Acute lung injury in children: from viral infection and mechanical ventilation to inflammation and apoptosis
Bern, R.A.

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Depletion of Resident Alveolar Macrophages Does Not Prevent Fas-mediated Lung Injury in Mice

Reinout A. Bem
Alex W. Farnand
Venus Wong
Amy Koski
Michael E. Rosenfeld
Nico van Rooijen
Charles W. Frevert
Tom R. Martin
Gustavo Matute-Bello

1 Research Service of the VA Puget Sound Health Care System and 2 Center for Lung Biology, Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington, Seattle (WA); 3 Department of Pathobiology, Department of Medicine, University of Washington, Seattle (WA); 4 Department of Cell Biology, Faculty of Medicine, Free University, Amsterdam, The Netherlands

Activation of the Fas/FasL system in the lungs results in a form of injury characterized by alveolar epithelial apoptosis and neutrophilic inflammation. Studies in vitro show that Fas activation induces apoptosis in alveolar epithelial cells and cytokine production in alveolar macrophages. The main goal of this study was to determine the contribution of alveolar macrophages to Fas-induced lung inflammation in mice, by depleting alveolar macrophages using clodronate-containing liposomes. Liposomes containing clodronate or PBS were instilled by intratracheal instillation. After 24 hr, the mice received intratracheal instillations of the Fas-activating monoclonal antibody Jo2 or an isotype control antibody, and were studied 18 hours later. The Jo2 mAb induced increases in broncholalveolar lavage fluid (BALF) total neutrophils, lung caspase-3 activity, BALF total protein, and worsened histological lung injury in the macrophage-depleted mice. Studies in vitro showed that Fas activation induced the release of the cytokine KC in a mouse lung epithelial cell line, MLE-12. These results suggest that the lung inflammatory response to Fas activation is not primarily dependent on resident alveolar macrophages and may instead depend on cytokine release by alveolar epithelial cells.
INTRODUCTION

Acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS) are acute-onset inflammatory lung conditions characterized by hypoxemia and bilateral lung infiltrates, in the setting of a normal cardiac preload. Each year, more than 175,000 patients develop ALI/ARDS in the United States, and of these approximately 74,000 die. However, the initial pathogenic events leading to ALI/ARDS remain unclear, and treatment is limited to supportive measures.

The receptor-ligand system Fas/FasL has been implicated in the pathogenesis of ALI in humans and animals. Fas (CD95) is a cell surface receptor which in the lungs is expressed in airway and alveolar epithelial cells, in neutrophils, and in alveolar macrophages. The natural ligand of Fas, FasL (CD178) exists in a soluble form and in a membrane-bound form. In the lungs, the membrane-bound form of FasL is expressed in the airway and alveolar epithelia, as well as in resident and migrating leukocytes. The soluble form of FasL (sFasL) results primarily from proteolytic cleavage of membrane-bound FasL by MMP-7 (matrilysin), although it can also be released by activated monocytes. Initial studies had suggested that sFasL was primarily a negative regulator of membrane FasL, but subsequent studies showed that sFasL retains its bioactivity in the lungs, both in vivo and in vitro.

The Fas/FasL system has been traditionally considered a prototypical pro-apoptotic system. Binding of Fas to FasL triggers activation of a series of cysteine proteases collectively known as caspases, which eventually leads to apoptosis. However, studies performed in vitro have established that binding of Fas to FasL can also lead to activation of pro-inflammatory pathways, activation of the nuclear factor κB, and release of pro-inflammatory cytokines, including the neutrophil chemoattractant IL-8 (CXCL8). The role of the pro-apoptotic function of the Fas/FasL system in human and experimental lung injury has been extensively studied (reviewed in), but the role of the pro-inflammatory function of Fas in the pathogenesis of lung injury remains less clear.

Earlier studies investigating activation of the Fas/FasL system in the lungs consistently found evidence of an inflammatory response characterized by cytokine release and neutrophil recruitment into the lungs. The relevance of the pro-inflammatory function of the Fas/FasL system was confirmed in studies demonstrating that mice deficient in Fas have an impaired neutrophilic response to inhaled LPS, and that silencing of Fas in the lungs protected against lung inflammation in a model of hemorrhagic shock followed by cecal ligation and puncture. Furthermore, blockade of the Fas/FasL system by specific pharmacologic inhibitors or by the lpr mutation resulted in reduced BALF neutrophil counts and lower concentrations of TNF-α and MIP-2 48 hr after intratracheal instillation of S. pneumoniae. Taking together, these studies suggest that the Fas/FasL system may play an important role not just in apoptosis, but also in the development of inflammation.
of an inflammatory response in the lungs following exposure to LPS, live bacteria, and sepsis.

