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Depletion of Alveolar Macrophages Exacerbates Respiratory Mycoplasmosis in Mycoplasma-Resistant C57BL Mice but Not Mycoplasma-Susceptible C3H Mice

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Indirect evidence suggests that innate immune mechanisms involving alveolar macrophages (AMs) are of major importance in antimycoplasmal defense. We compared the effects of AM depletion on intrapulmonary killing of *Mycoplasma pulmonis* during the early phase of infection in mycoplasma-resistant C57BL/6NCr (C57BL) and mycoplasma-susceptible C3H/HeNCr (C3H) mice. More than 80% of AMs were depleted in both strains of mice by intratracheal insufflation of liposome-encapsulated dichloromethylene bisphosphonate (L-Cl_2MBP), compared to no significant AM depletion in either strain following insufflation of liposome-encapsulated phosphate-buffered saline (L-PBS), PBS alone, or no treatment. AM-depleted (L-Cl_2MBP) and control (L-PBS) mice were infected intranasally with 10^5 CFU of *M. pulmonis* UAB CT, and their lungs were quantitatively cultured to assess intrapulmonary killing at 0, 8, 12, and 48 h postinfection. AM depletion exacerbated the infection in C57BL mice by reducing killing of the organism to a level comparable to that in C3H mice without AM depletion. In contrast, AM depletion did not alter killing in C3H mice. These results directly identify the AM as the main effector cell in early pulmonary antimycoplasmal defense and suggest that differences in mycoplasmal killing by AMs may explain the resistance of C57BL mice and the susceptibility of C3H mice to mycoplasmal infection.

Pneumonia is the sixth leading cause of death in the United States (34), and *Mycoplasma pneumoniae* accounts for 20 to 30% of all pneumonias in the general population (8, 20). Current evidence suggests that resistance to respiratory mycoplasmal infections consists of two components: innate resistance associated with the alveolar macrophage (AM) (5, 6, 12, 13) and a late contribution by humoral immunity (19).

Mycoplasma pulmonis infection in mice provides an excellent animal model that reproduces the essential features of human respiratory mycoplasmosis (23). Mouse strains differ markedly in resistance to *M. pulmonis* (15), with C57BL and C3H mice representing the extremes in response to this infection (15). C57BL mice have a 100-fold higher 50% lethal dose, 50% pneumonia dose, and 50% microscopic lesion dose than C3H mice (15). During the first 72 h postinfection (p.i.), the numbers of mycoplasmas decrease by more than 83% in the lungs of C57BL mice but increase by 18,000% in the lungs of C3H mice (29). In C57BL mice, maximum mycoplasmacidal activity occurs within 8 h p.i., but there is no increase in the number of macrophages, neutrophils, or lymphocytes in the lungs until after 72 h p.i. (15, 29, 30). Mechanical clearance of mycoplasmas does not differ between the two strains of mice (29). Specific antibody cannot be demonstrated during the first 72 h p.i. (4). Thus, nonspecific intrapulmonary killing of *M. pulmonis* occurs and is most likely mediated by rapidly activated resident AMs (13).

Macrophage depletion has been used to investigate the protective roles of AMs in the lungs (1, 34, 35) and resident macrophages in the liver and spleen (2, 7, 33, 41–44). To further delineate the role of the AM in early clearance of mycoplasmas from the lungs, we gave liposome-encapsulated dichloromethylene bisphosphonate (L-Cl_2MBP) by intratracheal insufflation to selectively deplete AMs in mice. We observed that AM depletion prior to infection with mycoplasmas reduced mycoplasma killing in resistant C57BL mice to a level comparable to that in susceptible C3H mice without AM depletion. In contrast, AM depletion did not alter the killing of mycoplasmas in the lungs of infected C3H mice. We concluded that AMs have a central role in early antimycoplasmal defense of the lungs and that differences in antimycoplasmal activity of AMs may explain the respective resistance and susceptibility of C57BL and C3H mice to respiratory mycoplasmal infection.

