Volutrauma, but not atelectrauma, induces systemic cytokine production by lung-marginated monocytes.

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ABSTRACT

Objective: Ventilator-induced lung injury has substantive impact on mortality of patients with acute respiratory distress syndrome. Although low tidal volume ventilation has been shown to reduce mortality, clinical benefits of open lung strategy are controversial. In this study, we investigated the impact of two distinct forms of ventilator-induced lung injury, i.e. volutrauma vs. atelectrauma, on the progression of lung injury and inflammation, in particular alveolar and systemic cytokine production.

Design: Ex vivo study.

Setting: University research laboratory.

Subjects: C57/BL6 mice.

Interventions: Isolated, buffer-perfused lungs were allocated to one of three ventilatory protocols for 3 hours: *control* group received low tidal volume (7ml/kg), PEEP (5cmH₂O) and regular sustained inflation; *high-stretch* group received high tidal volume (30-32ml/kg) with PEEP (3cmH₂O) and sustained inflation; and *atelectasis* group received the same tidal volume as control but neither PEEP nor sustained inflation.

Measurements and Main Results: Both injurious ventilatory protocols developed comparable levels of physiological injury and pulmonary edema, measured by respiratory system mechanics and lavage fluid protein. High-stretch induced marked increases in pro-inflammatory cytokines in perfusate and lung lavage fluid, compared to control. In contrast, atelectasis had no effect on perfusate cytokines compared to control, but did induce some upregulation of lavage cytokines. Depletion of monocytes margined within the lung microvasculature, achieved by pretreating mice with intravenous liposome-encapsulated clodronate, significantly attenuated perfusate cytokine levels, especially tumor necrosis factor, in the high-stretch, but

not atelectasis group.

Conclusions: Volutrauma (high-stretch), but not atelectrauma (atelectasis), directly activates monocytes within the pulmonary vasculature, leading to cytokine release into systemic circulation. We postulate this as a potential explanation why open lung strategy has limited mortality benefits in ventilated critically-ill patients.

INTRODUCTION

Mechanical ventilation can cause or aggravate lung injury leading to significant morbidity and mortality in critically-ill patients, a concept known as ventilator-induced lung injury (VILI). Various aspects of ventilation have been proposed as injurious based primarily on animal experiments, including overstretch of lungs, termed volutrauma, and epithelial shear stress resulting from repetitive collapse and re-opening of half-atelectatic lung units, termed atelectrauma (1). In the light of the preclinical evidence, 'protective ventilation' was designed to reduce VILI, by limiting volutrauma through the use of low tidal volumes (V_T) as well as preventing atelectrauma through the use of high positive end-expiratory pressure (PEEP) or recruitment maneuvers ("open-lung" strategy) (2), and has now been embraced by clinical practice (3).

Although the use of low V_T has convincingly been shown to improve mortality in patients with acute respiratory distress syndrome (ARDS) (4, 5), the clinical benefits of open lung strategy are still controversial (6-11). This lack of benefits is often ascribed to the deleterious physiological interactions of high PEEP such as end-inspiratory overstretch, hemodynamic changes, and impaired lymphatic drainage (12). Alternatively, this may be related to our current inadequate understanding of the optimal way to prevent atelectasis: for example, more sophisticated, personalized titration of PEEP or recruitment maneuvers may be essential rather than the protocolized approaches used in clinical trials (13). However, this lack of mortality benefits could also originate from fundamental differences between volutrauma and atelectrauma in the pathophysiology of VILI, particularly in propagation of lung inflammation to systemic organs, which plays a crucial role in the development of multi-organ failure (MOF) and hence considerably

affects mortality in ARDS patients (14).