An important question is whether the inflammatory response associated with the Fas/FasL system results from a direct pro-inflammatory effect of Fas signaling in specific lung cells, or instead is secondary to an initial apoptotic injury in the lungs. Studies by Park et al. showed that human macrophages incubated with human recombinant sFasL or the agonistic antibody CH-11 in vitro do not become apoptotic, but instead release pro-inflammatory cytokines such as TNF-α and IL-8. Interestingly, in the Park study macrophages released similar amounts of IL-8 in response to 500 ng/mL of sFasL and to 1 μg/mL of LPS. In contrast, the responses of alveolar epithelial cells to FasL in vitro include both apoptosis and release of IL-8. These in vitro studies led to the initial hypothesis that Fas-induced lung injury resulted from a combination of pro-inflammatory responses in macrophages, leading to cytokine release and neutrophil migration, and alveolar epithelial apoptosis, leading to disruption of the epithelial barrier. This hypothesis was tested in vivo using chimeric mice lacking Fas in either myeloid or non-myeloid cells, and the prediction was that following Fas activation, the mice expressing Fas in macrophages would develop an inflammatory response, and the mice expressing Fas in their epithelium would develop alveolar epithelial apoptosis and enhanced lung permeability. However, the prediction was wrong; the mice expressing Fas only in their myeloid cells showed little response to Fas activation, while the mice expressing Fas in their epithelium showed evidence of both inflammation and apoptosis, suggesting that the inflammatory response to Fas in the lungs was independent of Fas activation in macrophages. It is possible that in the chimera study, resident alveolar macrophages might have been activated in response to exposure of the basement membrane resulting from apoptosis of alveolar epithelial cells, or alternatively in response to phagocytosis of apoptotic epithelial cells. Therefore, the question of whether macrophages were responsible for cytokine production and inflammation following Fas activation remained unclear.

The goal of the present study was to determine whether resident alveolar macrophages are required for the development of Fas-induced lung inflammation in mice, using a model of clodronate depletion of lung alveolar macrophages. Furthermore, we investigate whether murine alveolar epithelial cells release cytokines in response to Fas activation. The main findings are that macrophage-depleted mice developed a neutrophilic inflammatory response following Fas activation with the Fas-activating antibody Jo2 in vivo, and that the murine alveolar epithelial cell line MLE-12 releases the neutrophil chemoattractant KC in response to Fas activation in vitro.
METHODS

Reagents
Clodronate (clod, dichloromethylene diphosphonate)-encapsulated liposomes and phosphate buffered saline (PBS)-encapsulated liposomes were prepared as described before 27. Clodronate was a kind gift of Roche diagnostics GmbH, Mannheim, Germany. The liposomes were stored up to 2 weeks at 4°C in sealed tubes containing N2. Purified hamster anti-mouse Fas mAb Jo2, LPS-free, azide free, was purchased from BD PharMingen (San Diego, CA). Purified hamster anti-keyhole limpet hemocyanin IgG2, also from BD PharMingen, was used as isotype control mAb. Antibodies used for immunohistochemistry included rat anti-mouse mac-2 (Accurate Laboratory Research Products, Westbury, NY), rat anti-F4/80 mAb (Serotec Inc, Raleigh, NC), rabbit anti-CX3CR1 Ab (Anaspec, San Jose, CA), goat anti-CCR2 Ab (Abcam, Camebridge, UK), biotinylated mouse-anti rat IgG, chicken anti-rat IgG-Alexa Fluor 647, chicken anti-rabbit IgG- Alexa Fluor 488 and donkey anti-goat IgG-Alexa Fluor 546 (all Zymed, Invitrogen, Carlsbad, CA).

Animal protocols
The animal protocols were approved by the Animal Care Committee of the VA Puget Sound Healthcare System, Seattle, WA. Briefly, male C57BL/6 mice weighing 25-30 g (Jackson Laboratories, Bar Harbor, ME) were anesthetized with inhaled isoflurane and placed on an inclined surface. The larynx was visualized and the trachea was intubated with a gavage tube attached to a 1.0 ml syringe containing 100 μl of water. Intubation of the trachea was verified by movement of the water meniscus in the syringe during the animal’s respiratory efforts. After endotracheal intubation, each mouse received 100 μL of liposomes in a single aliquot through the endotracheal tube. The tube was removed and the mice were allowed to recover from anesthesia and return to their cages with free access to food and water.

The mice were re-anesthetized and re-intubated 24 hr after instillation of the liposomes, and received instillations of Jo2 mAb or an isotype control IgG, 2.5 μg/g. After the instillations, the mice were allowed to recover from anesthesia and returned to their cages with free access to food and water. Eighteen hours later, the mice were euthanized with an intraperitoneal injection of pentobarbital (120 mg/kg) and exsanguinated by closed intracardiac puncture. The thorax was opened and the trachea cannulated and secured. The left hilum was clamped and the left lung was removed and placed in a tube containing sterile H2O plus protease inhibitor (Roche Applied Science, Indianapolis, IN) for homogenization. After removing the left lung, the right lung was lavaged with a 0.6 ml aliquot of 0.9 % NaCl containing 0.6 mM EDTA, followed by three separate 0.5 ml aliquots. The BAL aliquots were pooled for further analysis. Immediately after the BAL procedure, the right lung was fixed with 4% paraformaldehyde at 15 cm H2O for histological analysis.
**Experimental design**

First, to determine the extent and duration of alveolar macrophage depletion induced by clodronate, mice were treated with intratracheal PBS- or clodronate liposomes and then studied after 24, 48 or 72 hr (n=3/group per time).

