INFECTION AND IMMUNITY, Apr. 1997, p. 1139–1146 Vol. 65, No. 4
0019-9567/97/$04.00+0

Alveolar Macrophages Are Required for Protective Pulmonary Defenses in Murine *Klebsiella* Pneumonia: Elimination of Alveolar Macrophages Increases Neutrophil Recruitment but Decreases Bacterial Clearance and Survival

ELLEN BROUG-HOLUB,[1]* GALEN B. TOEWS,[2] J. FREEK VAN IWAARDEN,[1] ROBERT M. STRIETER,[2] STEVEN L. KUNKEL,[2] ROBERT PAINE III,[2] AND THEODORE J. STANDIFORD[2]

Department of Cell Biology and Immunology, Vrije Universiteit, Amsterdam, The Netherlands,[1] and Division of Pulmonary and Critical Care Medicine, The University of Michigan Medical School, Ann Arbor, Michigan[2]

Received 24 September 1996/Returned for modification 25 November 1996/Accepted 3 January 1997

To study the in vivo role of alveolar macrophages (AM) in gram-negative bacterial pneumonia in mice, AM were eliminated by the intratracheal (i.t.) administration of dichloromethylene diphosphonate encapsulated liposomes. Subsequently, the AM-depleted mice were infected i.t. with 100 CFU of *Klebsiella pneumoniae*, and the effects of AM depletion on survival, bacterial clearance, and neutrophil (polymorphonuclear leukocyte [PMN]) recruitment were assessed. It was shown that depletion of AM decreases survival dramatically, with 100% lethality at day 3 postinfection, versus 100% long-term survival in the control group. This increased mortality was accompanied by 20- to 27- and 3- to 10-fold increases in the number of *K. pneumoniae* CFU in lung and plasma, respectively, compared to those in nondepleted animals. This decreased bacterial clearance was not due to an impaired PMN recruitment; on the contrary, the *K. pneumoniae*-induced PMN recruitment in AM-depleted lungs was sevenfold greater 48 h postinfection than that in control infected lungs. Together with an increased PMN infiltration, 3- and 10-fold increases in lung homogenate tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein 2 (MIP-2) levels, respectively, were measured. Neutralization of TNF-α or MIP-2, 2 h before infection, reduced the numbers of infiltrating PMN by 41.6 and 64.2%, respectively, indicating that these cytokines mediate PMN influx in infected lungs, rather then just being produced by the recruited PMN themselves. Our studies demonstrate, for the first time, the relative importance of the AM in the containment and clearance of bacteria in the setting of *Klebsiella* pneumonia.

Alveolar macrophages (AM) are strategically situated at the air-tissue interface in the alveoli and alveolar ducts and are therefore the first cells that encounter inhaled organisms and antigens in the lower respiratory tract. AM not only act in their traditional role as phagocytes but also function as potent secretory cells. Upon appropriate stimulation, AM can release a wide variety of biologically active products (25, 35), thereby playing an important role in regulating inflammatory reactions within the lung. A great deal about the in vivo immunoregulatory capacities of AM has been learned from studies in which AM were eliminated by the intratracheal (i.t.) administration of liposomes containing the drug dichloromethylene diphosphonate (DMDP-liposomes). The depletion of macrophages is based on accumulation of the drug in phagolysozomes via avid phagocytosis of the DMDP-liposomes, resulting in selective killing of the phagocytes without damage to surrounding tissues or other cell types (50). Elimination of AM by this method is >80% and lasts for >5 days (4, 43). By using this method, it was shown that AM play a very important role in downregulation of local T-cell proliferation upon antigen deposition in the lower lung (43) and in suppression of the antigen-presenting cell activity of lung dendritic cells in situ (13). Furthermore, AM depletion results in an attenuated initial polymorphonuclear leukocyte (PMN) recruitment and cytokine production in the lung in response to i.t.-administered lipopolysaccharide (LPS) or *Pseudomonas aeruginosa* infection (4, 11). From these studies, it appears that the presence of AM in the lower lung is indispensable for an adequate response to stimuli threatening pulmonary homeostasis.

The mechanism(s) by which AM mediate PMN influx and activation in response to inflammatory stimuli is unclear but is likely dependent on the expression of specific macrophage-derived PMN chemotactic and activating cytokines. Two cytokines that have been shown to play a crucial role in innate immunity against bacterial pathogens are tumor necrosis factor α (TNF-α) and macrophage inflammatory protein 2 (MIP-2). An important role for TNF-α in host defense against bacterial invasion is indicated by several studies (12, 24, 52). TNF-α activates macrophages to kill intracellular pathogens and is directly involved in neutrophilic inflammation of the airways (4, 16, 49, 53). This involvement is twofold: TNF-α activates endothelial cells, resulting in upregulation of intracellular adhesion molecule 1 expression, a vital component for PMN recruitment (22, 23), and it induces the production of chemokines in an autocrine and paracrine manner (19, 21, 36). MIP-2 is a C-X-C chemokine that possesses chemotactic activity for PMN both in vitro and in vivo (1, 8, 54). Murine MIP-2 is speculated to perform the functions of human interleukin-8 (IL-8) in mice (33). Recently, an important role was ascribed to MIP-2 in mediating lung PMN recruitment in LPS-induced lung injury in rats (34) and in lung PMN recruitment and bacterial clearance in murine *Klebsiella* pneumonia (10). The cellular source for MIP-2 is predominantly the AM. However, infiltrating PMN also ap-

* Corresponding author. Mailing address: Department of Cell Biology and Immunology, Faculty of Medicine, Vrije Universiteit, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. Phone: (31)(20)4448079. Fax: (31)(20)4448081. E-mail: E.Holub.Cell@med.vu.nl.

pear to represent a cellular source of MIP-2 in the setting of pneumonia (55).

In this study, we sought to further elucidate the in vivo role of the AM in long-term gram-negative bacterial pneumonia. Therefore, we selectively removed AM from the lungs by i.t. instillation of DMDP-liposomes, providing animals deficient in functional AM. These AM-depleted mice were subsequently infected with 100 CFU of *Klebsiella pneumoniae*, an inoculum size which is normally sublethal and causes little illness in normal mice. The relative importance of AM in the setting of gram-negative pneumonia was assessed by studying the effect of AM depletion on survival, bacterial clearance, and PMN recruitment following this mild infection.

MATERIALS AND METHODS

Reagents. Murine recombinant TNF-α and MIP-2 used for the generation of antibodies and standards for enzyme-linked immunosorbent assay (ELISA) were purchased from R&D Systems (Minneapolis, Minn.). Polyclonal antimurine TNF-α and MIP-2 antisera used in ELISA were produced by immunization of rabbits with recombinant murine TNF-α or MIP-2 in multiple intradermal sites with complete Freund's adjuvant. The soluble TNF receptor-immunoglobulin (Ig) fusion protein (sTNFR:Fc) was a generous gift from Immunex Corporation, Seattle, Wash. sTNFR:Fc is composed of soluble chimeric human p80 TNF receptor linked to the Fc region of human IgG1, which results in a greater efficacy in neutralizing TNF bioactivity than that of monomeric soluble TNF receptor (27).

Animals. Specific-pathogen-free CBA/J mice (6- to 8-week-old females; Charles River Breeding Laboratories, 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.

