LUNG INFLAMMATION INDUCED BY ENDOTOXIN IS ENHANCED IN RATS DEPLETED OF ALVEOLAR MACROPHAGES WITH AEROSOLIZED CLODRONATE

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Clodronate liposomes were given to rats via intratracheal inhalation to investigate the importance of alveolar macrophages (AMs) in inhaled endotoxin-induced lung injury. When AM depletion was maximal (87% to 90%), rats were exposed to lipopolysaccharide (LPS) or saline. Neither clodronate nor saline liposomes induced an influx of neutrophils (PMNs) into the lungs. However, depleted LPS-exposed rats had 5- to 8-fold higher numbers of lavage PMNs and greater lavage cell reactive oxygen species release compared to undepleted rats. Although AM depletion by itself did not significantly increase inflammatory cytokine expression in lung tissue, LPS-induced

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message levels for interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α were approximately 2-fold higher in AM-depleted rats compared to undepleted rats. These results indicate that cells other than AMs can recruit inflammatory cells into the lungs during acute LPS-induced injury and that AMs play an important suppressive role in the innate pulmonary inflammatory response.

**Keywords** alveolar macrophage, clodronate, depletion, endotoxin, inflammation, lipopolysaccharide (LPS), liposomes, lung injury

Macrophages represent a first line of defense against inhaled particles in the alveolar regions of the lung. These cells remove debris and have the capacity to release proinflammatory and chemotactic molecules in response to a variety of stimuli, resulting in recruitment of other inflammatory cells and an amplification of the initial response. Alveolar macrophages (AMs) also contribute to the subsidence of inflammation and tissue repair through their release of anti-inflammatory mediators and removal of damaged cells [1–3]. In addition to AMs, alveolar epithelial cells and polymorphonuclear neutrophils (PMNs) can also release many of the same mediators [4–6], and are potentially important contributors to acute pulmonary inflammatory responses to inhaled toxicants or toxins. One method of examining the importance of AMs relative to other cell types in response to specific stimuli is to deplete these phagocytic cells by pharmacologic means, such as via the administration of clodronate-containing liposomes prior to the initiation of inflammatory lung injury.

Clodronate is a bisphosphonate that induces apoptosis and necrosis in target cells [7, 8]. By encapsulating this drug in liposomes, phagocytic cells in the lung can be successfully targeted and depleted [9–12]. Because the principal phagocytic cell type in the alveolar space of naïve animals is typically the AM, these cells are preferentially targeted by inhaled or tracheally instilled clodronate liposomes. We have previously developed an intratracheal inhalation (ITIH) system to maximize the alveolar deposition of clodronate liposomes and remove a high percentage of lavagable AMs from rats [10, 13]. Maximal depletion of AMs from the alveolar region occurs at 3 days post delivery of clodronate liposomes by ITIH and is not accompanied by the increased recruitment of PMNs or immature macrophages [10]. This method of delivering clodronate liposomes was used here to examine the importance of AMs in the inflammation and recruitment of PMNs into the lungs that follows exposure to aerosolized endotoxin (lipopolysaccharide, LPS).

LPS is a known to cause pulmonary inflammation, including the production of proinflammatory cytokines, chemokines, cell adhesion molecules, reactive oxygen species, and stress proteins [14–21]. However,
AM-specific responses to LPS have not been quantitated relative to those associated with other resident and nonresident pulmonary cells. In the current study, we hypothesized that cells other than AMs are important in the pulmonary inflammatory response to moderate levels of aerosolized LPS. The dose of LPS investigated was chosen to induce an inflammatory response with <30% PMNs in bronchoalveolar lavage (BAL) without obvious damage to epithelial cells (i.e., no substantial increases in protein concentration, lactate dehydrogenase [LDH] activity, or β-glucuronidase activity in BAL). Rats were given clodronate-filled liposomes by ITIH to deplete AMs and were exposed to aerosolized LPS 3 days later when depletion was maximal. Leukocytes in BAL and cytokine message levels in lung tissue were assessed 4 and 24 hours following LPS exposure to examine early and later events in acute LPS-induced lung inflammation.