The present study was designed to explore the nature of lung injury and inflammation induced by these two different components of VILI, i.e. volutrauma vs. atelectrauma. It is in general very difficult to reliably or reproducibly model atelectrauma in intact animals without using a 'pre-insult' to the lungs, and this experimental difficulty to create 'pure' atelectrauma in vivo has significantly hindered our understanding of its pathophysiology. We therefore developed an ex vivo model of VILI using an isolated perfused lung (IPL) system, which enabled us to study not only volutrauma but also atelectrauma in healthy mouse lungs. The results provide clear evidence that volutrauma, but not atelectrauma, directly activates monocytes marginated within the lung vascular compartment to secrete pro-inflammatory cytokines into the systemic circulation. Our findings offer a previously unappreciated hypothesis as to why preventing atelectasis apparently has a much less beneficial impact on ARDS patients than reducing V_T .

MATERIALS AND METHODS

All protocols were approved by the Ethical Review Board of Imperial College London, and carried out under the authority of the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK. We used male C57BL/6 mice (Charles River, Margate, UK) aged 9-12 weeks and weighing 25–30g.

Isolated perfused lung

The IPL model was prepared using a commercially available miniature organ perfusion system (Isolated Perfused Lung Size 1 Type 839; Hugo-Sachs Elektronik,

March-Hugstetten, Germany), in a similar fashion to our previous reports (15, 16). Mice were anesthetized with intraperitoneal injection of ketamine (130mg/kg) and xylazine (13mg/kg), tracheostomized and mechanically ventilated with air. A midline laparotomy was performed and 100 IU of heparin was intravenously injected. After exsanguination from the inferior vena cava, the ventilation gas was changed to 5% CO₂ in air, the lower half of the body below the liver was severed, and a midline thoracotomy was performed with the sternum removed to ensure visibility for vascular cannulation. The animal body was placed in the IPL chamber, and the pulmonary artery and left atrium were cannulated with stainless steel cannulae. Perfusate was drained from the left atrium to a reservoir, and either drained perfusate (recirculating perfusion) or fresh perfusate (non-recirculating perfusion) was returned to the pulmonary artery through a heat exchanger and bubble trap, using constant-flow peristaltic pumps. The chamber was water-sealed, humidified, and temperature-regulated at 37°C throughout the experiments.

Lungs were perfused at 25ml/kg/min with RPMI-1640 without phenol red (Invitrogen, Paisley, UK) supplemented with 4% low-endotoxin bovine serum albumin, with left atrial pressure maintained at 2.5mmHg (zero referenced at the level of the left atrium) by adjustment of the height of the reservoir. All lungs were initially perfused for 10 minutes (equilibration period) in a non-recirculating manner to wash out the residual blood within the pulmonary circulation, and then perfused at the same flow rate in a recirculating manner for the subsequent 3 hours. Complete and thorough perfusion was confirmed by the appearance of the lungs during the equilibration period: if the level of 'whiteness' was inhomogeneous across the lungs, the preparation was discarded (in ~5% of the preparations, due mainly to accidental displacement of cannulae). Every hour, perfusate samples were collected for

cytokine determination by ELISA (R&D Systems, Abington, UK), and the perfusate within the IPL system was exchanged for fresh medium. At the end of experiments, lung lavage was performed using 750µl of saline as described previously (17), and the lavage fluid samples were used for cytokine analysis and protein assay (Bio-Rad, Hertfordshire, UK). Some animals were intravenously injected with 200µl of liposome-encapsulated clodronate (FormuMax Scientific, Palo Alto, CA) 24 hours prior to the IPL experiments, to deplete lung-marginated monocytes.

Mechanical ventilation protocol

Lungs were ventilated with 7ml/kg V_T and 5cmH₂O PEEP during the equilibration period. Lungs were then recruited with a sustained inflation (SI; 25cmH₂O for 5 seconds) to minimize any atelectasis developed during the surgical procedure and standardize volume history, and allocated to one of three ventilatory protocols: control, atelectasis, or high-stretch. In the control group, lungs were ventilated with low V_T (7ml/kg) with PEEP (5cmH₂O), and regular SI (every 15 minutes) was applied to prevent atelectasis. In the high-stretch group, lungs were ventilated with high V_T (30-32ml/kg) with both PEEP (3cmH₂O) and SI. A lower level of PEEP was chosen in the high-stretch group than in the control group, because at PEEP of 5cmH₂O the lungs ventilated with high V_T tend to hit the inner surface of the chest cavity (e.g. the incised edge of rib cage as well as remaining diaphragm) at end-inspiration. This generates significant changes in surface pressure over the lungs, making it difficult to standardize the true transpulmonary pressure. The atelectasis group received the same low V_T as the control group but neither PEEP nor SI. Respiratory rate was maintained at 80/min with an inspiratory-expiratory time ratio of 1:2 in all groups. At the end of the experiments, SI was applied to all the lungs, before respiratory