Liposomes. The liposomes were composed of egg phosphatidylcholine and cholesterol and contained either phosphate-buffered saline (PBS) or DMDP (a kind gift of Boehringer GmbH, Mannheim, Germany) dissolved in H_2O (0.25 g/ml) as described previously (51). Briefly, 86 mg of egg phosphatidylcholine (Sigma) and 8 mg of cholesterol were dissolved in 10 ml of chloroform; a lipid film was produced by low-vacuum rotary evaporation and dispersed in either 10 ml of PBS or DMDP solution. The suspension was kept at room temperature for 2 h, after which it was sonicated for 3 min in a water bath sonicator and again kept for 2 h at room temperature. The liposome solution was diluted to 50 ml with PBS and centrifuged at 15,000 × *g* for 30 min to remove free DMDP. Liposomes were resuspended in 4 ml of PBS. Liposome size was always checked by dark-field microscopy.

Intratracheal administration of liposomes. Mice were anesthetized with approximately 1.8 to 2 mg of pentobarbital per animal intraperitoneally (i.p.). The trachea was exposed, and 30 μl of either DMDP- or PBS-liposomes or saline was administered via a sterile 26-gauge needle. The skin incision was closed with a suture to make the wound accessible for a second *K. pneumoniae* inoculation 24 h later.

***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, 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 660 nm. A standard of absorbancies based on known CFU was used to calculate the inoculum concentration. Bacteria were pelleted by centrifugation at 10,000 rpm for 30 min, washed twice in saline, and resuspended at the desired concentration. Each animal was anesthetized with approximately 1.8 to 2 mg of pentobarbital i.p. The trachea was exposed, and 30 μl of inoculum containing 100 CFU of *K. pneumoniae* or saline was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples. In neutralizing experiments, 0.5 ml of specific anti-MIP-2 neutralizing antibody or sTNFR:Fc (100 μg/animal) was injected i.p. 2 h before bacterial inoculation.

Lung harvesting for cytokine analysis and histologic examination. At designated time points, the mice were anesthetized with inhaled methoxyflurane, blood was collected by orbital bleeding, and the animals were sacrificed. Whole lungs were then harvested for assessment of TNF-α and MIP-2 protein expression. Prior to lung removal, the pulmonary vasculature was perfused with 1 ml of PBS containing 5 mM EDTA, via the right cardiac 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 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. For histologic examination, lungs were excised en bloc without perfusion and inflated with 1 ml of 4% paraformaldehyde in PBS to improve resolution of anatomic relationships.

BAL. Bronchoalveolar lavage (BAL) was performed to obtain intra-alveolar cells. The trachea was exposed and intubated by using a 1.7-mm-outer-diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1-ml aliquots with a final volume of 5 ml. Cytospins were prepared from BAL cells and stained with Diff-Quick (Baxter, McGaw Park, Ill.).

Lung MPO assay. Lung myeloperoxidase (MPO) activity was quantitated as described previously (29). Briefly, whole lungs were homogenized in 2 ml of 50 mM potassium phosphate (pH 6.0) with 5% hexadecyltrimethylammonium bromide and 5 mM EDTA. The resultant homogenate was sonicated and centrifuged at 3,000 × *g* for 15 min. The supernatant was mixed 1:15 with assay buffer and read at 490 nm. MPO units were calculated as the change in absorbance over time.

Determination of plasma and lung CFU. At the time of sacrifice, plasma was collected, and then 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 plasma and 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, whereafter colonies were counted.

Cytokine ELISAs. Murine TNF-α and MIP-2 were quantitated by using a modification of a double ligand method as previously described (39). Briefly, each well of flat-bottom 96-well microtiter plates (Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) was coated with 50 μl of either rabbit anti-TNF-α antibody or rabbit anti-MIP-2 antibody (each at a concentration of 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 50-μl aliquots of cell-free supernatants (neat and 1:10 diluted) 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-α or anti-MIP-2 antibody (3.5 mg/ml in PBS [pH 7.5]–0.05% Tween 20–2% fetal calf serum) was added to each 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. The plates were washed again four times, and chromogen substrate (Bio-Rad) 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 dilutions of recombinant murine TNF-α or MIP-2 from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine TNF-α or MIP-2 concentrations above 25 pg/ml. The ELISA did not cross-react with IL-2, IL-4, IL-6, or gamma interferon. In addition, the ELISA did not cross-react with other members of the murine chemokine family, including murine JE/MCP-1, RANTES, KC, GROα, or ENA-78.

Isolation and reverse transcription-PCR amplification of whole-lung mRNA. Whole lungs were harvested at specific times after inoculation with *K. pneumoniae*. Total cellular RNA was isolated by homogenizing the lungs with a tissue homogenizer in 3 ml of Trizol solution (Gibco). After homogenization, RNA was extracted with phenol-chloroform and precipitated with isopropanol. The RNA pellet was washed with alcohol and dissolved in diethylpyrocarbonate-treated water. Total RNA was determined by spectrometric analysis at a wavelength of 260 nm. Five micrograms of total RNA was reverse transcribed into cDNA by using 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-α, with β-actin primers serving as a control. The TNF-α sense and antisense primers used had the sequences 5′-CCTGTAGCCCACGTAGTAGC-3′ and 5′-TTGACCTCAGCGCTGAGTTG-3′, respectively, giving an amplified product of 380 bp. The β-actin sense and antisense primers used had the sequences 5′-ATGGATGACGATATCGCTC-3′ and 5′-GATTCCATACCCAGGAAGG-3′, respectively, giving an amplified product of 812 bp. The amplification buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2 mM MgCl. Specific oligonucleotide primers were added (200 ng/sample) to the buffer, along with 5 μl of the reverse-transcribed cDNA samples. The mixture was first incubated for 5 min at 94°C, cycled 30 (TNF) or 32 (actin) times at 93°C for 45 s and at 50°C (TNF) or 52°C (actin) for 45 s, and elongated at 72°C for 80 s. After amplification, the sample (8 μl) was separated on a 2% agarose gel containing ethidium bromide (0.3 mg/ml [0.003%]), and bands were visualized and photographed by using UV transillumination.

Statistical analysis. Data were analyzed on a Macintosh II computer, using the Statview II statistical package (Abacus Concepts, Inc., Berkeley, Calif.). Survival data were compared by using chi-square analysis. All other data are expressed as means ± standard errors of the means (SEM) and compared by using a two-tailed Student *t* test. Data were considered statistically significant if *P* values were less than 0.05.

RESULTS

Elimination of AM in vivo by i.t. instillation of DMDP-liposomes. In mice, elimination of AM can be accomplished by a single i.t. injection of 100 μl of DMDP-liposomes. It was

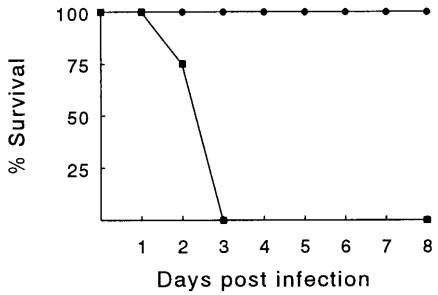

FIG. 1. Effect of AM depletion on survival in *Klebsiella* pneumonia. CBA/J mice were depleted of AM by i.t. administration of 30 μl of DMDP-liposomes (closed squares) or received saline (closed circles) i.t. 24 h prior to inoculation with 100 CFU of *K. pneumoniae* ($n = 9$ per group).

previously shown that within 2 to 3 h after administration of 100 μl of DMDP-liposomes, >80% of the AM were depleted and depletion lasted for >5 days (5). Since we wished to study the effects of AM depletion on the outcome of bacterial infection, we decreased the dose of DMDP-liposomes to limit the impact of nonphagocytosed liposome remnants on newly recruited phagocytic cells while effectively reducing AM number and function. Using the same DMDP-liposomes, we injected mice i.t. with 30 μl of liposome suspension; 24 h later, the extent of depletion was assessed by BAL. Administration of DMDP-liposomes resulted in a 65% reduction in AM numbers compared to those in animals receiving either saline or PBS-liposomes. Furthermore, together with a significant decrease in the number of AM, administration of DMDP-liposomes resulted in an increase of PMN in the lung, ranging between 40 and 65% of total BAL cells (data not shown).