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MATERIALS AND METHODS

Animals. Pathogen-free 8- to 12-week-old C57BL (C57BL/6NCr) and C3H (C3H/HeNCr) mice and retired breeder Sprague-Dawley rats were obtained from the Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, Md. Rats and mice were subsequently maintained in autoclaved Microisolator cages (Lab Products, Maywood, N.J.) and provided food (Agway, Inc., Syracuse, N.Y.) and water ad libitum. Mice were tested and shown to be negative for murine pathogens (17). Surgical anesthesia was induced by intramuscular injection with ketamine (8.7 mg/100 g of body weight; Aveco, Fort Dodge, Iowa) and xylazine (1.3 mg/100 g of body weight; Haver, Shawnee, Kans.).

Media and chemicals. Clodronate (Cl_2MBP) was generously provided by Boehringer Mannheim GmbH (Mannheim, Germany). Egg phosphatidylcholine and cholesterol were premixed, dissolved in chloroform, and distributed to single-use vials (Avanti Polar Lipids, Alabaster, Ala.). BBL Mycoplasma Broth Base (Becton Dickinson Microbiology Systems, Cockeysville, Md.), phosphate-buffered saline (PBS; Mediatech, Inc., Herndon, Va.), saline (Abbott Laboratories, Abbott Park, Ill.), horse serum (Gibco BRL Laboratories, Grand Island, N.Y.), and Diff Quik stain kits (Baxter Diagnostics Inc., McGaw Park, Ill.) were ob-

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tained from various sources. All other chemicals were from Sigma Chemical Co., St. Louis, Mo.

Liposomes. Liposomes were prepared by using sterile techniques as described previously (3). Briefly, 86 mg of egg phosphatidylcholine and 8 mg of cholesterol were dissolved in 5 ml of chloroform in a round-bottom flask. The chloroform was removed by using a low-vacuum rotary evaporator at 37°C to form a thin lipid film around the flask. The lipid was dispersed in sterile PBS, with or without Cl_2MBP, and allowed to incubate at room temperature for 2 h. The suspension was sonicated for 3 min and incubated again for 2 h at room temperature. Liposomes were washed and centrifuged three times to remove free Cl_2MBP. The final pellet was resuspended in sterile PBS and used immediately or stored under nitrogen at 4°C for use within 7 days. Immediately prior to use, the amount of Cl_2MBP entrapped in the liposomes was determined on the basis of the competition for calcium between Cl_2MBP and murexide (7). Liposomes were lysed with 5% Triton X-100 before measurement of Cl_2MBP content.

Liposome insufflation. Animals were anesthetized and placed in dorsal recumbency. A longitudinal incision was made through the skin over the trachea. Liposomes (100 μl per mouse and 1 ml per rat), with or without Cl_2MBP, were injected through the tracheal wall into the lumen, followed by room air (200 μl per mouse and 2 ml per rat). Skin incisions were closed with Nexaband (Veterinary Products Labs, Phoenix, Ariz.).

Lung lavage. Bronchoalveolar lavage (BAL) samples were collected as described previously (12). Briefly, mice were anesthetized, and a sterile 19-gauge intravenous catheter (Deseret Medical, Sandy, Utah) was inserted 5 mm caudally into the lumen of the trachea. The lungs were then lavaged in situ with four separate 1-ml washes of sterile saline. The BAL fluid was centrifuged, and the cellular fraction was gently resuspended in sterile saline. Total leukocyte count was determined by using a hemocytometer and trypan blue (0.4%) exclusion to assess viability. Numbers of viable AMs and polymorphonuclear cells (PMNs) were calculated from these totals, using a differential cell count of Diff-Quik- or nonspecific esterase (22)-stained cytocentrifuge preparations. BAL supernatants were stored at −70°C prior to analysis of protein content. Rats were lavaged similarly except that a 14-gauge catheter and 10-ml washes with sterile saline were used to collect BAL fluids.

Mycoplasma infections. The UAB CT strain of *M. pulmonis* was used in all experiments (11). Groups of six mice were inoculated intranasally with 10^5 CFU of *M. pulmonis* in 50 μl of broth. Control mice received the same volume of broth A (11). The number of CFU in each inoculum was confirmed by enumeration after standard dilution, inoculation of agar plates, and incubation for 7 days at 37°C in room air with 95% humidity (14). Prior to each infection, representative animals from L-Cl_2MBP-, liposome-encapsulated PBS (L-PBS)-, or PBS-treated groups were euthanized at 24 h, and the numbers of AMs and PMNs were determined to ensure that AM depletion had been successful.