mechanics were evaluated by end-inflation pause technique (17, 18). This SI was applied to separate out unrecruitable pulmonary edema from reversible derecruitment. Ruptured lungs were identified by lack of pressure plateau during the end-inflation occlusion, and excluded from analysis.

Flow cytometry

In a separate group of animals, we used flow cytometry to assess the localization and number of leukocytes within the lung tissue. To determine the localization of leukocytes, at the end of the equilibration period, CD45-PE antibody (eBioscience, Hatfield, UK) was injected into the pulmonary arterial cannula and incubated for 2 minutes to stain leukocytes within the lung vasculature, while the lungs were kept inflated with 5cmH₂O of positive airway pressure. The unbound residual CD45 antibody was then washed out by 1-minute non-recirculating perfusion, before harvesting lung tissues and preparing single cell suspensions. Alveolar macrophages were all found negative for CD45 after this procedure, indicating that the antibody exclusively stains the cells within the intravascular compartment.

The leukocyte quantification by flow cytometry was performed as previously described (16, 19). Briefly, lung single cell suspensions were prepared by mechanical chopping of the harvested lung tissues, and then stained for 30 minutes in the dark at 4°C with antibody cocktail containing; F4/80-FITC, Ly6G-APC (Biolegend, London, UK), CD11c-AlexaFluor780 (eBioscience), CD11b-PE-CF594, and Ly6C-PE-Cy7 (BD Biosciences, Oxford, UK). Cell samples were then analyzed by a CyAn flow cytometer and Summit software (Beckman Coulter, High Wycombe, UK), and further data analysis was performed by FlowJo software (Tree Star, Ashland, OR). Absolute cell counts in samples were determined using microsphere

beads (Invitrogen).

Statistical analysis

Statistical analyses were performed using SPSS version 20 (IBM, Portsmouth, UK). Data were analyzed using t-test, 1-way or 2-way ANOVA, following which the normality of model residuals was assessed by QQ plot and Shapiro-Wilk test ($p < 0.05$ considered as skewed distribution). If the distribution was not normal, data was log-transformed and analyzed by the above parametric tests. If the data distribution remained skewed after transformation, non-parametric tests were used, either Kruskal-Wallis test or Mann-Whitney's U test as appropriate. For assessing the time-course data, when only 2 treatment groups were compared we tested the interaction between time and treatment by 2-way ANOVA. For plasma cytokine data, we performed end-point analysis by 1-way ANOVA. A p-value < 0.05 was considered significant, and to allow for multiple pairwise comparisons we used a Bonferroni-corrected cut off of $0.05/n$, where n is the number of pairwise comparisons.

RESULTS

Both high-stretch and atelectasis caused lung injury in IPL

Both the high-stretch and atelectasis protocol led to substantial increases in peak inspiratory pressure (PIP) during the 3-hour ventilation protocol, although the pattern of these changes was qualitatively very different (Fig 1A; $n=5-6$ each). In the high-stretch group, PIP was stable (or slightly decreased presumably due to the recruitment of any part-collapsed lung regions) for the initial 1.5-2 hours, and then exhibited a progressive increase toward the end of experiment. In contrast, in the

atelectasis group, a rapid increase in PIP was apparent within the first hour, coinciding with a progressive decrease of the visual lung size both at inspiration and expiration, reflecting an ongoing atelectasis of the lungs. After this, PIP remained relatively stable (potentially indicating a balance point at which the delivered tidal volume was sufficient to prevent further derecruitment) until the last 30 minutes, when it started to increase again. In the control group, PIP was stable throughout the protocol.

