Role of Lung-Marginated Monocytes in an In Vivo Mouse Model of Ventilator-Induced Lung Injury

Michael R Wilson¹, Kieran P O’Dea¹, Da Zhang¹, Alexander D Shearman¹, Nico van Rooijen², Masao Takata¹

¹ Department of Anaesthetics, Pain Medicine and Intensive Care, Faculty of Medicine, Imperial College London, Chelsea and Westminster Hospital, London, United Kingdom
² Vrije Universiteit, Vrije Universiteit Medisch Centrum, Department of Molecular Cell Biology, Faculty of Medicine, 1081 BT Amsterdam, Netherlands

Requests for reprints should be addressed to: Dr Masao Takata, Department of Anaesthetics, Pain Medicine and Intensive Care, Imperial College London, Chelsea and Westminster Hospital, 369 Fulham Road, London SW10 9NH, UK.

Corresponding author: Dr Masao Takata

Email: m.takata@imperial.ac.uk
Fax: 44 20 8237 5109, Tel: 44 20 8746 8816.

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject: Recruited leukocytes are known to play an important role in ventilator-induced lung injury (VILI), although studies have focused on neutrophils. Recent studies implicate lung-marginated monocytes in the development of other forms of acute lung injury.

What This Study Adds to the Field: This study demonstrates for the first time that inflammatory subset Gr-1$^{\text{high}}$ monocytes are recruited to the lung during high stretch mechanical ventilation, and contribute to the progression of pulmonary edema during VILI.
ABSTRACT

Rationale: Recruited leukocytes play an important role in ventilator-induced lung injury, although studies have focused predominantly on neutrophils. Inflammatory subset ‘Gr-1<sup>high</sup>’ monocytes are recruited to sites of inflammation, and have been implicated in acute lung injury induced by systemic endotoxin. Objectives: To investigate the recruitment and role of Gr-1<sup>high</sup> monocytes in an in vivo mouse model of ventilator-induced lung injury. Methods: Anesthetized mice were ventilated with low or high stretch. Flow cytometry was used to quantify monocyte subset margination to the lungs, and to assess their in situ cellular activation in response to mechanical stretch. To investigate monocyte involvement in lung injury progression, a ‘2-hit’ model was employed, with a sub-clinical dose of lipopolysaccharide (intraperitoneal) given 2 hours prior to high stretch ventilation. In some animals, monocytes were depleted using intravenous clodronate-liposomes. Development of lung injury was assessed in ventilated animals by peak inspiratory pressure and respiratory system mechanics. Measurements and Main Results: High stretch ventilation induced significant pulmonary margination of Gr-1<sup>high</sup>, but not Gr-1<sup>low</sup> monocytes compared to non-ventilated mice. These monocytes displayed increased activation status, with higher CD11b (versus non-ventilated mice) and lower L-selectin expression (versus low stretch ventilation). Lipopolysaccharide challenge led to enhanced lung margination of Gr-1<sup>high</sup> monocytes and neutrophils, and sensitised the lungs to high stretch-induced pulmonary edema. Clodronate-liposome pretreatment depleted lung monocytes (but not neutrophils) and significantly attenuated lung injury. Conclusions: High stretch mechanical ventilation promotes pulmonary margination of activated Gr-1<sup>high</sup> monocytes, which play a role in the progression of ventilator-induced lung injury.

248 words

Key words: Gr-1<sup>high</sup> monocytes, acute lung injury, stretch, inflammation, mechanical ventilation
INTRODUCTION

The development of ventilator-induced lung injury (VILI), produced by excessive lung stretch during mechanical ventilation, is known to have a significant impact on the outcome of clinical acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) (1). VILI, both clinically and experimentally, is characterised by pulmonary edema and inflammation (2-4). Historically, analysis of the roles of leukocytes in VILI has focused predominantly on alveolar macrophages (5, 6) and recruited neutrophils (7-11), at least in part because of the ease of recovery and visual identification of these cells.