**METHODS**

**Animals**

All animal experiments were done according to protocols approved by the University Committee on Animal Resources at the University of Rochester. Thirty-five pathogen-free male Fischer 344 rats (8 weeks; 240 ± 25 g) were obtained from Harlan (Indianapolis, IN). Rats were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility in wire-bottom cages and were allowed to acclimate for at least one week prior to use in experimental protocols. All animals were given free access to Purina rodent chow and water and were housed in an air conditioned barrier facility with a 12-hour light-dark cycle. On day 0, 2 groups of rats (16 rats/group) were given either clodronate-filled or saline-filled liposomes by ITIH (Figure 1) (see liposomes delivery methods below). Three days later, 8 rats from each group (AM-depleted or AM-undepleted) were then exposed to aerosolized LPS or saline. The 3-day interval between clodronate treatment and LPS aerosol exposure was chosen based on our prior work showing that AM depletion is maximal at this time [10]. At 4 and 24 hours post exposure to LPS or saline aerosols, 4 rats within each group were euthanized and examined for cellular and cytokine responses (see below). A summary of the experimental groups (n = 8 for each) is as follows: saline liposomes (undepleted) + aerosolized saline; clodronate liposomes (depleted) + aerosolized saline; saline liposomes (undepleted) + aerosolized LPS; and clodronate liposomes (depleted) + aerosolized LPS. In addition, 3 naïve (not liposome pretreated) rats were exposed to LPS and examined at 4 hours as another comparison group for cytokine message assessments.
Preparation of Clodronate Liposomes

Lipid liposomes incorporating clodronate were prepared according to the method of Hashimoto and colleagues [11]. Egg phosphatidylcholine (egg PC) and bovine brain phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol was purchased from Sigma Chemical (St. Louis, MO) and clodronate was a gift from Boehringer-Mannheim (Mannheim, Germany). The liposomes were composed of egg PC, PS, and cholesterol (6:1:4 by mole), and each batch contained 75 mg of egg PC, 13.3 mg of PS, and 25.4 mg of cholesterol. Clodronate (1.0 disodium clodronate) was solubilized initially in 4 mL phosphate-buffered saline without calcium (PBS) and 9.6 mL distilled water. The lipid mixture in chloroform was dried under nitrogen and redissolved in 4 mL of diethyl ether, followed by addition of 2 mL of the clodronate solution. The resultant biphasic mixture was then emulsified by probe sonication on ice using 4 10-second bursts (Heat Systems, Plainview, NY; Model W-220F). The emulsified mixture was placed on a rotary evaporator under reduced pressure at 30°C to slowly remove diethyl ether; any gel phase formed was broken by vortexing. After complete removal of ether, the liposome suspension was extruded three times through cellulose acetate syringe-type filters (0.2 μm; Corning Glass Works, Corning, NY). Drug-containing liposomes were pelleted by centrifugation at 10,000 × g for 40 minutes, and resuspended in 0.5 mL of sterile saline. Final clodronate content in liposomes was measured via competition for calcium with murexide (typical incorporation = 18.3 ± 4.5 mg clodronate per sample, representing ~10% recovery of starting material) as described by Claassen and Van Rooijen [22]. Control saline-filled liposomes were prepared in an identical manner except that clodronate was omitted.

FIGURE 1 Time line for exposures to clodronate or saline liposomes and subsequent LPS or saline aerosol exposures. On day 0, 16 rats were exposed to clodronate liposomes (AM-depleted) and 16 to saline liposomes (undepleted). Three days later, 8 rats from each group were exposed to LPS or saline aerosols. At 4 and 24 hours post aerosol exposure, rats from each group (n = 4) were euthanized and BAL fluid and lung tissue samples were obtained. See text for discussion.


**Intratracheal Inhalation of Liposomes**

Groups of 8 rats were given clodronate or saline liposomes via ITIH as previously described [10, 13]. After induction of anaesthesia (halothane, 3%; Halocarbon Laboratories, River Edge, NJ), the trachea of each rat was cannulated transorally with the plastic sheath of a 14-gauge catheter and the rats were connected to a constant flow rodent respirator system. Inspiratory pressure was held at 20 cm H2O. Using a timer-activated solenoid valve, the inspiratory and expiratory phases were set for 1.5 and 0.5 seconds, respectively. Anaesthesia was maintained with halothane (1%, mixed in-line with the liposome aerosol) throughout the exposures. Liposomes (0.8 to 0.9 mL of suspension; 15 mg clodronate) were mixed with sterile saline to a total volume of 4 mL and then aerosolized using a low-flow jet nebulizer (Lovelace Respiratory Research Institute, Albuquerque, NM; 1.67 liters per minute [LPM], 50 psi). The entire volume of the liposome suspension was used, resulting in an estimated alveolar deposited dose of 186 μg clodronate per rat during the 55-minute exposure. Using a multistage cascade impactor, the liposome particles were determined to have a mass median aerodynamic diameter of 1.1 μm (geometric standard deviation [GSD] = 1.9 μm). The aerosol concentration was monitored via a real-time aerosol sensor (RAS-2; Monitoring Instruments for the Environment, Bedford, MA), the output of which was displayed on a chart recorder. At the end of the exposure, the halothane was turned off to allow the rats to regain consciousness while on the respirator. Rats were removed from the inhalation system and the cannulae removed when spontaneous breathing was apparent; this typically occurred within 5 minutes of halothane cessation. The righting reflex was typically restored within 5 minutes of cannula removal.