At the end of the experiment, respiratory system elastance and resistance increased substantially in both the high-stretch and atelectasis groups, with atelectasis inducing greater percentage increases (compared to control) than high-stretch (Fig 1B; n=5-6 each). Lavage fluid protein was also dramatically increased to comparable levels following both injury protocols (Fig 1C; n=5-6 each), indicating that alveolar-capillary barrier permeability increased to a similar extent following either high-stretch or atelectasis. The development of pulmonary edema in both injury groups was further supported by the presence of endotracheal foamy fluid, which was never seen following control non-injurious ventilation. The pulmonary arterial pressure (PAP) following the injurious ventilation protocols was not higher than that in the control group (Fig 1D; n=5-6 each), suggesting that the physiological changes observed were not a result of hydrostatic edema.

Comparison of volutrauma and atelectrauma demonstrated a distinct inflammatory pattern

The production and release of inflammatory mediators have been postulated to play a highly important role in VILI, in terms of increasing not only pulmonary but also extra-pulmonary organ dysfunction (20-22). To clarify the impact of the different

injurious ventilation strategies on this, we measured levels of keratinocyte-derived chemokine (KC) and tumor necrosis factor (TNF) in the vascular compartment (i.e. IPL perfusate) as well as in the alveolar compartment (i.e. lavage fluid). Perfusate cytokines showed marked increases during the 3-hour protocol in the high-stretch model (Fig 2A; n=5-6 each). However, in sharp contrast, the atelectasis model showed only minor increases in perfusate cytokines, which were almost identical to those observed in the control group. The pattern of intra-alveolar cytokine production generally followed that of the perfusate data (Fig 2B; n=5-6 each). High-stretch ventilation induced a very substantial upregulation of lavage fluid KC, and a more modest, but still significant upregulation of TNF, compared to control ventilation. The atelectasis group showed smaller upregulations of lavage fluid KC and TNF compared to high-stretch, but still higher than control (albeit not significant in TNF with 4 fold increase compared to control). It is important to note that atelectasis did not exhibit the same marked increase in perfusate TNF as seen in high-stretch, despite comparable levels of lavage fluid TNF (geometric mean [95%CI]; 152 [62-372] pg/ml for high-stretch vs 101 [66-153] pg/ml for atelectasis) and similarly increased alveolar-capillary barrier permeability. This suggests that increased perfusate TNF during high-stretch was not a consequence of systemic spill-over of TNF produced within the alveolar compartment, but rather, originated from an extra-alveolar source.

Monocytes in the lung peripheral pool are responsible for the systemic cytokine production following high-stretch

We have previously demonstrated that monocytes within the lung microvasculature play an important role in the evolution of lung injury of various etiologies (16, 19, 23,

24). In buffer-perfused IPL models, such marginated leukocyte populations have generally been considered to be almost completely washed out from the pulmonary circulation, along with red blood cells. To investigate this, we utilized flow cytometric techniques to determine the number of monocytes and neutrophils remaining within the vasculature, after the initial 10 minute wash-out period (i.e. just before the start of the injurious ventilation protocols). Unexpectedly, we found substantial numbers of intravascular (stained by CD45 antibody infused into the IPL system), lung-marginated leukocytes remaining within the lung, typically in the range of 5×10^5 to 1×10^6 cells. To address whether monocytes among these lung-marginated leukocyte populations are responsible for the high-stretch induced cytokine production observed, we pretreated mice in vivo with liposome-encapsulated clodronate. As expected, this procedure almost completely depleted intravascular Ly6C^{high} and Ly6C^{low} subsets of monocytes, but was without effect on neutrophils (Fig 3; n=4 each).