In a second set of experiments, mice were assigned to one of four groups: PBS-liposomes + control IgG mAb (PBS + IgG, n=6); clodronate liposomes + control IgG mAb (Clod + IgG, n=6); PBS-liposomes + Jo2 mAb (PBS + Jo2, n=6); and clod-liposomes + Jo2 mAb (Clod + Jo2, n=6).

**Sample processing**

The BALF aliquots from each mouse were pooled, and an aliquot was processed immediately for total cell counts and differentials. The remainder of the BALF was spun at 200 x g and the supernatants were stored in individual aliquots at -70°C. Each left lung was homogenized in 1.0 ml of sterile H₂O with protease inhibitors (Roche Diagnostics Corporation, Indianapolis IN). The lung homogenate was divided into aliquots for later cytokine and myeloperoxidase (MPO) measurements. For cytokine and caspase-3 activity measurements, an aliquot of the lung homogenate was vigorously mixed with a buffer containing 0.5% Triton-X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl₂ and 1mM MgCl₂, pH 7.40, incubated for 30 min at 4°C, and then spun at 10,000 x g for 20 min. The supernatants were stored at -70°C. For MPO measurements, the homogenate was vigorously mixed with 50 mM potassium phosphate, pH 6.0, with 5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich, St. Louis, MO) and 5 mM EDTA. The mixture was sonicated and spun at 12,000 x g for 15 min at 25°C, and the supernatant was stored at -70°C.

**Measurements**

**Total cell counts and differentials.** Total cell counts were performed on an aliquot of the BALF, using a hemacytometer. Differential cell counts were performed on cytopsin preparations stained with the Diff-quick method (Andwin Scientific, Addison IL).

**Myeloperoxidase** was measured in lung homogenates using the Amplex Red fluorometric assay, following instructions from the manufacturer (Molecular Probes, Eugene, OR) \(^2\)

**Permeability measurements.** The total protein concentration in BALF was measured using the bicinchoninic acid method (BCA assay, Pierce Co., Rockford, IL). The concentration of IgM in BALF was measured with a specific mouse immunoassay (R&D Systems, Minneapolis MN). After dilution of the samples, the lower limit of detection of the IgM assay was 20 ng/mL.

**Cytokine measurements.** The cytokines TNF-α, IL-1β, MIP-2, KC, GM-CSF, VEGF, IFN-γ and IL-6 were measured in lung homogenates using the Fluorokine MultiAnalyte Profiling kits (R&D systems, Minneapolis, MN) for a multiplex fluorescent bead assay (Luminex, Austin, TX). After dilution of the samples, the lower limits of detection were 18.7 pg/mL for TNF-α, 130.1 pg/mL for IL-1β, 29.1 pg/mL for KC, 21.1 pg/mL for MIP-2, 23.1 pg/mL for GM-CSF, 13.2 pg/mL for VEGF, 63.1 pg/ml for IFNγ and 27.2 pg/mL for IL-6.
**Caspase-3 activity** in lung homogenates was measured with the caspase-3/CPP32 Fluorometric Assay kit (Biovision Inc., Mountain View, CA). Lung homogenate aliquots were diluted 1:2 in assay reaction buffer containing 10mM DTT and incubated for 2 hr at 37°C with the caspase-3 specific substrate DEDV-AFC (50μM). Fluorescence was read with a fluorometer using 400 nm excitation and 505-nm detection filters. Results are shown as arbitrary fluorescence units.

**Histopathology and immunohistochemistry**

The sections were deparaffinized by heating to 57°C for 60 min and rehydrated by washing twice in xylene for 5 min, twice in 100% ethanol for 3 min, twice in 95% ethanol for 3 min, and once in dH2O for 5 min. The slides were then washed three times in PBS for 5 min and treated with 0.3% Triton X-100 for 30 min at room temperature. After washing in PBS three times for 5 min, endogenous peroxidases were blocked with Peroxo-Block (Zymed, Invitrogen, Carlsbad, CA) for 90 seconds at room temperature. Next, the slides were washed again in PBS, treated with boiling 10 mM citric acid, 0.05% Tween-20, pH 6.0 for 15 min and blocked 30 min at room temperature with Dako Serum-Free Protein Block (Dako, Carpinteria, CA). The tissues were then labelled with rat anti-mac-2 mAb overnight at 4°C in a moist chamber. After washing in PBS for three times, the tissues were labelled with biotinylated mouse anti-rat mAb for 30 min at room temperature for mac-2 and washed in PBS three times. The slides were then labelled with streptavidin horseradish peroxidase conjugate (Zymed) for 10 min at room temperature, rinsed three times with PBS, and developed with AEC Peroxidase Substrate (Zymed) for 7.5 min. The slides were rinsed with running deionized H2O for 5 min, counterstained with 1% methyl green for 6 min and mounted with glycerol-vinyl-alcohol (Zymed).