Quantitative mycoplasma culture of lungs. Mice were euthanized at various times from 0 to 48 h p.i. Lungs were removed aseptically, individually minced, and sonicated for 30 s in broth A. Tenfold serial dilutions were plated onto mycoplasmal agar, and the total number of CFU in the lungs of each animal was determined after incubation for 7 days as described previously (14).

Lung histopathology. Lungs were removed and fixed by intratracheal infusion of 10% formalin in 70% ethanol until the lungs reached approximately normal distention (4). Sections (5 μm) of paraffin-embedded tissue were stained with hematoxylin and eosin.

BAL protein assay. All BAL samples were analyzed simultaneously by the Bradford Micro-Method protein assay (Bio-Rad, Rockville Centre, N.Y.), using a standard curve prepared from assaying known amounts of bovine serum albumin in 0.9% NaCl (12).

Statistical analysis. All experiments were performed with six animals per group and were repeated to ensure reproducibility. Parametric culture data were analyzed by analysis of variance followed by Tukey's multigroup comparison for parametric data or by Mann-Whitney *U* tests with Bonferroni-adjusted probabilities for nonparametric data (36). Mycoplasma CFU counts were first converted to common logarithms, and results were expressed as means ± standard errors of the means. Probabilities (*P*) of 0.05 or less were considered significant.

RESULTS

Depletion of AMs by L-Cl_2MBP. The concentration of Cl_2MBP in our L-Cl_2MBP preparation was 16.66 ± 1.3 mM (mean ± standard error of the mean, $n = 8$). Because different preparations of L-Cl_2MBP are known to be highly variable in efficacy of depleting AMs and previous studies using L-Cl_2MBP to deplete AMs have used rats, we tested each preparation of liposomes in rats to ensure consistency of AM depletion, following the dosage regimen of Berg et al. (1). Rats were inoculated intratracheally with a 1-ml liposome preparation containing 1.61 ± 0.10 μM Cl_2MBP and euthanized 72 h later. For each of our L-Cl_2MBP preparations, we found >75% depletion of AMs in BAL samples of treated rats (Fig. 1A), consistent with previous reports (1).