**Endotoxin (LPS) Aerosol Exposure**

Three days after liposome administration, groups of 8 rats were exposed to LPS from *Pseudomonas aeruginosa* (Sigma Chemical; lot 87F4009; 4×10⁶ endotoxin units [EU]/mg) via whole-body inhalation in a compartmentalized exposure system. Aerosols (mass median aerodynamic diameter = 0.72 μm; GSD = 1.61 μm) were generated at 30 psi using an Aerotech nebulizer (CIS-US, Bedford, MA) and were then delivered at an airflow of 15 LPM into the 31-L exposure system. Aerosol concentration was monitored by an aerosol sensor (RAS-2) as described above for the liposomes. Exposures to aerosols of LPS or pyrogen-free saline as a control lasted for ~12 minutes. The alveolar deposited dose was estimated to be 358 EU per rat.
Bronchoalveolar Lavage

At 4 and 24 hours after LPS or saline aerosol exposure, 4 rats from each group (AM-depleted or undepleted) were anaesthetized with sodium pentobarbital (intraperitoneal [IP], 20 mg/100 g body weight [BW]) and exsanguinated. A small incision was made in the trachea below the 5th cartilagenous ring and the plastic sheath from an intravenous (IV) catheter was tied into the trachea. The lungs, trachea, and heart were removed en bloc and excess tissue were removed. The lungs were lavaged with sterile, pyrogen-free normal saline (5-mL aliquots × 10 times) with gentle massaging. Cells were pelleted by centrifugation at 300 × g for 12 minutes, with the supernatant from the first two lavage aliquots kept separate for protein and enzymatic analyses. Pelleted cells from all aliquots of lavage fluid for a given animal were pooled and resuspended in saline for viability determination, enumeration, differential analysis, and chemiluminescence. The right lung was frozen in liquid nitrogen for mRNA analaysis.

Cellular and Biochemical Parameters in BAL

Cells in BAL were counted with a bright-line hemocytometer, and viability was assessed by trypan blue exclusion. A hematoxylin-eosin variant (Diff-Quik; Baxter Scientific, Edison, NY) was used to stain cytocentrifuged cells for differential analysis at 250 ×. At least 500 cells per sample were examined in determining percentages and numbers of AMs, neutrophils, and lymphocytes. The magnitude of AM depletion in clodronate-treated rats was calculated relative to rats given saline liposomes and exposed to aerosolized saline and was adjusted for cell viability. The total protein concentration in cell-free BAL was measured by a bicinchoninic acid assay (Pierce Chemical, Rockford, IL) as an indicator of permeability injury to the alveolocapillary membrane. Lactate Dehydrogenase (LDH) activity in cell-free BAL fluid was measured based on its ability to oxidize NADH using a commercially available kit (DG1340-UV; Sigma Chemical). The activity of the lysosomal enzyme β-glucuronidase was measured in cell-free BAL from the production of p-nitrophenol from 4-nitrophenylglucuronide using reagents from Sigma Chemical.

Myeloperoxidase Staining

A myeloperoxidase stain was used to identify newly-arrived macrophages in lavage [23–25]. Cells pelleted by cytocentrifugation of BAL fluid were fixed (formaldehyde [10%] in 100% ethanol), washed in tap water, and then incubated with a benzidine dihydrochloride-based myeloperoxidase stain according to the method of Kaplow [26]. Cells were counted
and categorized according to size and shape, nuclear morphology, and the presence of blue cytoplasmic granules (myeloperoxidase-positive).