Effects of AM depletion on survival in *Klebsiella* pneumonia. Our initial experiments were designed to characterize the in vivo role of AM in the outcome of *Klebsiella* pneumonia in mice. Therefore, AM were depleted by the i.t. instillation of 30 μl of DMDP-liposomes 24 h prior to the inoculation of 100 CFU of *K. pneumoniae*. Control animals received a similar volume of saline i.t. A low inoculum size of *K. pneumoniae* was chosen to maximize the chances for detecting differences between depleted infected and nondepleted infected mice. As shown in Fig. 1, survival in AM-depleted animals decreased substantially 48 h postinoculation, with 100% lethality noted by day 3 postinoculation. In contrast, all of the nondepleted infected animals overcame the pneumonia, with no mortality observed during the 8 day postinfection follow-up. Importantly, animals treated with DMDP-liposomes alone did not appear ill, and no lethality was observed. This finding indicates to an important role for the AM in the initial bacterial clearance from the respiratory tract and in the onset of an inflammatory reaction.

Effects of AM depletion on bacterial clearance. Having found striking increases in mortality in AM-depleted infected animals, we determined whether AM depletion resulted in impaired lung bacterial clearance. Mice received DMDP-liposomes i.t. 24 h before *K. pneumoniae* inoculation. To ensure that the observed effects were not due to the liposomes themselves, separate groups of mice received either 30 μl of PBS-liposomes or saline i.t. as a control. Forty-eight hours after *K. pneumoniae* administration, plasma was collected, lungs were harvested, and serial dilutions were made from plasma and lung homogenates and plated on tryptic soy agar plates. As shown in Table 1, there were 20- to 27-fold and 3- to 10-fold increases in the numbers of *K. pneumoniae* CFU isolated from lung homogenates and plasma, respectively, of AM-depleted animals compared to lung and plasma samples obtained from either PBS-liposome- or saline-treated mice. A modest increase in the number of CFU in lung obtained from PBS-liposome-treated mice compared to control mice was observed; however, this was not significant, demonstrating that liposomes themselves did not influence bacterial clearance. Furthermore, six of nine saline-treated and seven of nine PBS-liposome-treated animals had no *K. pneumoniae* in plasma isolated 48 h postinoculation, whereas six of nine AM-depleted animals were bacteremic at this time point (data not shown).

TABLE 1. Effects of depletion of AM on *K. pneumoniae* CFU in plasma and lung 48 h after i.t. inoculation with 100 CFU of *K. pneumoniae*

Pretreatment	Mean log CFU ± SEM in:	
	Plasma	Lung
Saline	0.36 ± 0.25	1.71 ± 0.62
PBS-liposomes	0.11 ± 0.08	2.34 ± 0.46
DMDP-liposomes	1.12 ± 0.32[a]	4.56 ± 0.59[a]

[a] $P < 0.03$ compared with CFU in animals pretreated with saline or PBS-liposomes. Each group contained nine animals.

Effect of AM depletion on lung PMN recruitment. To determine whether the decreased bacterial clearance observed in AM-depleted mice was a result of an impaired recruitment of PMN, AM were eliminated 24 h prior to the i.t. administration of *K. pneumoniae*. Lungs were harvested 48 h after inoculation and assayed for lung MPO activity. The i.t. inoculation with *K. pneumoniae* in AM-depleted mice resulted in a nearly threefold increase in lung MPO activity at 48 h ($P < 0.01$) compared to animals which received saline i.t. (Fig. 2). At this time point, no enhanced MPO activity was demonstrated in lungs from control *K. pneumoniae*-infected lungs compared to noninfected lungs, confirming previous observations that an inoculum size of 100 CFU represents a sublethal challenge, inducing minimal inflammation in normal lungs. Although the i.t. instillation of DMDP-liposomes alone consistently resulted in a slight increase in MPO activity, this was not significantly different from the result for the saline controls. These data suggest that the decreased survival and bacterial clearance noted in AM-depleted mice does not result from an impaired capacity to recruit PMN to the lung. On the contrary, based on MPO data, the recruitment of PMN in AM-depleted lungs was greater than that observed in control lungs.

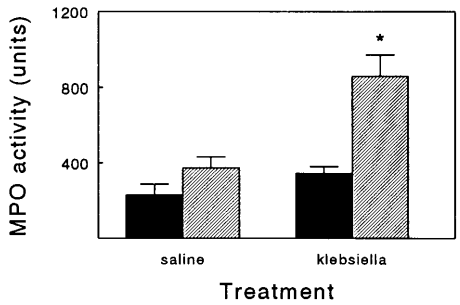

FIG. 2. Effect of AM depletion on lung MPO activity in *Klebsiella* pneumonia. CBA/J mice received 30 μl of DMDP-liposomes (hatched bars) or saline (solid bars) i.t. 24 h prior to inoculation with 100 CFU of *K. pneumoniae* or saline. MPO activity was measured in total lung homogenates 48 h after challenge with either i.t. saline or *K. pneumoniae* ($n = 9$ per group). Data are expressed as means ± SEM. *, $P < 0.01$ compared with infected animals pretreated with saline.

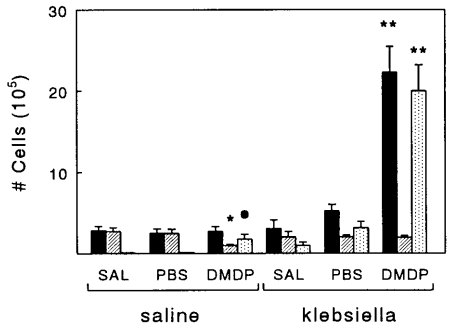

FIG. 3. Effect of AM depletion on *K. pneumoniae*-induced inflammatory cell recruitment to the lung. CBA/J mice received 30 μl of DMDP-liposomes (DMDP), PBS-liposomes (PBS), or saline (SAL) i.t. 24 h prior to inoculation with 100 CFU of *K. pneumoniae* (klebsiella) or saline (saline). BAL was performed 48 h postinfection, total cell numbers (solid bars) were counted, and numbers of AM (hatched bars) and PMN (dotted bars) were calculated from Diff-Quick-stained cytospins ($n = 7$ per group). Data are expressed as means ± SEM. *, $P < 0.02$, and ●, $P < 0.03$ compared with noninfected animals pretreated with saline or PBS-liposomes; **, $P < 0.005$ compared with *K. pneumoniae*-infected animals pretreated with saline or PBS-liposomes.