We then evaluated the impact of this monocyte depletion on the inflammatory and physiological characteristics of the injurious ventilation models. The release of cytokines into the vascular compartment (into the IPL perfusate) was significantly attenuated in monocyte-depleted lungs exposed to high-stretch ventilation, in particular TNF, which was virtually ablated (Fig 4A; n=3-5 each). Interestingly, monocyte depletion also significantly attenuated the change in PIP over time during high-stretch ventilation (Fig 4B; n=3-5 each), and tended to decrease the lavage protein, although this did not reach statistical significance (Fig 4C; n=3-5 each). In contrast, monocyte depletion had no effect on either inflammation or physiological deterioration following atelectasis (Fig 5A-C; n=3-5 each).

DISCUSSION

Propagation of lung inflammation to systemic organs and subsequent MOF is a major determinant of the mortality of ventilated ARDS patients. The data of the present study strongly suggest that monocytes margined within the lung vasculature are responsible for secretion of TNF into the circulation during VILI, which only occurs in response to lung overstretch, not atelectasis. This finding may have major clinical relevance with regards to the mechanisms as to how mechanical ventilation upregulates inflammatory mediators within the systemic circulation.

In this study, we have taken advantage of 'zero' pleural pressure (relative to atmospheric pressure) in the open-chest IPL setting to develop an atelectasis-related VILI model in healthy mouse lungs, and compared it with a high-stretch induced VILI model. Although atelectasis is common in postoperative and critical care settings (25-27), the pathological role and mechanism of atelectasis-related lung injury have not been thoroughly investigated, mainly due to difficulties in modeling 'pure' atelectrauma experimentally. Inducing significant atelectasis is difficult in large animals with intact thorax, because negative pleural pressure and normal surfactant function maintain the end-expiratory lung volume above the closing capacity, thereby preventing the lungs from developing severe atelectasis. While the lungs of small animals, in particular mice which have very little elastic recoil of the chest wall, are highly prone to derecruitment during mechanical ventilation in vivo, such models induce considerable hemodynamic instability due to impaired gas exchange before the development of permeability edema (28). For example, ventilation with zero PEEP and low V_T in rats in vivo has been shown to result in high mortality mainly due to right heart failure induced by hypoxia and pulmonary vasoconstriction, rather

than permeability-related ARDS (29, 30). Therefore, previous experimental atelectrauma models have used pre-injury such as surfactant depletion (31, 32) or relatively high tidal volume (33, 34) to enhance the atelectasis-related lung injury, but these additional insults make mechanistic analyses extremely difficult. Using the IPL model, we were able to overcome such limitations and produce homogenous atelectasis in a reproducible fashion. We found substantial changes in respiratory mechanics and increased barrier permeability (as indicated by increased levels of lavage fluid protein) purely by use of low V_T with no PEEP or recruitment maneuvers. Indeed, barrier permeability deteriorated in the atelectrauma model to a similar extent to the high-stretch model. Although the high-stretch ventilation used in the current study ($V_T=30\text{ml/kg}$) is much higher than is generally considered 'clinically-relevant' in humans, we have recently demonstrated that such high tidal volumes are required to produce 'stretch-induced' VILI in healthy mouse lungs (28, 35).

While the high-stretch model clearly increased the cytokine production both in the alveolar (lavage fluid) and vascular compartment (IPL perfusate), atelectrauma increased these cytokines only in the alveolar compartment. Intriguingly, we found that atelectasis induced similar levels of barrier permeability increase and intra-alveolar TNF upregulation, but much lower levels of TNF within the vascular compartment, as compared with high-stretch. This observation provides evidence against the generally accepted 'decompartmentalization' theory of systemic propagation of lung inflammation during VILI (36). The standard 'biotrauma' hypothesis proposes that mechanical ventilation promotes injury in non-pulmonary organs through the production of cytokines (including TNF) within the alveolar compartment, which then leak into the pulmonary and then systemic circulation as the alveolar-capillary barrier becomes more permeable (37-40). While such a

mechanism may have contributed to some extent to the perfusate cytokine levels in the high-stretch group, our data clearly indicate that the source of TNF released into the pulmonary vasculature is primarily extra-alveolar. Indeed, Dreyfuss' pioneering work of VILI reported lung endothelial bleb formation after high V_T ventilation of just 5 minutes (41), suggesting that high-stretch VILI can directly injure and activate 'extra-alveolar' cells within the pulmonary circulation. Furthermore, our data also demonstrate that the mechanism for this extra-alveolar TNF production is specifically activated in response to high-stretch, but not atelectasis. The difference between volutrauma and atelectrauma observed in this study is potentially consistent with the recent clinical findings in ARDS patients, showing that metabolic activity of lung lesions measured by positron emission tomography was positively correlated with plateau pressure and regional tidal volume, but not associated with cyclic recruitment-derecruitment (42).

