p., or control peptide i.t. 7 days prior to *K. pneumoniae* inoculation. As previously noted, survival was significantly increased in animals pretreated with the TNF peptide i.t. compared to animals receiving the control peptide (Fig. 5) ($P \leq 0.01$). Interestingly, significant increases in early lethality were ob-

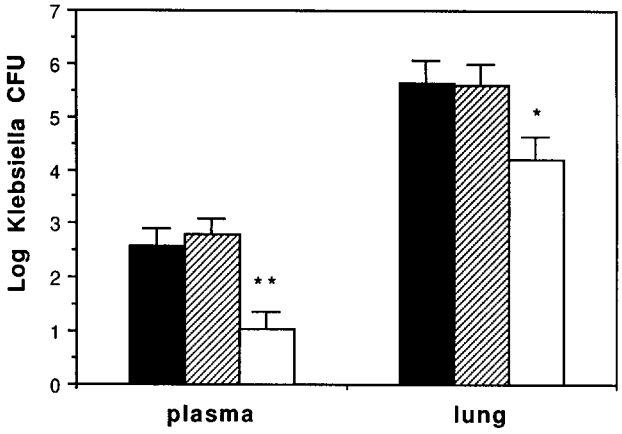

FIG. 2. Log CFU in plasma or lungs of mice challenged with either control peptide or TNF_{70-80} (10 μg). Values shown for lung CFU represent the total quantity of bacteria per whole lung, whereas those for plasma CFU represent the total quantity of bacteria per milliliter of plasma. n = 16 for the control peptide group (■), and n = 19 for the groups given TNF_{70-80} concomitantly with (▨) or 7 days prior to (□) *K. pneumoniae* administration. *, $P < 0.05$; **, $P < 0.01$ (compared to control peptide-treated animals and animals treated with TNF_{70-80} concomitantly with *K. pneumoniae*). Error bars indicate SEM.

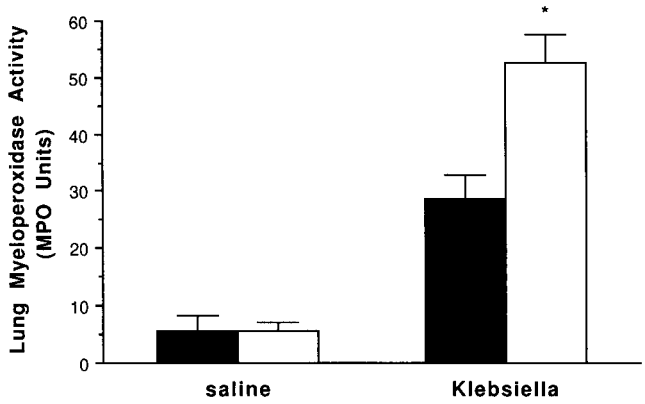

FIG. 3. Effect of TNF_{70-80} on lung MPO activity in *Klebsiella* pneumonia. CBA/J mice were pretreated 7 days prior to *K. pneumoniae* inoculation (3×10^3 CFU) with either control peptide (■) or TNF_{70-80} (□). Whole lung MPO activity was assayed 48 h after *K. pneumoniae* inoculation ($n = 19$ for TNF_{70-80}, $n = 16$ for control peptide, and $n = 6$ for saline). *, $P < 0.05$ compared to control peptide-treated animals. Error bars indicate SEM.

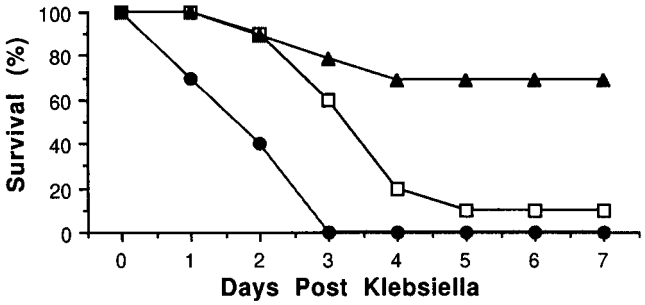

FIG. 5. Effect of i.p. versus i.t. administration of TNF_{70-80} on survival in *Klebsiella* pneumonia. CBA/J mice were passively immunized with either control peptide i.t. (□), TNF_{70-80} i.t. (▲), or TNF_{70-80} i.p. (●) 7 days prior to *K. pneumoniae* inoculation. Animals were inoculated i.t. with 3×10^3 CFU of *K. pneumoniae* ($n = 10$ per group).