Development of pulmonary inflammation after i.t. inoculation of *K. pneumoniae*. To confirm the increased PMN influx induced by *K. pneumoniae* in AM-depleted mice, we performed BAL 48 h postinfection to determine the total number and phenotype of the inflammatory cells. To exclude a nonspecific effect of liposomes on inflammatory cell recruitment, control mice received either PBS-liposomes or saline i.t. prior to infection. As shown in Fig. 3, the total number of BAL cells obtained from AM-depleted, *K. pneumoniae*-infected mice was approximately 7- or 4-fold higher than the amount of BAL cells obtained from the infected controls, pretreated with saline or PBS-liposomes, respectively.

Total BAL cell numbers did not differ significantly between saline- and PBS-liposome-pretreated groups, demonstrating that the observed increased inflammatory response in the DMDP-treated mice is not due to nonspecific effects of liposomes themselves. The increased BAL cell numbers were entirely due to increased numbers of PMN, as assessed by examination of Diff-Quick-stained cytospin preparations. Histologic examination of the lungs of saline-, PBS-liposome-, and DMDP-liposome-treated animals revealed the presence of substantial numbers of intra-alveolar and interstitial PMN (Fig. 4A to C). However, only in the AM-depleted animals were large numbers of *K. pneumoniae* organisms present, filling the alveolar spaces (Fig. 4C). Furthermore, cytospin preparations of BAL cells from AM-depleted lungs showed that the recruited PMN were actively phagocytosing, as they were filled with *K. pneumoniae* organisms (Fig. 4F). Although *K. pneumoniae* organisms were abundantly present in the cytoplasm of both PMN and AM in the BAL cells from depleted animals 48 h postinfection, no intracellular bacteria were detected in BAL cells retrieved from saline- or PBS-liposome-treated mice at this time point (Fig. 4D and F).

Production of proinflammatory cytokines and chemokines within the lung after i.t. inoculation of *K. pneumoniae*. Since a striking difference in the recruitment of inflammatory cells was found between lungs of depleted and control *K. pneumoniae*-infected mice, we next performed experiments to characterize the production of important activating and chemotactic cytokines within the lung during the development of *Klebsiella* pneumonia. As shown in Fig. 5, a significant increase in the levels of TNF-α and MIP-2 was noted in homogenates from AM-depleted lungs 48 h after the i.t. administration of 100 CFU of *K. pneumoniae*. With this low inoculum of *K. pneumoniae*, no enhanced levels of these cytokines were detected in homogenates of nondepleted infected lungs. Furthermore, administration of DMDP-liposomes alone did not increase the basal levels of cytokines and chemokines. These studies indicate that the greater magnitude of lung inflammation in AM-depleted mice correlates with the enhanced expression of proinflammatory cytokines within the lung.

Time-dependent mRNA expression in lung homogenates. Our initial studies revealed significant increases in the expression of TNF-α and MIP-2 in AM-depleted lungs 48 h after induction of *Klebsiella* pneumonia, accompanied by high numbers of infiltrated PMN. However, these recruited PMN may also be the source of the detected proinflammatory cytokines. To ascertain the mechanism of PMN recruitment in the infected AM-depleted lungs, we isolated mRNA from total lung homogenates 6 and 24 h after the administration of *K. pneumoniae* and assessed the presence of TNF-α and MIP-2 mRNAs by reverse transcription-PCR. The i.t. administration of *K. pneumoniae* resulted in significant increases in TNF-α mRNA in homogenates from AM-depleted lungs by 24 h (Fig. 6). In contrast, the level of TNF-α mRNA in control *K. pneumoniae*-infected lungs was not greater than in normal noninfected lungs, nor did depletion of AM by itself increase the total lung TNF-α mRNA level. At these time points, MIP-2 mRNA was undetectable in either control or DMDP-treated mice infected with *K. pneumoniae* (data not shown). The enhancement in TNF-α message in AM-depleted lungs 24 h postinfection suggests that TNF-α may play a role in the observed PMN recruitment.

Effect of anti-MIP-2 serum or sTNFR:Fc on PMN recruitment. Although the enhanced TNF-α and MIP-2 mRNA and/or protein levels in AM-depleted infected lungs suggest a possible causal role of these cytokines in PMN recruitment, it could not be excluded that these infiltrated PMN themselves were the source of TNF-α or MIP-2. To determine the biologic relevance of TNF-α and MIP-2 as mediators of PMN influx in *Klebsiella* pneumonia in AM-depleted mice, animals were injected i.p. with 0.5 ml of either sTNFR:Fc (100 μg/animal) or anti-MIP-2 serum 2 h before i.t. administration of *K. pneumoniae*. Lungs were lavaged 48 h postinfection, total BAL cells were counted, and differentials were made. The results (Fig. 7) show that systemic neutralization of TNF-α and neutralization of MIP-2 in AM-depleted mice significantly reduces the number of infiltrating PMN in BAL by 41.6% ($P < 0.05$) and 64.2% ($P < 0.05$) respectively, compared to DMDP-liposome-treated animals receiving control IgG. A slight but nonsignificant increase in AM was observed in the lungs of these animals. These AM were small and morphologically resembled blood monocytes, suggesting that these AM were recently derived from circulating monocytes. These data confirm that TNF-α and MIP-2 mediated the initial recruitment of PMN in AM-depleted infected lungs, rather than just being produced by the infiltrated PMN themselves.

DISCUSSION

Effective host defense against lung bacterial infection is primarily dependent on rapid clearance of the organisms from the respiratory tract. AM are considered to play a prominent role in this process by phagocytosis of airborne microorganisms and by orchestrating the inflammatory response against these incoming foreign particles. So far, however, evidence for this regulatory role in pulmonary immune responses is provided in an indirect manner. In this study, a direct approach was used to examine the role of AM in *Klebsiella* pneumonia. Therefore,