We have recently demonstrated that lung-marginated monocytes, and their interaction with the pulmonary endothelium, are important during acute lung injury of various etiologies including VILI (16, 19, 23). In fact, evidence from large animal experiments indicated that the lung has arguably the largest peripheral pool of leukocytes within its microcirculation, and that trafficking behavior of leukocytes is unique within lung microvasculature, where they are tentatively 'trapped' even in the healthy lung because the size of leukocytes is bigger than the lung capillary diameter (43). Previous studies have reported between 1 (16) and 10 million (44) leukocytes within the vasculature in healthy mouse lungs. Even following 10 minutes of perfusion to wash out blood, we found approximately 0.5 million leukocytes retained within the lung vasculature. This population has been almost completely ignored in the previous literature of IPL, but has the potential to play an important role in the

pathogenesis of lung injury in the IPL models, particularly as a putative source of soluble inflammatory mediators.

To clarify whether monocytes margined within the lung microvasculature play a role in the production of cytokines found in the IPL perfusate, we depleted monocytes by in vivo pretreatment with liposome-encapsulated clodronate. In the monocyte-depleted lungs, we demonstrated a clear attenuation of TNF and KC production in response to high-stretch. The almost complete abrogation of systemic (perfusate) TNF production suggests that intravascular monocytes are likely to be the major source of TNF, while the partial attenuation of KC upregulation may suggest a secondary source. It has been demonstrated that pulmonary endothelial cells in isolation are capable of producing IL-8 (i.e. human homologue of KC) (45, 46). Our data suggest either both monocytes and endothelial cells producing KC, or endothelial KC production being regulated by interaction with monocytes within the vasculature. Interestingly, monocyte depletion also induced a significant attenuation of pulmonary edema in the high-stretch model, a consistent finding with our previous in vivo high-stretch model (19). This may suggest that the lung-margined monocytes activated by stretch can also exacerbate pulmonary edema, either through direct cellular interaction or producing soluble edemagenic factors. Recent literature showed that the mouse IPL produced soluble edemagenic factors in response to injurious ventilation with high V_T plus low end-expiratory transpulmonary pressure (47), but did not attempt to identify a cellular source. Therefore, It is possible that stretch-activated lung-margined monocytes produced such edemagenic factors in our high-stretch model. The absence of impact of monocyte depletion in the atelectrauma model is compatible with the current proposed mechanism of atelectrauma; i.e. repetitive collapse and opening of the lung units,

which is primarily an intra-alveolar event (1, 31).

CONCLUSIONS

In summary, we successfully developed two different ex vivo VILI models of volutrauma and atelectrauma, and comparison of these models revealed a distinct inflammatory pattern of systemic cytokine release. We have demonstrated that lung-marginated, intravascular monocytes were activated following high-stretch ventilation alone, leading to pro-inflammatory cytokine production and contributing to pulmonary edema, potentially challenging the current decompartmentalization paradigm. Several physiological and technical reasons have been suggested as to why prevention of atelectasis has not been as successful as reducing tidal volume in previous clinical trials, and more trials are currently underway in this area. Our study provides another piece of novel mechanistic insight to the puzzle, indicating that systemic inflammation, which has been postulated to play a major role in determining multiple organ failure and death during ARDS, occurs to a much greater extent in response to high-stretch than atelectasis.