Recent evidence has however suggested that lung recruited monocytes may also play a significant role in the pathogenesis of ALI (12, 13). Monocytes are a pluripotent and heterogeneous population of cells of the mononuclear phagocytic system, capable of releasing a variety of inflammatory mediators. Our understanding of these cells has dramatically increased over the last 5 years with the identification of phenotypically distinct monocyte subsets (14, 15). Immature monocytes (defined in mice as Gr-1$^{\text{high}}$/CCR2+/CX3CR1$^{\text{low}}$) enter the circulation from the bone marrow, and have been shown to migrate to local sites of inflammation and injury (15-17). Hence, these Gr-1$^{\text{high}}$ cells have been termed ‘inflammatory subset’ monocytes. During maturation of monocytes there is a change to a less inflammatory (Gr-1$^{\text{low}}$/CCR2-/CX3CR1$^{\text{high}}$) phenotype (18), cells of which are committed to differentiate into tissue macrophages and dendritic cells (15, 19). We have previously demonstrated using mouse models that monocytes are rapidly recruited to the lung microvasculature during systemic endotoxemia, and that these ‘marginated’ cells contribute to the development of ALI by promoting the activation of pulmonary endothelial cells (12). Furthermore, we have shown a clear role for lung-marginated inflammatory Gr-1$^{\text{high}}$ monocytes in the development of pulmonary edema in a lipopolysaccharide (LPS)/zymosan model of ALI (13).
Based on these findings, in the current study we investigated the potential role of monocytes in the development of VILI using an in vivo mouse model. We demonstrated that monocytes, specifically the inflammatory Gr-1\textsuperscript{high} subset, were recruited to the lungs and activated during high stretch mechanical ventilation. Depletion of Gr-1\textsuperscript{high} monocytes in a clinically relevant, ‘2-hit’ model of VILI attenuated the progression of VILI, suggesting a substantial role for lung-marginated monocytes in VILI development. Some of the results of these studies have been previously reported in the form of an abstract (20).

**METHODS**

*Monocyte recruitment/activation during mechanical ventilation*

All protocols were approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK. The in vivo mouse model of VILI has been described previously (11, 21), and additional details are provided in an online data supplement. In brief, anesthetized male C57BL6 mice (Charles River), aged 8-12 weeks were tracheostomized and ventilated using a custom-made jet ventilator (21, 22). Animals were ventilated with either high stretch (tidal volume (V\textsubscript{T}) 34-36ml/kg, zero PEEP) or low stretch (V\textsubscript{T} 7-8ml/kg, 2-3cmH\textsubscript{2}O PEEP) protocols. High stretch ventilation with the same constant V\textsubscript{T} was continued until peak inspiratory pressure (PIP) increased by ~40\%, indicating substantial pulmonary edema, which took between 140-200 minutes (mean 160 minutes). Low stretch ventilation was continued for 180 minutes. A carotid artery cannula was used for blood pressure monitoring and blood gas analysis. Respiratory system compliance (Crs) and resistance (Rrs) were assessed periodically by end-inflation occlusion (22).
At termination, lung cell suspensions were prepared from excised lungs by mechanical disruption for flow cytometry analysis, as described in detail previously (11-13). Samples were stained with fluorophore-conjugated anti-mouse antibodies for CD11b, Gr-1 (Ly6C/G), F4/80, L-selectin or appropriate isotype-matched controls, and then analyzed using a FACSCalibur cytometer with CellQuest (Becton Dickinson) and Flowjo (Tree Star) software. Full details of the leukocyte identification procedure are provided in the online data supplement. In brief, monocytes were identified as CD11b+, F4/80+ events and subsets defined as expressing either low or high Gr-1, and were differentiated from F4/80-, Gr-1 very high neutrophils. Cells were quantified using microsphere counting beads (Caltag Medsystems) and activation assessed based on L-selectin and CD11b adhesion molecule expression.

Involvement of monocytes in pulmonary edema formation during VILI

The involvement of recruited monocytes in the progression of VILI was assessed in a clinically relevant two-hit model. LPS challenge (20ng/mouse, Ultrapure LPS, InVivoGen) was given intraperitoneally to induce a sub-clinical inflammation. In some animals, intravascular monocytes and macrophages were depleted by pretreatment with intravenous clodronate-loaded liposomes (200µl, gift from Roche Diagnostics GmbH (Mannheim, Germany)) given 24 or 48 hours prior to LPS challenge (13, 18, 23). 2 hours after LPS challenge (with/without clodronate-liposome pretreatment), some animals were sacrificed and neutrophil and Gr-1<sup>high</sup> monocyte numbers within the lungs determined, while other animals were anesthetised, instrumented, and ventilated with low or high stretch for 2 hours (see Fig. 1 and online supplement for full details of treatment groups). In this two-hit model ventilation parameters were similar to those described above, except that for high stretch, a slightly lower V<sub>T</sub> (28-30ml/kg) was used, because LPS challenge was expected to significantly exacerbate the degree of VILI. In order to further
delineate the potential role of systemic monocyte activation by LPS, depletion experiments were also carried out in the one-hit, pure VILI model using the ventilation parameters described above. VILI progression was evaluated by changes in PIP, Crs and Rrs.