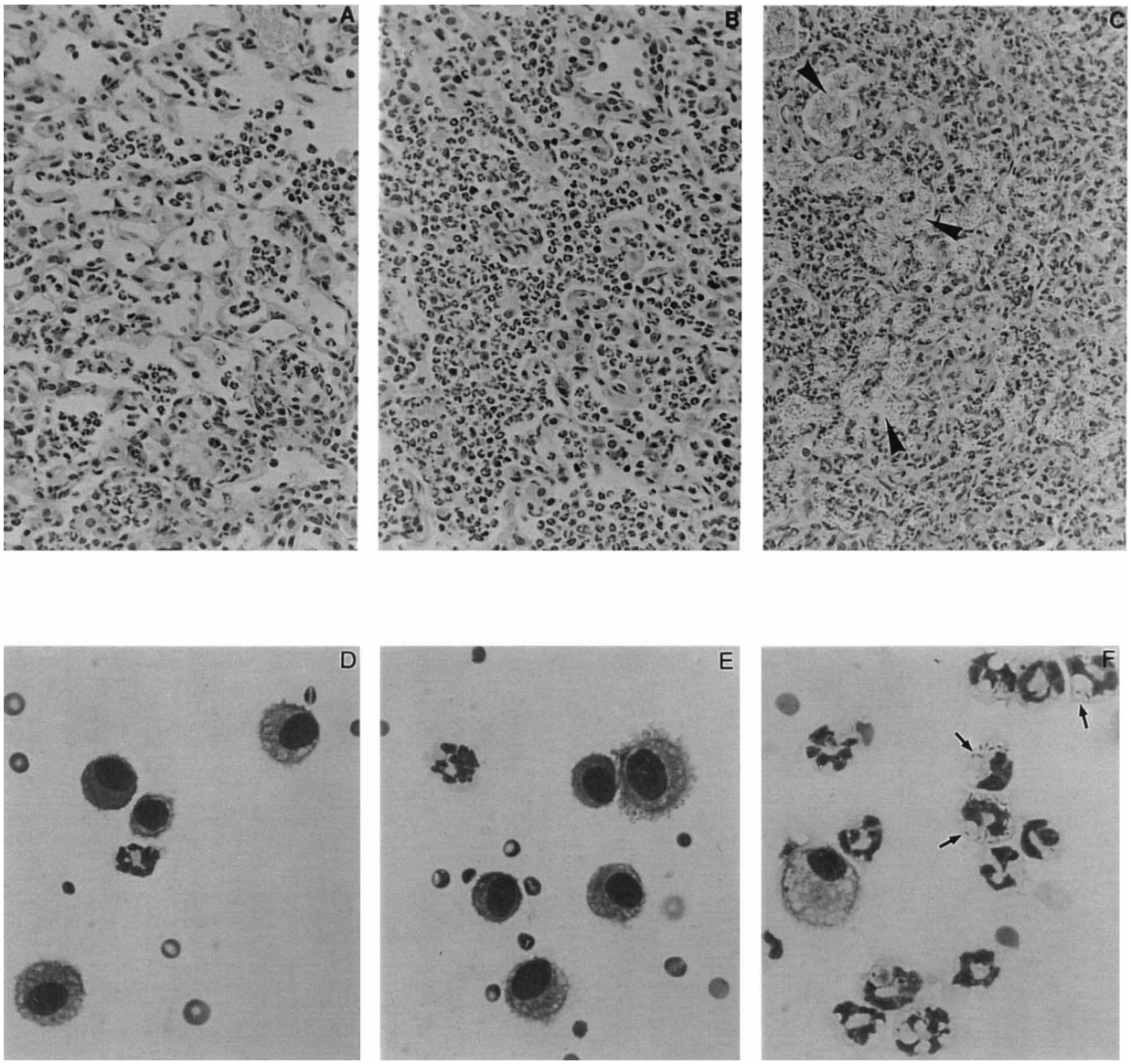

FIG. 4. Composite histologic sections and BAL differentials from *K. pneumoniae*-challenged mice pretreated i.t. with saline, PBS-liposomes, or DMDP-liposomes. (A to C) Histologic sections of lung 48 h after i.t. administration of *K. pneumoniae* (100 CFU) in saline-pretreated, PBS-liposome-pretreated, and AM-depleted mice, respectively. (D to F) BAL differentials from saline-pretreated, PBS-liposome-pretreated, and AM-depleted *K. pneumoniae*-challenged animals at 48 h postinfection, respectively. Arrows, intracellular presence of *K. pneumoniae* organisms in PMN and AM; arrowheads, intra-alveolar pockets of *K. pneumoniae* organisms.

mice were depleted of AM by the i.t. instillation of DMDP-liposomes, and subsequently the lungs were infected with a low dose of *K. pneumoniae.*

The results show that AM-depleted mice are unable to recover from this low-dose bacterial challenge and die within 72 h of inoculation, whereas 100% long-term survival is observed in the control group. This increased mortality is accompanied by an enhanced number of *K. pneumoniae* CFU in both plasma and lung 48 h postinfection. While this impaired bacterial clearance could have resulted from impaired PMN recruitment in AM-depleted infected lungs, the opposite was observed. Our results clearly show that even in the absence of functional AM, PMN are recruited in large numbers to the infected airspace. These PMN are actively phagocytosing as their cytoplasm is filled with vacuoles containing *K. pneumoniae* organisms. In this respect, it is noteworthy that PMN are neither morphologically nor functionally affected by DMDP-liposomes in vivo (28). Moreover, in vitro studies indicate that DMDP-liposomes do not alter phagocytosis and killing of bacteria by PMN (data not shown). Despite the presence of these large numbers of PMN, bacterial clearance from the lung was grossly inadequate in the absence of functional AM.

The mechanism(s) by which PMN are recruited to the lung in AM-depleted animals is unclear. It is commonly believed that the initiation of the inflammatory response after encounter of airborne pathogens begins with a cascade of AM-derived cytokines that subsequently leads to enhanced expression of both vascular and leukocyte adhesion molecules and to induction of local chemokine production, both indispensable conditions for efficient PMN recruitment (21–23, 35, 36, 45–48, 53, 55). However, even when the AM population was substantially

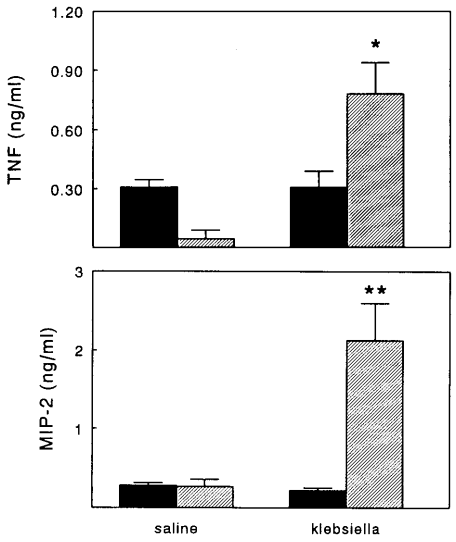

FIG. 5. Production of TNF-α and MIP-2 in total lung homogenates from AM-depleted and nondepleted mice 48 h after i.t. administration of *K. pneumoniae* (100 CFU). CBA/J mice received 30 μl of DMDP-liposomes (hatched bars) or saline (solid bars) i.t. 24 h prior to inoculation with 100 CFU of *K. pneumoniae* (klebsiella) or saline (saline). Lungs were homogenized 48 h postinfection, and the presence of cytokines was assessed by specific ELISA ($n = 9$ per group). Data are expressed as means ± SEM. *, $P < 0.03$, and **, $P < 0.004$ compared with *K. pneumoniae*-infected animals pretreated with saline.

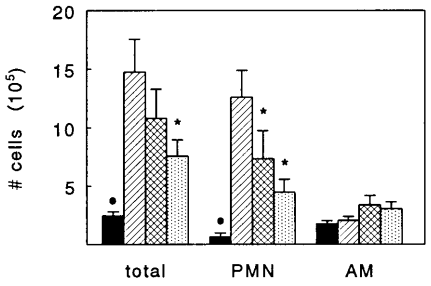

FIG. 7. Effect of soluble TNF-receptor:Fc (sTNFR:Fc) chimeras or anti-MIP-2 serum on *K. pneumoniae*-induced inflammatory cell recruitment to AM-depleted lungs. AM-depleted mice were pretreated with 0.5 ml of sTNFR:Fc (cross-hatched bars), anti-MIP-2 serum (dotted bars), or preimmune serum (hatched bars) 2 h prior to inoculation with 100 CFU of *K. pneumoniae*. Nondepleted control mice were pretreated with preimmune serum prior to inoculation with bacteria (solid bars). BAL was performed 48 h postinfection, total cell numbers were counted, and numbers of PMN and AM were calculated from Diff-Quick-stained cytospins ($n = 10$ per group). Data are expressed as means ± SEM. *, $P < 0.05$ compared with *K. pneumoniae*-infected animals pretreated with saline; ●, $P < 0.05$ compared with *K. pneumoniae*-infected animals pretreated with DMDP-liposomes with or without neutralization of TNF or MIP-2.