**Chemiluminescence for Assessing Production of Reactive Oxygen Species (ROS)**

Resting and activated respiratory burst activity were measured in BAL cells \((1 \times 10^5)\) as follows. Cells were mixed with luminol in Kreb’s-Ringer-HEPES buffer \((1.5 \text{ mM } \text{MgCl}_2 \cdot 6\text{H}_2\text{O}, 1 \text{ mM } \text{CaCl}_2, 1 \text{ mM } \text{K}_2\text{HPO}_4, 126 \text{ mM } \text{NaCl}, 5 \text{ mM } \text{KCl}, 10 \text{ mM } \text{dextrose}, 25 \text{ mM } \text{HEPES}, \text{pH } 7.3)\) such that the cell suspension equaled one half the reaction volume and the final concentration of luminol was \(10^{-4} \text{ M}\) (borosilicate glass, \(12 \times 75 \text{ nm})\). For activation of the cells, a phorbol methyl ester (phorbol myristate acetate, PMA) was added to some tubes such that its final concentration was \(1.5 \times 10^{-7} \text{ M}\). Chemiluminescence was measured using a TD-20e luminometer (Turner Designs; Sunnyvale, CA) immediately after addition of luminol or luminol + PMA and every 4 minutes thereafter up to 20 minutes. Respiratory burst activity was always measured within 3 hours of initial cell isolation from BAL. A time-response curve for each sample with and without PMA was constructed, and the area under these curves (AUC) was calculated using Sigmaplot (SPSS Science, Chicago, IL) to obtain total respiratory burst activity.

**Inflammatory Cytokine mRNA Analyses**

Total RNA was isolated from the right lung using TRIzol reagent (Life Technologies; Grand Island, NY) according to manufacturer’s directions. Steady state mRNA levels were quantitated by means of ribonuclease protection assays using a multiprobe template set from Pharmingen (rCK-1; San Diego, CA) \([27]\) to examine the production of pro- and anti-inflammatory cytokines associated with oxidative stress. The cytokines included in analysis were interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-β, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α, IL-2, and interferon (IFN)-γ. The intensities of cytokine bands were analyzed using a phosphorimager (ImageQuant, Molecular Dynamics), with each band normalized to the intensity of the L32 band to correct for RNA loading. All bands analyzed were within the linear range of the response of the phosphorimager. Details of the RNase protection assay methods used have been described previously by Johnston and colleagues \([17]\).

**Statistical Analysis**

Data are reported as mean ± standard error (SE). Data were analyzed for main effects of clodronate and LPS and for factor interactions by
2-way analysis of variance (ANOVA) using SigmaStat (SPSS) [28]. Data were appropriately transformed (e.g., base 10, natural logarithm) if an analysis of residuals suggested deviations from the assumptions of normality and equal variance; any outliers were replaced with group means. Differences between groups were further analyzed using Tukey multiple comparisons. All comparisons were considered statistically significant when \( p \) was \( \leq 0.05 \).

RESULTS

BAL Cell Numbers and Percentages from AM-Depleted and Undepleted Rats Exposed to Aerosolized Saline or LPS

Pretreatment with clodronate versus saline liposomes substantially reduced the numbers of total cells and AMs in rats subsequently exposed to saline aerosols (Table 1). Decreases in total BAL cells in clodronate-pretreated animals were due to depletion of AMs rather than neutrophils (PMNs) or lymphocytes (see Figure 2a and b for absolute numbers of AMs and PMNs, respectively). The numbers of AMs in BAL were reduced by 87.1\% \pm 3.3\% and 90.9\% \pm 0.9\% at 4 and 24 hours, respectively, in depleted versus undepleted rats exposed to aerosolized saline (Table 1). Both depleted and undepleted rats had very few PMNs in BAL at 4 and 24 hours after subsequent exposure to aerosolized saline (\( \sim 0.01-0.05 \times 10^7 \) PMNs; Figure 2b). Although the percentages per se of PMNs in depleted rats increased in comparison to undepleted rats (Table 1), this was due to decreases in total cell number. Absolute numbers of BAL PMNs were not increased in depleted versus undepleted rats exposed to saline (Figure 2b) and normalized percentages of PMNs in BAL were not significantly different between the two groups (Table 1). Lavaged cells from depleted versus undepleted rats exposed to saline aerosols had decreased viability and mildly increased levels of protein, LDH, and \( \beta \)-glucuronidase activities (Table 1). These changes, which were also present in depleted and undepleted rats exposed to LPS (see below), are consistent with ongoing phagocytic cell death and injury from the bisphosphonate.

BAL cellular responses in depleted versus undepleted rats exposed to LPS were substantially different from those described above for saline-exposed rats. Total cell numbers were significantly increased in both depleted and undepleted rats exposed to aerosolized LPS as compared to aerosolized saline (Table 1). Pretreatment with clodronate liposomes depleted 83.2\% \pm 4.0\% and 70.5\% \pm 3.7\% of AMs in LPS-exposed rats compared to undepleted rats exposed to saline aerosols (Table 1). Both depleted and undepleted rats had increased percentages of BAL PMNs (non-normalized and normalized) following exposure to aerosolized LPS.
TABLE 1  Cellular and Biochemical Parameters in Lavage Fluid from AM-Depleted and Undepleted Rats 4 and 24 Hours after Inhalation Exposure to Inhaled Endotoxin (LPS) or Saline