For triple labeling for F4/80, CX3CR1 and CCR2 the slides were blocked with Dako Serum-Free Protein Block containing 3% goat serum and donkey serum (Jackson Immunoresearch, West Grove, PA). The slides were incubated with the rat anti-F4/80 mAb (Serotec Inc, Raleigh, NC), rabbit anti-CX3CR1 Ab (Anaspec, San Jose, CA) and goat anti-CCR2 Ab (Abcam, Camebridge, UK) for 1 hr at room temperature, and with the secondary antibodies (chicken anti-rat IgG-Alexa Fluor 647, chicken anti-rabbit IgG-Alexa Fluor 488 and donkey anti-goat IgG-Alexa Fluor 546 (all Zymed, Invitrogen)) for 40 min at room temperature. For detection, the slides were treated with Sudan Black (Fisher, Pittsburgh, PA), rinsed with running deionized H2O for 5 min and mounted with ProLong Gold Anti-gade reagent (Zymed, Invitrogen). The fluorescence signal was visualized using a confocal microscope (LSM510, Carl Zeiss Inc, Thornwood, NY). For quantification, we counted the number of positive cells in 5 randomly selected high power fields (400x) per tissue section.

DNA nick-end labeling assays (TUNEL) (TACS In situ Apoptosis Detection Kit; Trevigen Inc., Gaithersburg, MD) were performed as previously described 18. For quantification of
the TUNEL assay, we counted the number of positive cells in each of the 12 randomly generated fields per tissue section, at a magnification of 400X.

**Cellular studies**

MLE-12 mouse lung epithelial cells (ATCC No. CRL-2210) were cultured at 37°C, 5% CO₂ in DMEM/F12 (with Ham formulation) (Invitrogen, Carlsbad, CA) supplemented with 2% fetal bovine serum (HyClone, Logan, UT) 1% penicillin/streptomycin (Invitrogen), 1% L-Glutamine (Invitrogen), 1% HEPES (Sigma-Aldrich, St. Louis, MO), 1% Insulin/Transferrin/Sodium Selenite (Invitrogen), 0.01% β-Estradiol (Sigma-Aldrich) and 0.01% Hydrocortisone (Sigma-Aldrich). MLE-12 cells are SV40-transformed mouse lung epithelial cells that show several features of type II cells, including the presence of microvilli, intracellular multimellar inclusion bodies in the cytoplasm, and expression of the surfactant proteins B and C. The cells were seeded in 96 well tissue-culture plates (Costar, Cambridge, MA) and incubated at 37°C, 5% CO₂ until reaching 70-80% confluence, at which point the media was replaced with fresh media supplemented with serial concentrations of either Jo2 mAb or an isotype control IgG, with or without the broad caspase-inhibitor zVAD-fmk (100μM) (Axxora, San Diego, CA). After 18 h, the supernatants were collected for measurement of KC concentration by ELISA (R&D systems, Minneapolis, MN), and cell survival was measured using alamar Blue (BioSource, Camarillo, CA) as described previously.

**Statistical analysis**

Comparisons between multiple groups were performed using one-way ANOVA. Significance between groups was determined with the Fisher’s Least Significant Difference (LSD) post-hoc test. A p value of <0.05 was considered statistically significant. Data are reported in the text as means ± SEM, and shown in figures as box and whisker plots depicting individual data points and the median, the interquartile ranges and the 10th and 90th percentiles. The data in figures 5 and 8 are shown as means ± SEM.

**RESULTS**

**Clodronate Liposomes induce macrophage depletion in mice**

To determine the extent of macrophage depletion induced by clodronate we administered intratracheal liposomes containing PBS or clodronate to normal mice, and then performed cell counts and differentials in BALF recovered at 24, 48 or 72 hr after liposome instillation (n = 3/group). Macrophage depletion was maximal 24 hr after treatment with clodronate liposomes, and the decrease in total alveolar macrophages was approximately one order of magnitude as compared to the mice treated with PBS-liposomes (0.2 ± 0.1 x 10⁵ vs 2.7 ± 0.3 x 10⁵ cells, respectively) (Figure 1A). The clodronate liposomes did induce a small degree
of neutrophil recruitment, in the order of $10^3$ total cells, that persisted for 72 hr (Figure 1B). Lipopolysaccharide was not detected in the clodronate- or PBS-liposomes using the Limulus amebocyte assay.

To determine whether the neutrophil response seen in the BALF of the clodronate-liposome treated mice was associated with tissue damage, we examined lung tissue sections from mice euthanized 24 hr after treatment with liposomes containing PBS- (Figure 1C) or clodronate (Figure 1D). These sections confirmed that clodronate liposomes induced macrophage depletion. Importantly, no neutrophilic infiltrates were seen in the alveolar spaces or the interstitium of the mice treated with clodronate liposomes, and the normal lung architecture was preserved.