served in animals pretreated with TNF_{70-80} i.p. compared to mice receiving control peptide or TNF_{70-80} i.t. followed by *K. pneumoniae* ($P < 0.05$).

DISCUSSION

The present study indicates that the intrapulmonary administration of TNF_{70-80} prior to lung bacterial challenge results in significant increases in lung bacterial clearance and survival in murine *Klebsiella* pneumonia. The mechanism by which pretreatment with TNF_{70-80} enhances lung innate immunity is probably multifactorial. The administration of TNF_{70-80} results in augmented production of the important activating and/or chemotactic cytokines TNF, MIP-2, IL-12, and IFN-γ. While the effect of TNF_{70-80} on individual cytokines is modest (1.5- to 1.7-fold increase), the collective influence of increases in these chemotactic and/or activating cytokines may be additive or even synergistic in the setting of pneumonia. As TNF_{70-80} has previously been shown to prime leukocyte effector cell activities, priming of lung immune cells, particularly alveolar macrophages, is a plausible explanation for the upregulation of cytokine expression. The source of IFN-γ is less clear, but this cytokine may be expressed by resident lung NK and/or T cells in response to TNF_{70-80} directly or indirectly through TNF_{70-80}-induced IL-12 production. TNF_{70-80} has previously been shown to prime human PMN for enhanced superoxide production and degranulation in response to *N*-formyl-Met-Leu-Phe (17). However, it is not likely that the effects observed in this study are due to the priming effects of TNF_{70-80} on PMN in vivo, as few PMN are found within the lung airspaces and interstitium of uninfected animals, and minimal influx of PMN occurs after the i.t. administration of TNF_{70-80} in the absence of bacterial challenge. Augmented intrapulmonary production of MIP-2 and TNF may account for the increased influx and activation of PMN (10, 18, 26), whereas IFN-γ, in addition to TNF, may result in enhanced ingestion and killing of *K. pneumoniae* by alveolar macrophages and infiltrating PMN (14, 22, 26).

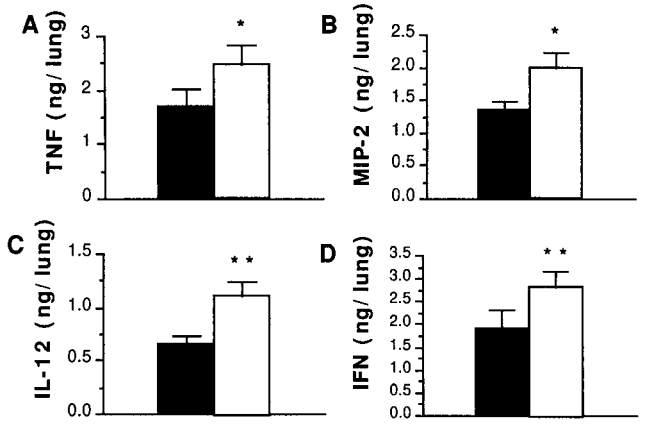

FIG. 4. Effect of TNF_{70-80} administration on lung TNF, MIP-2, IL-12, and IFN-γ levels in *Klebsiella* pneumonia. CBA/J mice were pretreated 7 days prior to *K. pneumoniae* inoculation (3×10^3 CFU) with either control peptide (■) or TNF_{70-80} (□), and lung cytokine levels were determined 48 h after *K. pneumoniae* inoculation ($n = 19$ for TNF_{70-80}, $n = 16$ for control peptide, and $n = 6$ for saline). *, $P < 0.05$; **, $P < 0.01$ (compared to control peptide-treated animals). Error bars indicate SEM.