<table>
<thead>
<tr>
<th></th>
<th>Total cells</th>
<th>% AM</th>
<th>% PMN (normalized%)</th>
<th>% Lymphocytes</th>
<th>Protein (mg/mL)</th>
<th>LDH (nmol/min/mL)</th>
<th>1 &amp; Glucuronidase (nmol/min/mL)</th>
<th>% AM depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
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<tr>
<td>liposomes + saline</td>
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<td></td>
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<tr>
<td>4 hours</td>
<td>1.69 ± 0.39</td>
<td>94.1 ± 2.5</td>
<td>1.73 ± 1.10 (1.57 ± 0.85)</td>
<td>4.17 ± 1.59</td>
<td>92.2 ± 0.9</td>
<td>0.18 ± 0.03</td>
<td>101 ± 12</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>24 hours</td>
<td>2.65 ± 0.39</td>
<td>94.4 ± 1.2</td>
<td>1.56 ± 0.66 (1.45 ± 0.60)</td>
<td>4.02 ± 0.70</td>
<td>95.3 ± 0.2</td>
<td>0.22 ± 0.03</td>
<td>125 ± 30</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td><strong>Clodronate</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>liposomes + saline</td>
<td>0.25 ± 0.05#</td>
<td>92.2 ± 3.79</td>
<td>4.86 ± 2.69 (0.55 ± 0.25)</td>
<td>2.94 ± 1.01</td>
<td>79.4 ± 3.9</td>
<td>0.39 ± 0.04#</td>
<td>187 ± 23#</td>
<td>6.9 ± 1.6#</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.34 ± 0.03#</td>
<td>77.5 ± 1.2</td>
<td>15.8 ± 1.1 (1.62 ± 0.13)</td>
<td>6.66 ± 0.45#</td>
<td>82.4 ± 3.1#</td>
<td>0.39 ± 0.02#</td>
<td>165 ± 9#</td>
<td>6.2 ± 0.9#</td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>liposomes + LPS</td>
<td>1.29 ± 0.06</td>
<td>66.9 ± 2.6</td>
<td>32.4 ± 2.58 (24.8 ± 2.0*)</td>
<td>0.70 ± 0.22</td>
<td>92.7 ± 1.7</td>
<td>0.16 ± 0.00</td>
<td>72 ± 5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>24 hours</td>
<td>1.33 ± 0.15</td>
<td>77.6 ± 0.6</td>
<td>23.7 ± 3.0 (12.0 ± 2.7*)</td>
<td>1.61 ± 0.16#</td>
<td>92.9 ± 0.6</td>
<td>0.15 ± 0.02#</td>
<td>111 ± 12</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td><strong>Clodronate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liposomes + LPS</td>
<td>2.46 ± 0.53#</td>
<td>11.0 ± 1.74*#</td>
<td>84.3 ± 1.2* (127 ± 30*#)</td>
<td>4.68 ± 0.58#</td>
<td>93.8 ± 2.1*</td>
<td>0.50 ± 0.04*#</td>
<td>254 ± 4*#</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>24 hours</td>
<td>3.65 ± 0.34*#</td>
<td>20.6 ± 1.3*#</td>
<td>71.0 ± 1.0* (95.4 ± 7.4*#)</td>
<td>6.88 ± 0.22#</td>
<td>93.2 ± 1.0*</td>
<td>0.55 ± 0.02*#</td>
<td>300 ± 12*#</td>
<td>8.1 ± 0.5*#</td>
</tr>
</tbody>
</table>

Note. Each group had a total n of 8, with n = 4 at each time point (mean ± SE). Exposures to LPS or saline aerosols were done 3 days after liposome administration by ITIH (see Methods and Figure 1), with BAL parameters assessed 4 and 24 hours later. *Significantly different from saline-exposed rats with the same depletion status, P < .05; #significantly different from undepleted rats that received same post exposure (saline or LPS), P < .05.

aThe normalized percentage of BAL PMNs and percent AM depletion were calculated using as a baseline the total cell number from rats receiving saline liposomes and aerosolized saline at the same time point adjusted for cell viability.
PMN numbers were maximally increased in depleted rats, increasing 5-and 8-fold at 4 and 24 hours, respectively, following exposure to LPS as
compared to saline (Figure 2b). Although percentages of lymphocytes in BAL were slightly increased, PMNs comprised the majority of the cellular response to LPS in depleted rats. In contrast, undepleted rats had much lower percentages of BAL PMNs and higher percentages of AMs following LPS exposure.