Based on these data, in the remaining experiments we administered In either a control IgG mAb or Jo2 mAb at 24 hr after liposome instillation. The mice were euthanized and

![Figure 1](image)

**Figure 1.** Total alveolar macrophages (A) and PMN (B) counts in the BALF of mice 24, 48 or 72 hr after intratracheal instillation of liposomes containing PBS (white) or clodronate (gray) (n=3/group for each time). Panel C and D show representative lung tissue sections from mice studied 24 hr after intratracheal instillation of liposomes containing PBS or clodronate respectively (H&E staining, magnification 400x). Note the absence of macrophages and neutrophils in the lungs from the mouse treated with clodronate liposomes (D).
studied 18 hours after administration of the antibodies. One animal in the PBS + IgG group died after PBS-liposome instillation.

The lung neutrophilic response to Jo2 mAb is not impaired by macrophage depletion

After treatment with clodronate liposomes, the BALF macrophage count decreased to 5.1 ± 1.1 x 10⁴ cells in the mice receiving the nonspecific IgG (p < 0.05 compared to the PBS + IgG and the PBS + Jo2 groups), and to 7.7 ± 1.8 x 10⁴ cells in the mice instilled with Jo2 mAb (p < 0.05 compared to the PBS + Jo2 group) (Figure 2A). Despite having a lower macrophage count, the mice treated with clodronate liposomes and Jo2 had a total BALF PMN count of 25.8 ± 4.4 x 10⁴ cells, which was significantly increased as compared to each of the other treatment groups (0.06 ± 0.06 x 10⁴ cells in the mice treated with PBS + IgG; 2.6 ± 1.6 x 10⁴ cells in the mice treated with PBS + Jo2; and 6.0 ± 3.1 x 10⁴ cells in the mice treated with clodronate + IgG, Figure 2B).

As an additional measurement of the neutrophil response, we assessed the total neutrophilic content in the lungs by measuring MPO activity in whole lung homogenates. Whole lung MPO activity was significantly increased in the mice treated with clodronate.

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**Figure 2.** Total BALF macrophage (A), PMN (B) and lymphocyte (C) counts and lung homogenate MPO activity (D) in mice treated with intratracheal liposomes containing PBS or clodronate followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 μg/g, and studied 18 hr later. n=6/group except for the PBS + IgG group, n=5.
liposomes and Jo2, as compared to the mice treated with clodronate liposomes and IgG (0.8 ± 0.1 vs 0.3 ± 0.0 cells, p < 0.05), but was similar to that of mice treated with PBS-liposomes and Jo2 (Figure 2D). Thus, the combined BALF and MPO data suggest that macrophage depletion did not prevent the neutrophilic response to Jo2, and may have even enhanced it.

The Jo2 mAb also induced a lymphocytic response in the BALF, which was highest in the mice treated with clodronate liposomes (Figure 2C).

The lung cytokine response to Jo2 mAb is not impaired by macrophage depletion
The administration of Jo2 mAb was associated with a trend towards an increase in all of the cytokines tested, and in almost all cases this increase was independent of macrophage depletion (Figure 3). These findings suggest that the cytokines tested did not originate in resident alveolar macrophages.

Clodronate treatment did not prevent Fas-mediated histologic lung injury
The mice in the PBS + IgG group exhibited normal lung histology (Figure 4A). The administration of Jo2 mAb to mice treated with PBS-liposomes resulted in focal areas of inflammatory infiltrates (Figure 4B). The lungs from mice treated with clodronate liposomes and IgG appeared normal, except for the absence of AM (Figure 4C). In contrast, the administration of Jo2 mAb to mice treated with clodronate liposomes was followed by inflammatory infiltrates and alveolar wall thickening (Figure 4D). Thus, the administration of Jo2 resulted in histologic lung injury regardless of the presence or absence of resident macrophages.