depleted, we consistently demonstrated high levels of TNF-α and MIP-2 protein in total lung homogenates, together with a massive PMN influx 48 h postinfection. Furthermore, TNF-α mRNA, but not MIP-2 mRNA, levels are enhanced in AM-depleted lungs 24 h after infection. Thus, there does not appear to be a deficiency in the production of activating and chemotactic cytokines in AM-depleted lungs. To exclude the possibility that these cytokines and chemokines are produced by the infiltrated PMN, rather than inducing their recruitment, neutralization experiments were performed. Systemic neutralization of either TNF-α or MIP-2 prior to infection resulted in a significant reduction in infiltrating PMN in the AM-depleted lungs, providing evidence that these cytokines are indeed required for PMN recruitment. The question remains as to which cells are the source for these elevated cytokine levels. Studies by Berg et al. (4) and Hashimoto et al. (11) showed that 4 h after i.t. administration of either LPS or *P. aeruginosa* in AM-depleted rat lungs, the number of infiltrating PMN was markedly reduced compared to that of nondepleted animals. This decreased influx was accompanied by lower levels of TNF-α, MIP-2, and CINC/*gro* (cytokine-induced neutrophil chemoattractant) in lavage fluid 4 h after induction of inflammation (4, 11), suggesting a critical role for the AM in the initial recruitment of PMN into infected lungs. However, from our studies, it is evident that if the initial response by AM fails to occur, other pulmonary cells adopt this function and take care of proper PMN recruitment. Furthermore, although TNF-α is predominantly produced by mononuclear phagocytes in vivo, a number of other cells, including endothelial cells, mast cells, smooth muscle cells, lymphocytes, B cells, and T cells, are capable of secreting TNF-α (9, 44). Moreover, interstitial macrophages, which are not affected by DMDP treatment (50), might be an important source of TNF-α. Likewise, the production of chemokines is not restricted to macrophage populations (33). Recent studies showed that alveolar epithelial cells could participate in inflammatory cell recruitment by secreting IL-8 (37), RANTES (49), and MCP-1 (26, 38) either upon stimulation with proinflammatory cytokines or directly in response to LPS (26). In addition to elaborating chemokines, alveolar epithelial cells can influence inflammation via synthesis and secretion of the complement components C2, C3, C4, and C5 (40), which can initiate and amplify chemotaxis of leukocytes, activation of phagocytes, and opsonization of bacteria.

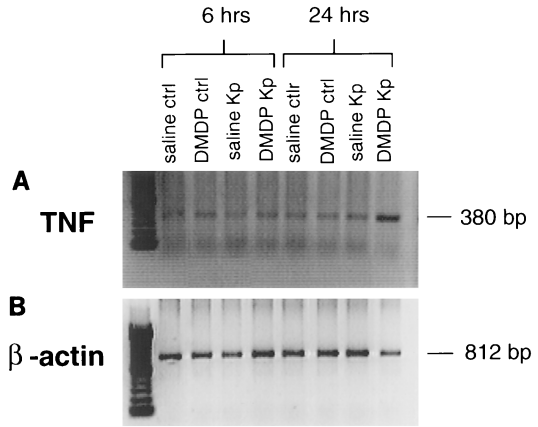

FIG. 6. Time-dependent production of TNF-α mRNA in AM-depleted and normal lung homogenates after inoculation with *K. pneumoniae* (100 CFU). (A) PCR product of TNF (30 cycles); (B) PCR product of β-actin (32 cycles). Positions of molecular size markers are indicated at the right. The lungs from three animals were combined at each specific time point assayed. Samples: saline ctrl (control), saline pretreated, noninfected lungs; DMDP ctrl, DMDP-liposome-pretreated, noninfected lungs; saline Kp, saline pretreated, *K. pneumoniae*-infected lungs; DMDP Kp, DMDP-liposome-pretreated, *K. pneumoniae*-infected lungs.

Since PMN recruitment in response to bacteria in AM-depleted lungs is not impaired, the question arises as to why these large numbers of infiltrated PMN are not able to clear the bacteria from the lung. Furthermore, one could argue that the recruited PMN induce enhanced tissue damage, thereby contributing to the detriment of the host response. The latter, however, seems unlikely since it was previously shown that systemic neutralization of either TNF-α or MIP-2 prior to

infection of mice with *K. pneumoniae* resulted in a significant decrease in PMN influx to the lung but also markedly decreased survival of the animals. This decreased survival was accompanied by an increased number of *K. pneumoniae* CFU in lung and plasma, suggesting that the increased mortality is merely due to decreased clearance of bacteria rather than resulting from enhanced tissue damage caused by the infiltrated PMN (10, 17). A possible explanation for the deficient bacterial clearance is that in the absence of AM, initial replication and dissemination are unimpeded, overwhelming the ability of PMN to clear the organisms. Enhanced penetrance of bacteria to the lung interstitium in AM-depleted mice seems unlikely, since it was demonstrated that administration of DMDP-liposomes does not affect the permeability of the alveolar epithelium (data not shown and reference 11). Another explanation for deficient clearance by infiltrated PMN is that the PMN may need additional AM-derived activation signals for optimal phagocytosis and killing of bacteria. It is known that the ability of PMN to kill some pathogens can be boosted substantially by cytokines and other humoral mediators. For example, gamma interferon and colony-stimulating factors can prime the PMN oxidative burst to soluble stimuli (31) and can amplify the PMN antibody-dependent cytotoxicity (3, 7). Pyrogenic cytokines, such as IL-1, IL-6, and TNF-α, all prime various pathways that contribute to the activation of NADPH oxidase, the major enzyme for generation of microbicidal reactive oxygen species (6, 15, 52). Furthermore, priming of PMN by these cytokines appears to be responsible for increased resistance of mice to bacterial infection (5). It is conceivable that AM are indeed important for activation of the infiltrating PMN, for a significant regulatory role in pulmonary immunity is ascribed to this cell type. Previous studies by Thepen et al. showed that elimination of AM has dramatic consequences for T-cell proliferation, local antibody production, and antigen-presenting capacity by dendritic cells (13, 41–43). AM-depleted animals become hyperresponsive to antigenic stimulation in the lung and mount large local and systemic immune responses to low levels of inhaled antigens which do not elicit responses in intact animals. This AM-mediated regulation of lung dendritic cell and T-cell function is proposed to be invoked both by secretory products, such as nitric oxide (13, 14), prostaglandins (20), and transforming growth factor β (32), and by cell contact-dependent mechanisms (30). Thus, communication between AM and other cell types within the lung is crucial for proper pulmonary immune processes.

In summary, depletion of AM in mice prior to an i.t. challenge with 100 CFU of *K. pneumoniae* results in a dramatic decrease in survival, due to a defective clearance of this small number of bacteria. This deficient clearance was not a consequence of reduced PMN recruitment. On the contrary, a massive infiltration of PMN in the AM-depleted infected lungs was demonstrated and proved to be induced, in part, by local production of the inflammatory cytokines TNF-α and MIP-2. Although an overzealous PMN infiltration in response to *K. pneumoniae* occurs, it remains unclear why these PMN are not able to clear the bacteria from the lungs completely. In conclusion, our study highlights the importance of the AM as critical effector cells in lung antibacterial host defense. While the PMN likely contribute to bacterial clearance, the AM appear to be an indispensable component of innate immunity in *Klebsiella* pneumonia.