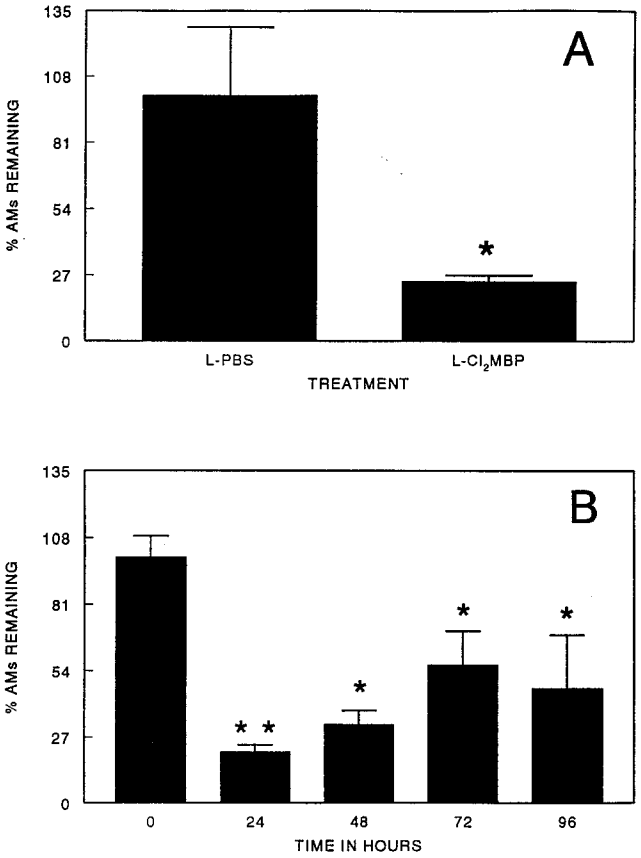


FIG. 1. Time course for liposome insufflation. (A) Adult Sprague-Dawley rats ($n \geq 12$) were insufflated intratracheally with 1.61 ± 0.10 μM L-Cl_2MBP and euthanized 72 h later for assessment of AM numbers. Bars indicate the percentages of total AMs remaining compared to animals receiving L-PBS (100%). (B) Adult C57BL mice ($n \geq 6$) were insufflated intratracheally with 0.425 μM L-Cl_2MBP and euthanized at 0, 24, 48, 72, and 96 h. All assessments were based on total cell counts with a hemocytometer and differential cell count using nonspecific esterase-stained cytospins. Results are means ± standard errors of means. Asterisks denote statistical significance of $P < 0.05$; double asterisks denote significance from all other treatment groups.

In contrast, mice given 0.425 μM L-Cl_2MBP had maximum AM depletion (79%) at 24 h (Fig. 1B). We therefore determined the dose response to L-Cl_2MBP insufflation of C57BL mice at 24 h. Insufflations of 0, 0.66, 0.99, and 1.33 μM L-Cl_2MBP resulted in a dose-dependent reduction in the number of AMs, with maximum depletion being 87% at a dose of 1.33 μM. This dose and treatment time were used in all mycoplasma infection studies of mice.

A dose-dependent increase in the number of PMNs was noted at the 24-h time point in mice given L-Cl_2MBP or control L-PBS (Fig. 2A). However, by 72 h, the numbers of PMNs had decreased to preinsufflation levels in control animals, while those receiving L-Cl_2MBP maintained slightly elevated PMN levels (13%) to 96 h (Fig. 2B).

Cytospin preparations of BAL cellular fractions from Cl_2MBP-depleted animals contained large, vacuolated AMs, PMNs, and modest cell debris. In comparison, cells from control animals were smaller, more uniform, and tended to form compact clusters. The cellular fraction was consistently >95% macrophages with >90% cell viability.

Lung histopathology. Lung sections from both C57BL and C3H mice were evaluated prior to infection with mycoplasmas