To confirm the extent of macrophage depletion induced by clodronate, we performed immunohistochemistry using the monocyte/macrophage marker Mac-2 (Figure 4, E-H). Mac-2 recognizes galectin-3, a β-galactoside-binding lectin which is highly expressed in macrophages. The lungs of mice treated with PBS-liposomes contained intra-alveolar cells staining for Mac-2 (arrows) (Figure 4, E-F). In contrast, the lungs from mice treated with clodronate liposomes and control IgG showed very few cells staining for Mac-2 (Figure 4H). However, the mice treated with clodronate liposomes and Jo2 showed a small number of cells staining for Mac-2, suggesting the presence of either residual resident macrophages or newly recruited macrophages (Figure 4D). Alternatively, Mac-2 may be expressed in type II cells in areas of severe inflammation. To further confirm the extent of macrophage depletion, we performed additional immunohistochemistry using F4/80 mAb, which recognizes a 160 kD glycoprotein expressed by murine monocytes/macrophages. Clodronate-liposome administration resulted in a decrease in cells staining for F4/80, and this decrease was similar in the mice treated with IgG and Jo2 mAb (Figure 5A).
Figure 3. Cytokine concentrations in lung homogenates from mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 μg/g, and studied 18 hr later. The cytokines were measured simultaneously using a multiplex assay, and include: TNF-α (A), IL1-β (B), MIP-2 (C), KC (D), GM-CSF (E), VEGF (F), IFN-γ (G) and IL-6 (H). n=6/group, except for the PBS + IgG group, n=5.
Figure 4. Lung tissue sections from mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 μg/g, and studied 18 hr later. The right column shows representative lung tissue sections stained with H&E (400x, insets 200x). The left column shows immunohistochemistry for AM (arrows), using anti mac-2 antibody.
Resident alveolar macrophages are thought to express low levels of the fractalkine receptor, CX3CR1, and variable levels of the MCP-1 receptor, CCR2. Newly recruited, highly inflammatory monocytes show high expression of CCR2, but low expression of CX3CR1. Therefore, we investigated the expression of CX3CR1 and CCR2 in cells expressing F4/80. In all mouse groups, the majority of cells expressing F4/80 co-expressed CX3CR1 and CCR2 (Figure 5B). We found no evidence for an increase in F4/80+, CX3CR1-, CCR2+ cells, which have been associated with increased inflammation and extensive recruitment to inflammatory sites. Thus, the data suggest that the lung injury

Figure 5. Number of cells staining for F4/80 in lung tissue sections from mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 μg/g, and studied 18 hr later (A). Panel B shows the percentage of F4/80 cells co-staining for CX3CR1 or CCR2 in the same sections. Data is shown as means ± SEM. * p < 0.05 as compared to percentages of CX3CR1 or CCR2 positive cells.

Figure 6. Lung homogenate caspase-3 activity (arbitrary fluorescence units) (A) or TUNEL positive cells per 12 high-power fields (B) in mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 μg/g and studied 18 hr later. n=6/group, except for PBS + IgG, n=5.
seen in the mice treated with clodronate liposomes and Jo2 did not result from increased recruitment of CX3CR1-, CCR2+ monocytes, and supports the idea that the inflammatory response caused by Jo2 was driven by activation of cells other than macrophages.

Apoptotic response to Fas activation in the setting of macrophage depletion

As mentioned in the introduction, activation of the Fas/FasL system can lead to inflammation and also apoptosis. To determine the extent of apoptosis in the lungs we measured caspase-3 activity in whole lung homogenates. Caspase-3 activity was highest in the mice treated with clodronate liposomes and Jo2 (1611.1 ± 400.8 arbitrary units), as compared to each of the other groups: 389.7 ± 14.2 in mice treated with PBS-liposomes and IgG; 758.9 ± 171.4 in mice treated with PBS-liposomes and Jo2; and 444.4 ± 40.5 in mice treated with clodronate liposomes and IgG (Figure 6A). As a separate measurement of apoptosis, we counted the number of cells staining positive by TUNEL in lung tissue sections (Figure 6B). There was a trend towards greater number of TUNEL-positive nuclei in the lungs of the mice treated with clodronate liposomes and Jo2, but this did not reach statistical significance.

We and others have postulated that alveolar epithelial cell apoptosis results in epithelial disruption and increase in the permeability of the epithelial barrier to proteins. The lung permeability response was assessed by measuring the BALF total protein and IgM concentrations. The BALF total protein concentration was 356.5 ± 60.0 μg/ml in the Clod + Jo2 mice, and this was significantly increased with respect to each of the other treatment groups (56.6 ± 2.3 μg/ml, PBS + IgG; 69.3 ± 11.8 μg/ml, PBS + Jo2; 165.3 ± 19.6 μg/ml, Clod + IgG, p < 0.05) (Figure 7A). The BALF total protein concentration was also significantly increased in the Clod + IgG group as compared to the PBS + IgG group (p < 0.05). The BALF concentration of IgM followed a similar pattern and was highest in the

![Figure 7](image_url)

**Figure 7.** Total BALF protein (A) and IgM (B) concentrations in mice treated with intratracheal liposomes containing PBS or clodronate, followed 24 hr later by intratracheal instillations of Jo2 mAb or control IgG mAb, 2.5 μg/g and studied 18 hr later. n=6/group, except for PBS + IgG, n=5.
Clod + Jo2 mice (Figure 7B). Contrary to our expectations, there was no apparent increase in the permeability markers in response to Jo2 mAb in the PBS-liposome treated mice.