**Myeloperoxidase in BAL Cells from AM-Depleted and Undepleted Rats Exposed to Aerosolized LPS or Saline**

Treatment with clodronate liposomes did not induce the recruitment of immature monocytic cells into the airspaces of saline-exposed rats as determined by myeloperoxidase staining (Table 2). However, rats pretreated with clodronate and subsequently exposed to LPS had higher percentages of peroxidase-positive AMs than any of the other groups (although total numbers of AMs were low in these depleted animals). Of those cells identified as macrophages from their size, shape, and nuclear morphology, 7.1% ± 1.7% were peroxidase positive 4 hours after LPS exposure and 26.4% ± 5.0% were positive 24 hours after exposure (Table 2). Undepleted, LPS-exposed rats also had a smaller increases in the percentage of peroxidase positive AMs 24 hours after LPS exposure (4.2% ± 1.0% compared to 0.5% ± 0.3% in saline-exposed rats, Table 2).

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Peroxidase-Positive Macrophages in Bronchoalveolar Lavage Fluid from AM-Depleted and Undepleted Rats Exposed to Inhaled Endotoxin (LPS) or Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Peroxidase-positive</td>
<td></td>
</tr>
<tr>
<td>Saline liposomes + saline</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>Clodronate liposomes + saline</td>
<td></td>
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<tr>
<td>4 hours</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Saline liposomes + LPS</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>24 hours</td>
<td>4.2 ± 1.0*</td>
</tr>
<tr>
<td>Clodronate liposomes + LPS</td>
<td></td>
</tr>
<tr>
<td>4 hours</td>
<td>7.1 ± 1.7*,#</td>
</tr>
<tr>
<td>24 hours</td>
<td>26.4 ± 5.0*,#</td>
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</tbody>
</table>

*Significantly different from saline-exposed rats with the same depletion status, P < .05.

*Significantly different from undepleted rats that received the same post exposure (saline or LPS), P < .05.
Production of ROS by BAL Cells from Depleted and Undepleted Rats Exposed to LPS or Saline Aerosols

The resting and PMA-stimulated release of ROS from total cell populations in BAL are shown in Figure 3. Clodronate liposomes did not affect resting or PMA-stimulated BAL cell chemiluminescence at either 4 or 24 hours in rats exposed to aerosolized saline. ROS production in BAL cells was, however, significantly increased after LPS aerosol challenge at both time points studied. As was found for changes in the numbers and percentages of PMNs in BAL, unstimulated and stimulated ROS release were highest in depleted rats that were exposed to LPS (Figure 3).

Levels of Cytokine mRNA in Lung Tissue from AM-Depleted and Undepleted Rats Exposed to LPS or Saline Aerosols

Message expression in whole lung tissue for cytokines involved in inflammation was assessed by RNase protection assays in AM-depleted and undepleted rats exposed to LPS or saline aerosols. Tissues from naïve rats (no liposome treatment) exposed to aerosolized LPS were also examined. For this latter group, responses were examined only at 4 hours, because it has been previously shown that cytokine mRNA levels are not elevated over controls 24 hours after exposure to LPS aerosols [17]. Figure 4 shows the appearance of specific bands from the RNase protection assays and Figure 5 quantifies changes in mRNA expression based on densitometry measurements. Densitometry data were normalized to L32 mRNA levels, and are shown for IL-1α, IL-1β, IL-6, and TNF-α (there were no measurable changes in IL-3, IL-4, IL-5, TNF-β, IL-10, IL-2, or IFN-γ message levels). All values are plotted as ratios relative to rats pretreated with saline liposomes and exposed to saline aerosols. Levels of cytokine mRNA were increased at 4 hours following aerosol exposure and returned to control levels 24 hr. AM depletion by itself did not significantly increase inflammatory cytokine expression (compare clodronate liposomes + saline and saline liposomes + saline groups, Figure 5). However, all groups exposed to LPS (LPS alone; clodronate liposomes + LPS, and saline liposomes + LPS) had significant increases in cytokine message expression. Depleted and undepleted rats exposed to LPS aerosols (clodronate liposomes + LPS and saline liposomes + LPS) had higher levels of cytokine mRNA expression at 4 hr than rats exposed to LPS alone. Increases in cytokine mRNA were greatest for AM-depleted rats (clodronate liposomes + LPS), consistent with the other markers of inflammation assessed (PMN number, ROS release).
FIGURE 3 Resting and PMA-stimulated respiratory burst activity in BAL cells from AM-depleted and undepleted rats exposed to inhaled LPS or saline. (a) Resting activity; (b) PMA-stimulated activity. Groups are the same as in the legends to Figure 2a and b. The ordinate is the area under the time-response curve (AUC) for BAL cells obtained at 4 hours (open bars) and 24 hours (filled bars). Each bar represents the mean ± SE of data from 4 rats. *Significantly different from saline-exposed rats with the same depletion status, $P < .05$; #significantly different from undepleted rats that received same post-exposure (saline or LPS), $P < .05$. 