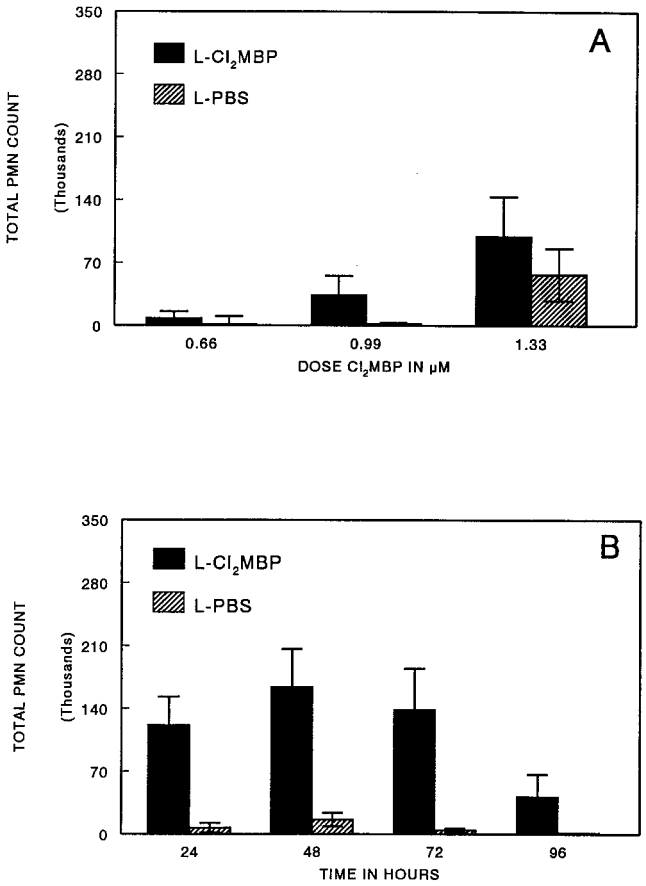


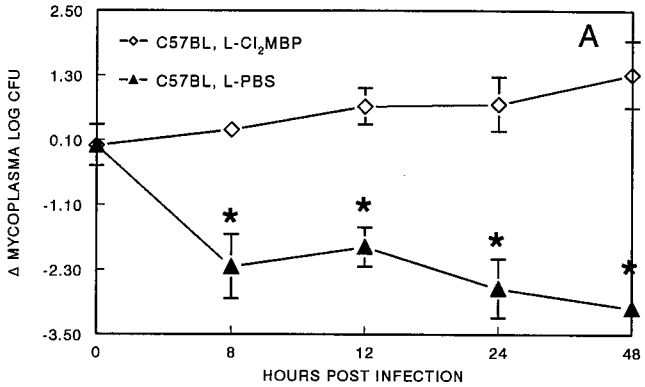
FIG. 2. PMN response to liposome insufflation. (A) Adult C57BL mice ($n \geq 6$) were insufflated intratracheally with 0, 0.66, 0.99, or 1.33 μM L-Cl_2MBP or L-PBS and euthanized 24 h later for assessment of total PMN numbers. (B) Adult C57BL mice ($n \geq 6$) were insufflated intratracheally with 0.425 μM L-Cl_2MBP or L-PBS and euthanized 0, 24, 48, 72, and 96 h later for assessment of total PMN numbers. The graphs plot the number of PMNs as a percentage of the total cell number determined from differential cell counts on cytospin preparations. Results are means ± standard errors of the means.

24 h after liposome insufflation. Lungs from mice of both strains given L-Cl_2MBP or L-PBS were histologically normal, with no increase of PMNs in airways or alveoli.

Protein concentration in BAL fluids. Increased protein in BAL fluids is a sensitive indicator of tissue injury and transudation of serum constituents into the lungs (12, 21, 35). Administration of L-Cl_2MBP had no significant effect on protein content of BAL samples compared to control mice given either L-PBS, PBS alone, or no treatment. Total protein contents of the BAL samples for C57BL mice were 181.40 ± 18.64 μg/ml after L-Cl_2MBP treatment, 168.68 ± 18.16 μg/ml after L-PBS treatment, 150.19 ± 32.6 μg/ml after treatment with PBS alone, and 129.48 ± 28.46 μg/ml with no treatment (means ± standard deviations). These levels were comparable to those from C3H mice: 160.01 ± 36.36 μg/ml after L-Cl_2MBP treatment, 127.65 ± 22.16 μg/ml after L-PBS treatment, and 146.79 ± 10.30 μg/ml with no treatment.

Effect of AM depletion on infection in C57BL mice. To determine the role of AMs in the early response to *M. pulmonis* infection, C57BL mice were insufflated with L-Cl_2MBP, L-PBS, or PBS alone and 24 h later infected with *M. pulmonis* UAB CT. Mice were euthanized at 0, 12, 24, and 48 h p.i., and their lungs were quantitatively cultured for mycoplasmas to assess killing. Similar numbers of mycoplasmas were recovered from the lungs of mice receiving L-PBS or PBS alone, with a reduction in mycoplasma CFU of almost 100% by 48 h. In contrast, mice given L-Cl_2MBP had significantly higher mycoplasma CFU by 8 h, and the CFU count continued to increase to 48 h p.i. (Fig. 3A).

Effects of AM depletion on mycoplasma infections in C57BL and C3H mice. Mice of both strains were insufflated with L-Cl_2MBP or L-PBS and 24 h later infected with mycoplasmas. The numbers of AMs and PMNs recovered from Cl_2MBP-treated C3H mice were comparable to those from Cl_2MBP-treated C57BL mice (data not shown). Following administration of L-PBS, resistant C57BL mice had significantly lower CFU counts ($P < 0.01$) beginning at 8 h p.i. than similarly treated susceptible C3H mice (Fig. 3B). This difference also was seen at 12, 24, and 48 h ($P < 0.001$). There was no difference in mycoplasma CFU between mouse strains when mice were given L-Cl_2MBP before infection with mycoplasmas (Fig. 4A). Additionally, there was no significant difference in mycoplasma CFU between C57BL mice given L-Cl_2MBP and C3H mice given L-PBS (Fig. 4B). The administration of L-Cl_2MBP to C3H mice had no effect on the number of my-