**Jo2 mAb causes KC release from mouse lung epithelial cells in vitro**

Because the *in vivo* studies suggested that Jo2 mAb induced a pro-inflammatory cytokine response in the macrophage depleted mice, we investigated the cytokine response of mouse lung epithelial cells to Jo2 stimulation. We used MLE-12 cells, a lung epithelial cell line with several type II features which expresses Fas. As a representative cytokine we measured KC, a murine functional homolog of IL-8. The KC concentrations were measured in supernatants from MLE-12 cells after 18 hr incubation with serial concentrations of Jo2 mAb. The release of KC increased proportionally in response to increasing concentrations of Jo2 (Figure 8A). Treatment with the pan-caspase inhibitor ZVAD-fmk did not abrogate KC release, indicating that the induction of KC release was independent of caspase activation. MLE-12 are relatively resistant to Fas-induced apoptosis. As expected, incubation of the MLE-12 cells with Jo2 mAb caused no effect on cell survival, as determined by Alamar blue assay (Figure 8B). Interestingly, TNF-α, IL-1β, MIP-2 and MCP-1 were all below the limit of detection of the assay.

**Figure 8.** Concentrations of KC in the supernatant of MLE-12 cells incubated with serial concentrations of a control IgG (black), Jo2 mAb (grey) or Jo2 mAb plus the broad caspase inhibitor zVAD (100μM) (dark gray) (A). Cells incubated with VP16 (100μM) serve as positive control. Panel B shows cell survival in the same cells, as, determined by the Alamar Blue assay. The figures show data from at least 3 independent experiments. Data is shown as means ± SEM; * p<0.05 as compared with cells incubated with control IgG at each time point.
DISCUSSION

The main goal of this study was to determine whether resident alveolar macrophages are required for the development of Fas-induced lung inflammation in mice. Our main findings are that Fas activation induces cytokine release and neutrophilic alveolitis in mice deficient in alveolar macrophages, and that mouse epithelial cells release the chemokine KC in response to Fas activation in vitro. These findings suggest that the pro-inflammatory function of the Fas/FasL system plays an important role in the development of Fas-mediated lung injury, and point towards a prominent role of the epithelium in the cytokine response to Fas activation.

Studies investigating the role of the Fas/FasL system in the development of acute lung injury have focused primarily on its role as a pro-apoptotic system (reviewed in 17). However, in addition to apoptosis, activation of Fas may trigger pro-inflammatory pathways through activation of NF-κB. 39. The importance of the pro-inflammatory properties of the Fas/FasL system has been highlighted by a number of independent studies demonstrating that activation of Fas in the lungs of mice by either recombinant sFasL or activating antibodies is followed 3 to 24 hr later by a neutrophilic alveolitis associated with increased concentrations of pro-inflammatory cytokines including TNF-α, IL-1β, KC, MIP-2, GM-CSF, IL-5, and IFN-γ 18-21,23,40. This inflammatory response requires the presence of a functioning Fas receptor in the lungs and is prevented by the administration of pharmacological inhibitors of the Fas/FasL system 19,22,40. To investigate the magnitude of the response, Wortinger et al. directly compared the lung cytokine response to intratracheal instillations of recombinant human FasL (500 ng/mouse) and E. coli LPS (2 μg/mouse) at 3, 6 and 24 hr after instillation, and found that at all times tested, the BALF concentrations of GM-CSF, IL-1β, IL-5, IFN-γ and TNF-α were higher or similar in the FasL-treated mice as compared to the LPS-treated mice 40. Although the amount of FasL used in the Wortinger study was several orders of magnitude higher than the amount of LPS, the Fas/FasL system appears to play a role in lung inflammation induced by a number of noxae, including immune complexes, cecal ligation and puncture, inhaled bacteria and surprisingly, LPS itself 20-22,24.

The observation that Fas activation in the lungs is associated with an inflammatory response led to the question whether Fas signaling can trigger cytokine release by lung cells. Initial studies showed that human and murine macrophages do not become apoptotic in response to Fas ligation, but instead release pro-inflammatory cytokines. Specifically, human monocyte-derived macrophages release TNF-α and IL-8 in response to the Fas-activating mAb CH11, and murine alveolar macrophages release KC and MIP-2 in response to human recombinant sFasL. 8,40. These findings led us and others to propose the hypothesis that the Fas/FasL system contributes to lung inflammation in vivo by inducing cytokine release by alveolar macrophages. However, this hypothesis has been challenged by two separate studies. First, in a study designed to test whether Fas induces cytokine release by macrophages in vivo, we created chimeric mice expressing Fas in either their myeloid cells or their non-myeloid cells 26. Contrary to the hypothesis, we
found that only those mice expressing Fas in non-myeloid cells developed inflammation in response to the Jo2 antibody, and this was true for both the neutrophilic response and the cytokine response. Second, in a later study, Perl et al. showed that intratracheal instillation of Jo2 mAb induces release of KC, MIP-2 and MCP-1 in mice carrying the CSF1op mutation, even though these mice are deficient in monocytes and macrophages because they lack expression of colony stimulating factor-1. However, these studies are not definitive, because the chimeric mice had been subject to whole body irradiation, which may have modify the populations of immune cells in the lungs; and the CSF1op mice have macrophages derived from non-monocytic populations, and show normal responses to inflammatory stimuli thought to depend on macrophages, such as LPS.