**Statistical analysis**

Data are expressed as mean±SD. Statistical comparisons were made by t-tests or ANOVA with Bonferroni tests using Prism software (version 4.0). Statistical significance was defined as p<0.05.

**RESULTS**

**Monocyte recruitment/activation during mechanical ventilation**

High stretch mechanical ventilation induced a substantial deterioration in lung function consistent with pulmonary edema formation, compared to animals exposed to low stretch non-injurious ventilation (table 1). Low stretch ventilation for 180 minutes did not induce significant changes in PIP or blood gases, and only very small changes in Crs and Rrs. In contrast, high stretch ventilation induced a substantial increase in PIP, associated with large changes in respiratory mechanics and deterioration in oxygenation.

Following the ventilation protocols, margination of monocytes and neutrophils to the lungs was determined using previously validated flow cytometry methods (11-13). High stretch ventilation promoted a significant increase in the number of Gr-1 high monocytes within the lungs, compared to non-ventilated animals (Fig. 2A). There was no change in the number of Gr-1 low monocytes (Fig. 2B), leading to a dramatic increase in the ratio of Gr-1 high to Gr-1 low monocytes within the lungs following high stretch ventilation (Fig. 2C). As anticipated, high stretch
ventilation also promoted a significant increase in the number of neutrophils within the lungs, compared to both non-ventilated and low stretch ventilated mice (Fig. 2D). As the recruitment of monocytes due to high stretch ventilation was effectively limited to the Gr-1^{high} subset, subsequent experiments focused on these cells.

The activation status of lung-marginated Gr-1^{high} monocytes was evaluated by assessing cell surface expression of L-selectin and CD11b. Lung marginated Gr-1^{high} monocytes displayed increased expression of CD11b compared to non-ventilated mice, indicating increased activation (Fig. 3A). Surface L-selectin expression was higher on lung-marginated Gr-1^{high} monocytes following either ventilation protocol (high or low stretch) compared to non-ventilated animals, suggesting an increase in the proportion of less mature (ie. bone-marrow derived, high L-selectin expressing) cells within the gated population. L-selectin expression was much lower on cells from high stretch than low stretch ventilated mice (Fig. 3B), consistent with L-selectin shedding and hence greater activation of these monocytes following high stretch, compared to low stretch ventilation.

**Involvement of monocytes in pulmonary edema formation during VILI**

Based on these findings of increased Gr-1^{high} monocyte margination and activation within the lungs during high stretch ventilation, we investigated whether these cells may play a role in the development of high stretch-induced pulmonary edema. To investigate this, we used a two-hit model of VILI consisting of an intraperitoneal LPS challenge (sub-clinical dose) followed by mechanical ventilation. We considered this model to have a strong clinical relevance, as many intensive care patients are likely to have underlying systemic inflammation, and consequently pre-existing lung monocyte margination when they first receive ventilatory support. LPS challenge for 2 hours induced a substantial increase in the numbers of both Gr-1^{high} monocytes
and neutrophils within the lungs (i.e. increased margination prior to the start of ventilation) (Fig. 4). The specific involvement of monocytes in this model was assessed through the use of intravenous injection of clodronate-liposomes, a standard method used for depletion of monocytes and resident intravascular macrophages (24). 24 hour clodronate-liposome treatment substantially reduced the numbers of marginated Gr-1\textsuperscript{high} monocytes within the lungs in LPS-challenged mice, but had no effect on neutrophil numbers. In addition, alveolar macrophages (determined in lavage fluid by hemacytometer and differential cytology) were not affected at this time point by the intravenous clodronate-liposome treatment (1.8±0.2\times10^4 vs. 1.9±0.5\times10^4 alveolar macrophages in LPS-challenged mice, in the presence and absence of clodronate-liposome treatment respectively). At 48 hours after clodronate-liposome treatment, marginated Gr-1\textsuperscript{high} monocyte numbers were returned to the ‘normal’ levels seen in LPS-challenged mice (Fig. 4). These effects of clodronate-liposomes on lung monocytes over time, i.e. effective ablation of LPS-induced monocyte margination at 24 hours but its restoration at 48 hours, are consistent with our previous findings with this method in C57BL6 mice (13).