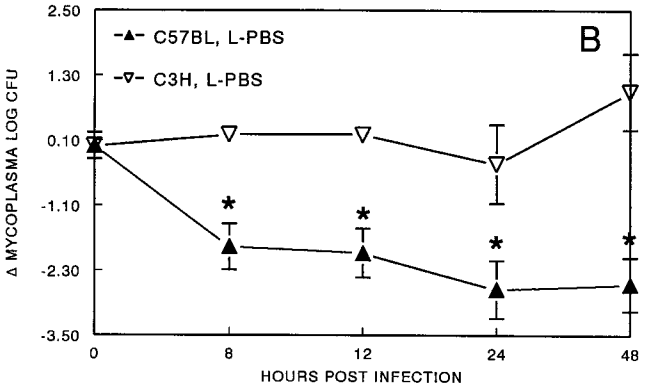


FIG. 3. Effect of intratracheal insufflation of liposomes on recovery of *M. pulmonis* from C57BL and C3H mice in vivo. Adult C57BL mice ($n \geq 12$) were insufflated intratracheally with L-Cl_2MBP or L-PBS and then infected with 10^5 CFU of *M. pulmonis* intranasally (A). Adult C57BL and C3H mice ($n \geq 12$) were insufflated intratracheally with L-PBS then infected with 10^5 CFU of *M. pulmonis* intranasally (B). All mice were euthanized 0, 8, 12, 24, and 48 h p.i., and the mean numbers of CFU (total recoverable mycoplasmas) were determined on whole lung homogenates. The graphs plot the change in mean log CFU from the 0-h time point. Asterisks denote statistical significance of $P < 0.05$. Results are means ± standard errors of the means.

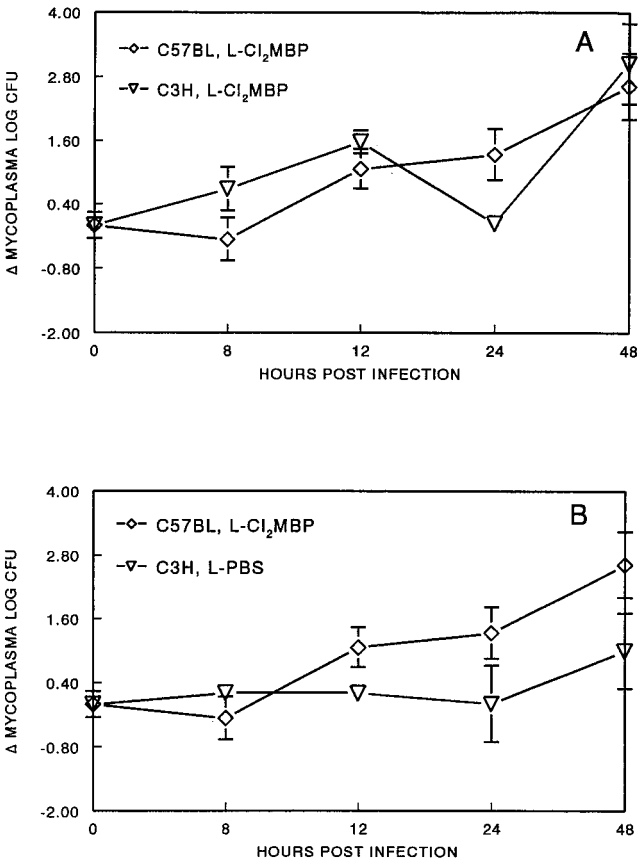


FIG. 4. Effect of AM depletion on intrapulmonary killing of *M. pulmonis* in resistant C57BL and susceptible C3H mice. C57BL mice ($n \geq 12$) were insufflated with L-Cl_2MBP and compared with C3H mice ($n \geq 12$) insufflated intratracheally with either L-PBS or L-Cl_2MBP. Both mouse strains were infected intranasally 24 h later with 10^5 CFU of *M. pulmonis*. Mice were euthanized 0, 8, 12, 24, and 48 p.i., and mean numbers of CFU (total recoverable mycoplasmas) were determined on whole lung homogenates. Graphs represent the mean change in log CFU for each time point for C57BL and C3H mice given L-Cl_2MBP (A) and for C57BL mice given L-Cl_2MBP and C3H mice given L-PBS (B). Results are means ± standard errors of the means.

coplasmas recovered at any time point in comparison to L-PBS-treated C3H mice.