ACKNOWLEDGMENTS

This research was supported in part by OM Laboratoires, Meyrin/Geneva, Switzerland, and by National Institutes of Health grants 1P50HL46487, HL50057, HL02401, HL31693, HL35276, HL02701, EA66180, HL52800, HL51243, and AA10571-01.

REFERENCES

1. **Appelberg, R.** 1992. Macrophage inflammatory proteins MIP-1 and MIP-2 are involved in T cell-mediated neutrophil recruitment. J. Leukocyte Biol. **52:**303–307.
2. **Bakker-Woudenberg, I. A. J. M., A. F. Lokerse, M. T. ten Kate, J. W. Mouton, M. C. Woodle, and G. Storm.** 1993. Liposomes with prolonged blood circulation and selective localization in Klebsiella pneumoniae-infected lung tissue. J. Infect. Dis. **168:**164–171.
3. **Baldwin, G. C., N. D. Fuller, R. L. Roberts, D. H. Ho, and D. W. Golde.** 1989. Granulocyte- and granulocyte-macrophage colony stimulating factors enhance neutrophil cytotoxicity toward HIV-infected cells. Blood **74:**1673–1677.
4. **Berg, J. T., S. T. Lee, T. Thepen, C. Y. Lee, and M. F. Tsan.** 1993. Depletion of alveolar macrophages by liposome-encapsulated dichloromethylene diphosphanate. J. Appl. Physiol. **74:**2812–2819.
5. **Cross, A. S., J. C. Sadoff, N. Kelly, E. Bernton, and P. Gemski.** 1989. Pretreatment with recombinant murine tumor necrosis factor α/cachectin and murine interleukin 1α protects mice from lethal bacterial infection. J. Exp. Med. **169:**2021–2027.
6. **Dularay, B., C. J. Elson, S. Clements-Jewery, C. Damais, and D. Lando.** 1990. Recombinant human interleukin-1 beta primes human polymorphonuclear leukocytes for stimulus-induced myeloperoxidase release. J. Leukocyte Biol. **47:**158–163.
7. **Ellis, M., S. Gupta, S. Galant, S. Hakim, C. VandeVen, C. Toy, and M. S. Cairo.** 1988. Impaired neutrophil function in patients with AIDS or AIDS-related complex: a comprehensive evaluation. J. Infect. Dis. **158:**1268–1276.
8. **Feng, L., Y. Xia, T. Yoshimura, and C. B. Wilson.** 1995. Modulation of neutrophil influx in glomerulonephritis in the rat with anti-macrophage inflammatory protein-2 (MIP-2) antibody. J. Clin. Invest. **95:**1009–1017.
9. **Gordon, J. R., and S. J. Galli.** 1991. Release of both preformed and newly synthesized tumor necrosis factor-α (TNF-α)/cachetin by mouse mast cells stimulated via the $Fc^{-}RI$. A mechanism for the sustained action of mast cell-derived TNF-α during IgE-dependent biological responses. J. Exp. Med. **174:**103–107.
10. **Greenberger, M. J., R. M. Strieter, S. L. Kunkel, J. M. Danforth, L. L. Laichalk, D. C. McGillicuddy, and T. J. Standiford.** 1996. Neutralization of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine Klebsiella pneumonia. J. Infect. Dis. **173:**159–165.
11. **Hashimoto, S., J.-F. Pittet, K. Hong, H. Folkesson, G. Bagby, L. Kobzik, C. Frevert, K. Watanabe, S. Tsurufuji, and J. Wiener-Kronish.** 1996. Depletion of alveolar macrophages decreases neutrophil chemotaxis to Pseudomonas airspace infections. Am. J. Physiol. **270:**L819–L824.
12. **Havell, E. A.** 1989. Evidence that tumor necrosis factor has an important role in antibacterial resistance. J. Immunol. **143:**2894–2899.
13. **Holt, P. G., J. Oliver, N. Bilyk, C. McMenamin, P. G. McMenamin, G. Kraal, and T. Thepen.** 1993. Downregulation of the antigen presenting cell function(s) by pulmonary dendritic cells in vivo by resident alveolar macrophages. J. Exp. Med. **177:**397–407.
14. **Kawabe, T., K. I. Isobe, Y. Hasegawa, I. Nakashima, and K. Shimokata.** 1992. Immunosuppressive activity induced by nitric oxide in culture supernatant of activated rat alveolar macrophages. Immunology **76:**72–78.
15. **Kharazmi, A., H. Nielsen, C. Rechnitzer, and K. Bendtzen.** 1989. Interleukin-6 primes human neutrophil and monocyte oxidative burst response. Immunol. Lett. **21:**177–184.
16. **Kips, J. C., J. H. Tavernier, and R. A. Pauwels.** 1992. Tumor necrosis factor causes bronchial hyperresponsiveness in rats. Am. Rev. Respir. Dis. **145:**332–336.
17. **Laichalk, L. L., S. L. Kunkel, R. M. Strieter, J. M. Danforth, M. B. Bailie, and T. J. Standiford.** 1996. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella* pneumonia. Infect. Immun. **64:**5211–5218.
18. **McColm, A. A., E. Shelley, D. M. Ryan, and P. Acred.** 1986. Evaluation of ceftazidime in experimental Klebsiella pneumoniae pneumonia: comparison with other antibiotics and measurement of its penetration into respiratory tissues and secretions. J. Antimicrob. Chemother. **18:**599–608.
19. **Ming, W. J., L. Bersani, and A. Mantovani.** 1987. Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. J. Immunol. **138:**1469–1474.
20. **Monick, M., J. Glazier, and G. W. Hunninghake.** 1987. Human alveolar macrophages suppress interleukin-1 activity via the secretion of prostaglandin E2. Am. J. Respir. Dis. **135:**72–78.
21. **Moser, R., B. Scheiffenbaum, P. Groscurth, and J. Fehr.** 1989. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. J. Clin. Invest. **83:**444–455.
22. **Mulligan, M. S., A. A. Vaporciyan, M. Miyasaka, T. Tamatani, and P. A. Ward.** 1993. Tumor necrosis factor regulates in vivo intrapulmonary expression of ICAM-1. Am. J. Pathol. **142:**1739–1744.
23. **Mulligan, M. S., A. A. Vaporciyan, R. L. Warner, M. L. Jones, K. E. Foreman, M. Miyasaka, R. F. Todd III, and P. A. Ward.** 1995. Compartmental-