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Figure Legends

Figure 1

Lungs receiving injurious ventilation protocols display considerable lung injury. (A) Time course of peak inspiratory pressure (PIP) in either control, high-stretch, or atelectasis ventilation protocol (mean $\pm$ SD). (B) Elastance (Ers) and resistance (Rrs) changes during each 3-hour protocol (shown as box-whisker plots; significant differences between 3 groups ($p<0.05$) found for both Ers and Rrs by Kruskal-Wallis 1-way ANOVA; * $p<0.05$ by pairwise Mann-Whitney U test with Bonferroni correction). The starting values of Ers were 21.3 $\pm$ 2.6 (control), 31.6 $\pm$ 0.5 (high-stretch) and 25.4 $\pm$ 2.7 cmH₂O/ml (atelectasis), while those of Rrs were 1.00 $\pm$ 0.09 (control), 0.98 $\pm$ 0.04 (high-stretch) and 1.00 $\pm$ 0.14 cmH₂O/ μ l/sec (atelectasis). (C) Lavage protein level (mean $\pm$ SD; significant difference between 3 groups ($p<0.05$) found by 1-way ANOVA; ** $p<0.01$ by pairwise Bonferroni post hoc test) and (D) pulmonary arterial pressure (PAP; perfusion pressure) at the end of each protocol (mean $\pm$ SD). n=5-6 each.

Figure 2

Lungs receiving injurious ventilation protocols display distinct cytokine profile. (A) Time course of KC (mouse homologue of IL-8) and TNF in the perfusate in either control, high-stretch, or atelectasis protocol (mean $\pm$ SD; significant differences between 3 groups ($p<0.05$) found for both KC and TNF by 1-way ANOVA; *** $p<0.001$, * $p<0.05$ vs. control, ^{†††} $p<0.001$, [†] $p<0.05$ vs. atelectasis by pairwise Bonferroni post hoc test). (B) Level of KC and TNF in the lung lavage fluid at the end of each 3-hour protocol (geometric mean and 95% confidence interval shown as the data required transformation to achieve normal distribution; significant difference

between 3 groups ($p<0.05$) found by 1-way ANOVA; $***p<0.001$, $*p<0.05$ by pairwise Bonferroni post hoc test). $n=5-6$ each.

Figure 3

A considerable number of leukocytes remain in the isolated perfused lung preparation after the initial 10 minute equilibration period with non-recirculating perfusion. Leukocytes within the lung vasculature were quantified using flow cytometry. CD45 antibody was infused into the pulmonary artery and incubated for 2 minutes before harvesting the lung sample. Intravascular leukocytes were identified as CD45 positive events. Leukocytes were further categorized by their CD11b and side scatter characteristics; R1 (high side scatter, CD11b positive events) and R2 (low side scatter, CD11b positive events). Neutrophils were identified as F4/80 negative, Ly6G positive population in R1, whereas monocytes were identified as F4/80 positive population in R2. Monocyte subsets were determined by their expression levels of Ly6C, 'inflammatory' monocytes being Ly6C high (R4), and 'resident' monocytes being Ly6C low (R5). Prior treatment of animals with liposome-encapsulated clodronate clearly decreased the number of both subsets of monocytes (geometric mean and 95% confidence interval shown as the data required transformation to achieve normal distribution; $***p<0.001$ by t-test). $n=4$ each.

Figure 4

Monocyte depletion attenuates systemic cytokine release and pulmonary edema in lungs receiving high-stretch ventilation. (A) Level of KC and TNF in the perfusate at 3 hours (mean+SD; $***p<0.001$, $*p<0.05$ by t-test). (B) Time course of peak

inspiratory pressure (PIP; mean $\pm$ SD; there is a significant interaction ($p<0.05$) between time and treatment by 2-way ANOVA). (C) Lavage protein level (mean+SD). n=3-5 each.

Figure 5

Monocyte depletion does not affect the progression of atelectasis-related lung injury. (A) Level of KC and TNF in the perfusate at 3 hour (mean+SD). (B) Time course of peak inspiratory pressure (PIP; mean $\pm$ SD). (C) Lavage protein level (mean+SD). The untreated data are those presented in figures 1 and 2. n=3-5 each.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

A

B

C