The i.t. administration of TNF_{70-80} was shown to be beneficial when given to mice 7 days prior to treatment with *K. pneumoniae* but not when given concomitantly with the administration of bacteria. There are several possible explanations to account for these differences. First, the effects of TNF_{70-80} as a priming agent appear to exceed direct activating effects on in vitro leukocyte respiratory burst and degranulation (17, 23). Therefore, pretreatment with TNF_{70-80} may allow for sufficient macrophage priming, whereas concomitant treatment may result in insufficient leukocyte-priming effects. Alternatively, leukocytes release proteolytic enzymes and other toxic substances in response to bacteria within the airspace, with the resultant inflammatory milieu leading to excessive peptide degradation and loss of biologic effects. Last, the administration of TNF_{70-80} concomitantly with bacterial challenge may induce excessive inflammation, resulting in enhanced lung injury and negating the beneficial effects on bacterial clearance. However, lung and blood *K. pneumoniae* CFU from the TNF_{70-80} concomitant treatment group were similar to those observed in animals receiving control peptide i.t. (data not shown), indicating that the bacterial clearances in the two groups were not different. Given this observation, we favor insufficient priming and/or excessive degradation as more likely explanations for the findings observed.

Treatment with TNF_{70-80}, as opposed to the intact TNF, has the distinct advantage of providing beneficial immunoadjuvant effects at doses that are well tolerated when administered locally or systemically. This peptide has been given i.p. to D-galactosamine-sensitized mice at concentrations of 500 mg/kg with no evidence of systemic toxicity, whereas the administra-

tion of native TNF i.p. at a concentration of 0.25 mg/kg resulted in substantial toxicity and in mortality rates as high as 70% (17). The low systemic toxicity of TNF_{70-80} is believed to be due, in part, to the failure of this peptide to stimulate endothelial cell effector activities, including adhesion molecule expression and cell-cell adhesion events. The present study indicates that TNF_{70-80} can be given by the i.p. or i.t. route in relatively large quantities. However, evidence of dose-limiting toxicity in the setting of bacterial challenge was observed, as the intrapulmonary administration of TNF_{70-80} at 50 μg i.t. concomitantly with *K. pneumoniae* administration resulted in increased early and late lethalities compared to those for animals receiving lower concentrations of TNF_{70-80} or control peptide followed by *K. pneumoniae* (data not shown). In addition, the i.p. administration of TNF_{70-80} accelerated lethality in animals challenged i.t. with *K. pneumoniae* compared to infected animals receiving control peptide or TNF_{70-80} i.t. The presence of TNF_{70-80} in the circulation may prime leukocytes such that upon exposure to bacteria or bacterial products (as would occur in pneumonia with bacteremia), an exaggerated systemic release of inflammatory mediators results, analogous to that observed in sepsis syndrome (28). In support of this possibility, animals pretreated with TNF_{70-80} i.p. were more susceptible to the lethal effects of i.p. lipopolysaccharide administration than animals pretreated with control peptide (data not shown). These findings contrast with those of Kumaratilake and colleagues, who found that the systemic administration of TNF_{70-80} resulted in improved clearance of bloodborne *P. chabaudi* in mice without obvious treatment-related deleterious effects (17). The disparity in the effects observed may relate to the propensity for bacteria and bacterial components from gram-negative organisms to induce the release of inflammatory mediators, which would be less likely to occur in the setting of parasitemia.

The most apparent potential clinical application of TNF_{70-80} would be as a prophylactic immunoadjuvant in patients at high risk for the development of bacterial pneumonia. The administration of TNF_{70-80} during an active infection was not effective. However, if mechanisms to improve the bioavailability of this peptide were identified, the applicability of TNF_{70-80} immunotherapy could be expanded to include active lung bacterial infection. Additional studies are required to further define mechanisms by which TNF_{70-80} enhances lung innate immune responses and to determine potential therapeutic applications to human disease.

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