To further investigate the role of alveolar macrophages in Fas-induced pulmonary inflammation, we used a model of macrophage depletion induced by instilling clodronate-containing liposomes into the lungs of mice. Clodronate liposomes cause macrophage depletion by a mechanism involving competitive inhibition of ADP/ATP translocase and subsequent apoptosis. In our study, treatment with Jo2 mAb was followed by neutrophilic inflammation and increased concentrations of several pro-inflammatory cytokines, despite the reduction in alveolar macrophages by clodronate-liposome instillations. Surprisingly, the neutrophil and cytokine responses to Jo2 mAb were actually enhanced by macrophage depletion. These findings suggest that the activation of pro-inflammatory pathways induced by Jo2 was not primarily dependent on alveolar macrophages.

The observation that lung cytokine release and neutrophilic inflammation in lung injury in vivo can occur when alveolar macrophages are depleted is important because it suggests that other cell type(s) in the lung can promote inflammation. In particular, the alveolar epithelium may be an important source of pro-inflammatory cytokines during lung injury. Studies performed in vitro show that primary human alveolar type II cells stimulated with LPS release CXC and CC chemokines, including MCP-1, GRO and IL-8. Additional experiments performed on rat primary type II cells and the human neoplastic type II cell line A549 confirm that alveolar epithelial cells can release IL-6 and IL-8, in response to IL-1β, TNF-α, and conditioned supernatants from LPS-treated macrophages. In the present study, mouse lung epithelial cells released the neutrophilic chemokine KC in response to Jo2 mAb in vitro. This further supports the hypothesis that the lung neutrophilic response in Fas-mediated lung injury may depend at least partly on cytokine release by alveolar epithelial cells.

An unexpected finding of the present study is that macrophage depletion seemed to worsen Fas-induced lung injury. Other investigators have found a similar enhanced lung inflammation and injury by macrophage depletion in different models of experimental lung injury. Using a rat model of aerosolized LPS, Elder et al. found that clodronate-containing liposome treatment led to a 5-fold increase in BALF PMN counts, as compared to saline liposomes. These findings have been reproduced by Beck-Schimmer et al. using a similar
rat model of LPS-induced lung injury and by Nakamura et al. in a rat model of ischemia-reperfusion. In addition, a delayed but more extensive lung neutrophilic response associated with increased mortality occurs in macrophage-depleted mice infected with Pseudomonas aeruginosa. Thus, several studies using models of lung injury that vary from activation of one single pathway (e.g. Fas in the present study) to instillation of LPS or live bacteria, have found that macrophage depletion can be associated enhanced lung injury. Knapp et al. have suggested that deficient phagocytosis and degradation of apoptotic PMNs due to macrophage depletion may be one mechanism of such enhanced lung injury. However, other studies have found that macrophage depletion with clodronate liposomes results in attenuation of lung injury following LPS administration, ischemia-reperfusion, experimental sepsis and mechanical ventilation. Thus, a possible explanation is that the relative contribution of the epithelium and macrophages to the production of pro-inflammatory cytokines is dependent on the initial injury to the lung, with the macrophages acting as immunomodulatory cells. Additional studies are needed to clarify the mechanisms linking macrophages and the inflammatory response in lung injury as well as the specific contribution of the Fas/FasL system to epithelial cytokine release.

Our study has several limitations. First, the administration of clodronate-liposomes did not result in a complete depletion of macrophages in the BALF. It could be argued that the residual macrophage population could be sufficient to induce a cytokine response. However, studies in which a comparable reduction in the of alveolar macrophages was achieved with clodronate liposomes treatment have shown impaired cytokine responses. As mentioned above, this suggests that the role of resident macrophages on cytokine production may depend on the cause of the lung injury, and further studies are needed to determine this issue. Another concern is that recruitment of highly pro-inflammatory monocytes could have explained the inflammatory responses to Jo2 mAb in the macrophage-depleted mice. However, we did not find evidence for an increase in the proportion of F4/80+, CCR2+ cells, which have been associated with increased pro-inflammatory activity. Finally, it is possible that the clodronate liposomes “primed” the lungs for additional injury. Our data shows that clodronate liposomes induced a mild neutrophilic response at baseline, which could have been magnified by the subsequent administration of Jo2. However, even if this was the case, our main conclusion that resident alveolar macrophages were not the primary source of pro-inflammatory cytokines remains valid.

In summary, depletion of alveolar macrophages by clodronate liposomes does not prevent, and may enhance, the lung cytokine and neutrophilic responses of mouse lungs to Fas activation in vivo. In addition, Fas activation with Jo2 mAb induces release of the chemokine KC by mouse lung epithelial cells in vitro. We conclude that the lung inflammatory response to Fas activation is not primarily dependent on alveolar macrophages and may instead depend on cytokine release by alveolar epithelial cells. These data are consistent with the interpretation that the alveolar epithelium may be an important source of pro-inflammatory cytokines during early acute lung injury.
REFERENCES