Effect of AM Depletion on Inflammation
DISCUSSION

This study has investigated BAL cellular responses and cytokine message expression in lung tissue from rats depleted of AMs by treatment with intratracheally inhaled clodronate-filled liposomes. Rats pretreated with clodronate liposomes had 87% to 91% depletion of lavage AMs at 4 and 24 hours post exposure to aerosolized saline compared to undepleted rats pretreated with saline liposomes (Table 1, Figure 2a). Despite AM depletion, these rats had significantly higher levels of lavaged PMNs (Table 1, Figure 2b), cellular ROS production (Figure 3), and mRNA for IL-1α, IL-1β, IL-6, and TNF-α in lung tissue (Figures 4, 5) following LPS exposure compared to undepleted rats. The observed increases in ROS production in BAL cells from AM-depleted rats exposed to LPS (Figure 3) were quantitatively larger than the increased percentage in PMNs in BAL. There were
3-fold increases in the percentage of PMNs in depleted and undepleted rats at 4 and 24 hours after LPS exposure, whereas PMA-stimulated ROS production was 4- and 6-fold higher at these times (Table 1, Figure 3). This increase in ROS production relative to BAL PMNs could reflect an increased activity of neutrophils or of newly recruited myeloperoxidase-positive monocytic cells in these animals (Table 2), the percentage of which was highest in depleted rats exposed to LPS. These data highlight the increased inflammatory response found in clodronate pretreated (AM-depleted) rats relative to undepleted rats exposed to LPS.

We have previously shown that ITIH is an effective method for delivering clodronate-containing liposomes to the lung to deplete AMs [10]. Administration of unencapsulated clodronate to deplete AMs is not desirable because other cell types are killed indiscriminately using this method.
Furthermore, the delivery of liposome-encapsulated clodronate promotes cell death via a predominantly apoptotic pathway [8], thus limiting the influx of inflammatory cells and their activation. To selectively target phagocytic cells, other investigators have delivered clodronate liposomes using whole-body inhalation [11] or intratracheal instillation [12, 29, 30]. However, the ITIH method of clodronate liposome administration used here combines the advantages of efficiency and low inflammatory potential to achieve a high degree of AM depletion without confounding variables [10]. One day after clodronate liposome administration, AMs are depleted by only about 42%; depletion is maximal (~90%) 3 days following ITIH of clodronate liposomes [10], consistent with levels found here (Table 1). In the present study, the ongoing cytotoxicity of inhaled clodronate liposomes against AMs was evident 3 to 4 days later based on decreases in cell viability and increases in protein, LDH, and β-glucuronidase levels in BAL (Table 1). However, despite this ongoing cytotoxicity, AM-depleted rats clearly mounted an increased LPS-induced inflammatory response with elevated levels of PMNs in BAL and cytokine mRNA in lung tissue compared to undepleted rats exposed to LPS (Figures 2b to 5).

Pulmonary inflammatory responses to LPS have been examined in naïve rats (not liposome pretreated) in our prior studies [15–17]. In this earlier work, rats receiving a similar dose of LPS to that delivered here had approximately 25% PMNs in BAL at 24 hours post exposure without accompanying changes in total protein concentration, cell viability, or LDH and β-glucuronidase activities. This is consistent with findings here for the percentages of PMNs in BAL from LPS-exposed rats pretreated with control saline liposomes (Table 1). Increases in BAL PMNs from clodronate pretreated LPS-exposed rats were evident at both 4 and 24 hours, whereas cytokine mRNA was elevated at 4 hours only. Increased numbers of PMNs in BAL at 24 hours are consistent with recruitment initiated by earlier increases in cytokine message (4 hr as examined here). The fact that levels of PMNs in BAL were also increased 4 hours after exposure to LPS (Table 2, Figure 2b) suggests that cytokine message levels were probably elevated at even earlier times, although additional experiments would be needed to define this more precisely.