DISCUSSION

Mechanisms of antimycoplasmal host defense in the lungs are poorly understood (23, 37), but specific immunity appears to have only a limited role. Pneumonia due to *M. pneumoniae* is not increased in severity in patients who have deficiencies in cell-mediated immunity (19, 26). Furthermore, patients with humoral immunodeficiencies have lung disease no more severe than do people without humoral immunodeficiencies during early stages of *M. pneumoniae* infection (19). Similarly, severe combined immunodeficient mice infected with *M. pulmonis* actually develop early lung disease less severe than that in infected immunocompetent controls (16). Collectively, these observations suggest that mechanisms of innate immunity have a key role in early antimycoplasmal defense (4, 8, 9, 19).

There is strong indirect evidence that innate immunity involving AMs is of major importance in antimycoplasmal defense of the lungs. After infection of resistant C57BL mice with *M. pulmonis*, maximum mycoplasmacidal activity occurs within 8 h p.i., long before recruitment of additional cells into the lungs or appearance of specific antibody in serum (15, 29, 30). In C57BL mice infected with *M. pulmonis* and exposed to nitrogen dioxide, intrapulmonary killing decreased as AM viability decreased and subsequently increased as AM viability was restored (13).

The purpose of this study was to determine the direct effects of AM depletion on the early phase of mycoplasmal infection in resistant C57BL and susceptible C3H mice. Liposomes of different cholesterol compositions have been shown to alter macrophage function by blocking or activating phagocytosis (10, 32). However, the insufflation of liposomes having the formulation that we used does not significantly alter macrophage function (44), a finding confirmed in the present study, as insufflation of L-PBS into C57BL mice did not alter mycoplasma killing. AMs ingest the L-Cl_2MBP which is degraded in lysosomes to release free Cl_2MBP into the cytoplasm, resulting in cell death (18, 40). The precise mechanism of Cl_2MBP cytotoxicity for macrophages is unknown, but it may be due to depletion of iron or other metal complexes in the cell (28) or a direct effect on ATP metabolism (31). L-Cl_2MBP has been shown to have little effect on alveolar epithelium or interstitial macrophages (31, 38, 39), although free Cl_2MBP has been shown to cause some edema of alveolar epithelium (1). The lack of increases in protein content of BAL fluids and discernible histologic effects in the lungs of our mice provided evidence that the L-Cl_2MBP caused little injury, except for depletion of AMs.

The insufflation of mice with L-Cl_2MBP or L-PBS resulted in modest increases in numbers of PMNs in BAL fluids, although no increase in PMNs was discernible in histologic sections. PMNs appear to be functionally and morphologically unaffected by L-Cl_2MBP in vivo and in vitro (33), presumably because of their low liposome ingestion (44). The increase in PMN numbers also may reflect blood contamination, which unavoidably occurs to a variable extent in BAL sample collection (27). Nevertheless, the presence of PMNs almost certainly had no effect on mycoplasmacidal activity, as we recovered significantly higher numbers of mycoplasmas from AM-depleted mice which had the highest level of PMNs in BAL fluids.

The administration of L-Cl_2MBP to resistant C57BL mice effectively abolished their early innate killing of mycoplasmas. This result indicates that unlike C3H mice, C57BL mice have a highly effective nonspecific pulmonary defense mechanism(s) that limits the extent of infection (17). Furthermore, our data show that the AM is the major contributor to early defense against mycoplasmas (13). The fact that mycoplasma numbers in AM-depleted C3H mice remained unchanged suggests that C3H mice have (i) a defective macrophage activation pathway (24, 25), (ii) a defect in nonspecific opsoninization in their lungs (e.g., surfactant protein A), or (iii) a functional defect in one or more of their AM subset populations, possibilities currently under investigation.

In summary, we have shown that depletion of AMs in resistant C57BL mice results in severe impairment of their mycoplasmacidal activity to a level comparable to that of susceptible C3H mice, while AM depletion in C3H mice does not alter their naturally impaired capacity to kill mycoplasmas. Thus, our results directly identify the AM as the main effector cell in early antimycoplasmal resistance of C57BL mice and defective AM function as the likely explanation for the susceptibility of C3H mice.

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