ized roles for leukocytic adhesion molecules in lung inflammatory injury. J. Immunol. **154:**1350–1363.
24. **Nakane, A., T. Minagawa, and K. Kato.** 1988. Endogenous tumor necrosis factor (cachetin) is essential to host resistance against *Listeria monocytogenes* infection. Infect. Immun. **56:**2563–2569.
25. **Nathan, C. F.** 1987. Secretory products of macrophages. J. Clin. Invest. **79:**319–326.
26. **Paine, R., III, M. W. Rolfe, T. J. Standiford, M. D. Burdick, B. J. Rollins, and R. M. Strieter.** 1993. MCP-1 expression by rat alveolar type II cells in primary culture. J. Immunol. **150:**4561–4570.
27. **Peppel, K., D. Crawford, and B. Beutler.** 1991. A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. J. Exp. Med. **174:**1483–1489.
28. **Qian, Q., M. A. Jutila, N. Van Rooijen, and J. E. Cutler.** 1994. Elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. J. Immunol. **152:**5000–5008.
29. **Remick, D. G., R. M. Strieter, M. K. Eskandari, D. T. Nguyen, M. A. Genord, C. L. Raiford, and S. L. Kunkel.** 1990. Role of tumour necrosis factor-a in lipopolysaccharide induced pathologic alterations. Am. J. Pathol. **136:**49–60.
30. **Rich, E. A., C. Cooper, Z. Toossi, M. L. Leonard, R. M. Stucky, R. T. Wiblin, and J. J. Ellner.** 1991. Requirement for cell-to-cell contact for the immunosuppressive activity of human alveolar macrophages. Am. J. Respir. Cell Mol. Biol. **4:**287–294.
31. **Roilides, E., K. Uhlig, D. Venzon, P. A. Pizzo, and T. J. Walsh.** 1993. Prevention of corticosteroid-induced suppression by human polymorphonuclear leukocyte-induced damage of *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. Infect. Immun. **61:** 4870–4877.
32. **Roth, M. D., and S. H. Golub.** 1993. Human pulmonary macrophages utilize prostaglandins and transforming growth factor beta-1 to suppress lymphocyte activation. J. Leukocyte Biol. **53:**366–371.
33. **Schall, T. J.** 1994. The chemokines, p. 419–460. *In* A. W. Thomson (ed.), The cytokine handbook, 2nd ed. Academic Press, San Diego, Calif.
34. **Schmal, H., T. P. Shanley, M. L. Jones, H. P. Friedl, and P. A. Ward.** 1996. Role for macrophage inflammatory protein-2 in lipopolysaccharide-induced lung injury in rats. J. Immunol. **156:**1963–1972.
35. **Sibille, Y., and H. Y. Reynolds.** 1990. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am. Rev. Respir. Dis. **141:**471–501.
36. **Smart, S. J., and T. B. Casale.** 1994. Pulmonary epithelial cells facilitate TNF-α-induced neutrophil chemotaxis. J. Immunol. **152:**4087–4094.
37. **Standiford, T. J., S. L. Kunkel, M. A. Basha, S. W. Chensue, J. P. Lynch III, G. B. Toews, J. Westwick, and R. M. Strieter.** 1990. Interleukin-8 expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. J. Clin. Invest. **86:**1945–1953.
38. **Standiford, T. J., S. L. Kunkel, S. H. Phan, B. J. Rollins, and R. M. Strieter.** 1991. Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. J. Biol. Chem. **266:**9912–9918.
39. **Strieter, R. M., S. L. Kunkel, M. D. Burdick, P. M. Lincoln, and A. Walz.** 1992. The detection of a novel neutrophil-activating peptide (ENA-78) using a sensitive ELISA. Immunol. Invest. **21:**589–596.
40. **Strunk, R. C., D. M. Eidlen, and R. J. Mason.** 1988. Pulmonary alveolar type II epithelial cells synthesize and secrete proteins of the classical and alternative complement pathways. J. Clin. Invest. **81:**1419–1426.
41. **Thepen, T., C. McMenamin, B. Girn, G. Kraal, and P. G. Holt.** 1992. Regulation of IgE production in pre-sensitized animals: in vivo elimination of alveolar macrophages preferentially increases IgE responses to inhaled allergen. Clin. Exp. Allergy **22:**1107–1114.
42. **Thepen, T., C. McMenamin, J. Oliver, G. Kraal, and P. G. Holt.** 1991. Regulation of immune response to inhaled antigen by alveolar macrophages: differential effects of in vivo alveolar macrophage elimination on the induction of tolerance vs immunity. Eur. J. Immunol. **21:**2845–2850.
43. **Thepen, T., N. van Rooijen, and G. Kraal.** 1989. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. J. Exp. Med. **170:**499–509.
44. **Tracey, K. J.** 1994. Tumor necrosis factor-alpha, p. 289–304. *In* A. W. Thomson (ed.), The cytokine handbook, 2nd ed. Academic Press, San Diego, Calif.
45. **Ulich, T. R., S. C. Howard, D. G. Remick, A. Wittwer, E. S. Yi, S. Yin, K. Guo, J. K. Welply, and J. H. Williams.** 1995. Intratracheal administration of endotoxin and cytokines. VI. Antiserum to CINC inhibits acute inflammation. Am. J. Physiol. **268:**L245–L250.
46. **Ulich, T. R., S. C. Howard, D. G. Remick, E. S. Yi, T. Collins, K. Guo, S. Yin, J. L. Keene, J. J. Schumke, C. N. Steiniger, J. K. Welply, and J. H. Williams.** 1994. Intratracheal administration of endotoxin and cytokines. VII. LPS induces E-selectin expression: anti-E-selectin and soluble-E-selectin inhibit acute inflammation. Inflammation **18:**389–398.
47. **Ulich, T. R., L. R. Watson, S. Yin, K. Guo, P. Wang, H. Thang, and J. del Castillo.** 1991. The intratracheal instillation of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1, and TNF-induced inflammatory infiltrate. Am. J. Pathol. **138:** 1485–1496.
48. **Ulich, T. R., S. Yin, D. G. Remick, D. Russel, S. P. Eisenberg, and T. Kohno.** 1993. The intratracheal administration of endotoxin and cytokines. IV. The soluble tumor necrosis factor receptor type I inhibits acute inflammation. Am. J. Pathol. **142:**1335–1338.
49. **VanOtteren, G. M., R. M. Strieter, S. L. Kunkel, R. Paine III, M. J. Greenberger, J. M. Danforth, M. D. Burdick, and T. J. Standiford.** 1995. Compartmentalized expression of RANTES in a murine model of endotoxemia. J. Immunol. **154:**1900–1908.
50. **Van Rooijen, N.** 1989. The liposome-mediated macrophage 'suicide' technique. J. Immunol. Methods **124:**1–6.
51. **Van Rooijen, N., and A. Sanders.** 1994. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J. Immunol. Methods **1174:**83–93.
52. **Williams, D. M., D. M. Magee, L. F. Bonewald, J. G. Smith, C. A. Bleicker, G. I. Byrne, and J. Schacter.** 1990. A role in vivo for tumor necrosis factor alpha in host defense against *Chlamydia trachomatis*. Infect. Immun. **58:** 1572–1567.
53. **Windsor, A. C. J., C. J. Walsh, P. G. Mullen, D. J. Cook, B. J. Fisher, C. R. Blocher, S. K. Leeper-Woodford, H. J. Sugerman, and A. A. Fowler III.** 1993. Tumor necrosis factor-α blockade prevents neutrophil CD18 receptor upregulation and attenuates acute lung injury in porcine sepsis without inhibition of neutrophil oxygen radical generation. J. Clin. Invest. **91:**1459–1468.
54. **Wolpe, S. D., B. Sherry, D. Juers, G. Davatelis, R. W. Yurt, and A. Cerami.** 1989. Identification and characterization of macrophage inflammatory protein 2. Proc. Natl. Acad. Sci. USA **86:**612–616.
55. **Xing, Z., M. Jordana, H. Kirpalani, K. E. Driscoll, T. J. Schall, and J. Gauldie.** 1994. Cytokine expression by neutrophils and macrophages in vivo: endotoxin induces tumor necrosis factor-α, macrophage inflammatory protein-2, interleukin-1β, and interleukin-6 but not RANTES or transforming growth factor-β1 mRNA expression in acute lung inflammation. Am. J. Respir. Cell Mol. Biol. **10:**148–153.

Editor: R. E. McCallum