The strategy of macrophage depletion using liposome-encapsulated clodronate has been employed previously to help define AM function in a variety of inflammatory and immune response. For example, we have shown that the inflammatory response to intratracheally instilled cytotoxic crystalline silica is significantly reduced in clodronate treated rats [10]. Hashimoto and colleagues [11] found that AM-depletion via inhalation of clodronate liposomes significantly reduced PMN chemotaxis to the lung in response to instilled live P. aeruginosa, a gram-negative bacterium that releases LPS. Our current observations of enhanced inflammatory
responses to inhaled endotoxin itself appear to conflict with findings from
two other studies in which animals were exposed to LPS. Koay and colleagues [31] found that the response to inhaled LPS was greatly reduced
in AM-depleted mice. However, the dose of LPS used produced a very
severe inflammatory reaction (~90% PMNs 4 hours after exposure as com-
pared to 32% PMNs in the present study). Furthermore, significant
decreases in response were not achieved by Koay and colleagues unless
the clodronate liposomes were both intratracheally instilled and intrave-
nously injected. Thus, the two depletion models are very different. Berg
and colleagues [9] found that the PMN response to instilled endotoxin
was also suppressed after depleting ~70% of AMs from the lung with
instilled clodronate liposomes. Again, however, the LPS dose was 25 times
higher than what was used in the present study (predicted alveolar dose of
200 ng, ~360 EU). Furthermore, both Berg [9] and Hashimoto [11] and
their colleagues reported substantial levels of BAL PMNs (~20% at the
time of subsequent exposures) following exposure to clodronate lipo-
somes. This contrasts with our results showing low numbers and normal-
ized percentages of BAL PMNs in saline-exposed rats receiving
clodronate liposomes by ITIH (Table 1). Higher levels of inflammation
from instilled clodronate liposomes may significantly alter responses to sub-
sequent injurious stimuli. Lastly, we conducted LPS exposures 3 days after
clodronate treatment, when AM depletion was maximal, whereas Berg and
colleagues [9] waited one day. The timing of exposures to inflammatory
stimuli is important because tolerance or adaptation can occur [15, 32–34].

AMs are known to be important in initiating and facilitating acute pul-
monary inflammatory responses to toxicants and other inducers of injury.
The spectrum of effects mediated by AMs in injured lungs can be suppress-
ive as well as stimulatory. Two groups have shown that antigen-presenting
activity in the lung is enhanced in macrophage-depleted rats [29, 30]. Tang
and colleagues [35] reported decreases in IFN-γ and increases in eosinophil-
ia, airway hyperreactivity, and IL-4/IL-5 production in response to ovalbu-
min challenge when AMs were depleted after allergic sensitization. A
suppressive role for AMs during some forms of infection has also been sug-
gested. For example, T cells have been shown to be present in greater num-
bers and to be more activated in AM-depleted versus undepleted mice with
Mycobacterium tuberculosis infections; depleted mice also had higher numbers
of lavage PMNs in the early stages of infection [36]. Likewise, the response
to influenza virus is reported to be enhanced in AM-depleted mice [37]. The
increases observed in the present study in PMNs in BAL, cellular ROS pro-
duction, and cytokine mRNA in lung tissue also point to a suppressive role
for AMs in this model. The LPS response was not simply unaffected by AM
depletion, but was instead amplified when AMs were absent. It could be
argued that the liposomes themselves primed the lung for its subsequent
response to LPS because cytokine mRNA levels in depleted and undepleted rats were higher than for rats exposed to LPS alone (Figure 5). However, this is countered by the fact that depleted and undepleted rats exposed to saline had low cytokine mRNA levels. Furthermore, the highest levels of mRNA expression were found in lung tissue from depleted rats exposed to LPS.

In addition to AM-related suppressive activity, other factors could also have contributed to our findings of significant inflammation and inflammatory cell recruitment in AM-depleted rats exposed to moderate levels of aerosolized LPS. It is very probable that pulmonary and/or leukocytic cells other than AMs are also involved in LPS-induced inflammatory responses. Early signals for the recruitment of inflammatory leukocytes are known to be elaborated from alveolar type II epithelial cells, interstitial macrophages, and PMNs. Also, the possibility that activated, undepleted AMs contribute to the observed responses to LPS cannot be ruled out. However, if remaining macrophages were solely responsible for the PMN influx, this would represent a huge biological reserve (i.e., the 71% to 91% AMs that were depleted were not needed at all). Aside from inflammatory cells, lung epithelial cells also respond to LPS exposure by increasing expression for CD14 [38, 39], a receptor for LPS, and for cytokines and chemokines [4, 40, 41]. Additional studies conducted during the earlier phases of AM depletion and during recovery to characterize the morphology and cytokine/chemokine profiles of remaining macrophages and epithelial/interstitial cells in the alveolar regions could better identify the cell types that control responses to inhaled LPS and related inflammatory stimuli in AM-depleted lungs.

REFERENCES

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