Having confirmed that LPS challenge substantially enhanced leukocyte margination to the lungs and that clodronate-liposome treatment had the desired effects, separate groups of treated animals were subjected to mechanical ventilation. Immediately following surgical instrumentation, baseline physiological parameters were assessed in these mice (table 2). Neither LPS challenge nor clodronate-liposome pretreatment had any effect on the parameters measured, despite the differences in lung-marginated leukocyte numbers at this point. We then mechanically ventilated animals for 2 hours with high stretch or low stretch ventilation. Since a slightly smaller V\textsubscript{T} (28-30ml/kg) was used in this model, the progression of VILI due solely to high stretch ventilation (in the absence of LPS) was slower than in the previously described one-hit experiments, i.e. producing only mild deterioration in respiratory function with small changes
in PIP (Fig. 5) and respiratory mechanics (Fig. 6) within the 2-hour protocol. LPS challenge, however, caused a sensitisation of the lung to high stretch ventilation, as shown by greater PIP increase over time and larger mechanics changes (Fig. 5,6). LPS challenged animals ventilated with low stretch ventilation, on the other hand, showed no substantial increase in PIP with negligible changes in respiratory mechanics (ΔCrs 1.1±6.3%, ΔRrs -10.4±6.8%; N=4), indicating that LPS challenge (and the consequent systemic inflammation) per se did not affect lung function in this model. Animals pretreated with clodronate-liposomes for 24 hours prior to LPS demonstrated significantly attenuated VILI in response to high stretch ventilation (compared to mice without clodronate-liposome pretreatment) in terms of final PIP, ΔCrs and ΔRrs (Fig. 5,6). In contrast, animals pretreated with clodronate-liposomes for 48 hours before LPS challenge showed no significant attenuation of injury (Fig. 5,6).

Finally, to address the probability that lung margination, rather than systemic activation of monocytes by LPS, was primarily responsible for the exacerbation of VILI in the two-hit model, the impact of monocyte depletion was also assessed in the one-hit, ‘pure VILI’ model. At 24 hours after clodronate-liposome treatment, Gr-1\textsuperscript{high} monocyte numbers within the lung (in the absence of LPS) were substantially lower (60-70%) than those in untreated mice, again with no effect on lung neutrophil number (Fig. 7). Separate groups of untreated and monocyte-depleted animals were then subjected to high stretch ventilation. PIP increased in response to high stretch in both groups of animals, but the increase in PIP over time showed a small but significant attenuation in animals pretreated with clodronate-liposomes compared to untreated mice (Fig. 8, p <0.05 for interaction between treatment and ventilation time). Changes in Rrs at the end of the protocol, though not Crs, were also significantly attenuated by clodronate-liposome pretreatment.

**DISCUSSION**
The purpose of the current study was to investigate the role of lung-marginated monocytes in the development of VILI. The potential involvement of monocytes in ALI in general has been largely overlooked, partly because they are less easy to identify in biological tissues than neutrophils for example, and partly because it is only in the last few years that the importance of monocyte subset functional heterogeneity has started to become fully appreciated (14, 25). The flow cytometric method used in the current study enables quantification of lung-marginated monocytes, and their categorization into ‘inflammatory’ and ‘resident’ subsets (13, 15, 18), thus allowing the involvement of these cells in VILI to be evaluated for the first time.

We found that mechanical ventilation, in particular high stretch ventilation associated with substantial deterioration in pulmonary function, induced the margination of significant numbers of monocytes to the lung. The newly recruited monocytes were of the Gr-1\textsuperscript{high} subset and there was no change in the numbers of Gr-1\textsuperscript{low} monocytes within the lungs. These findings are consistent with our previous observation that specifically Gr-1\textsuperscript{high} monocytes ‘marginate’ to the pulmonary microcirculation in response to systemic endotoxin (13). The number of monocytes recruited to the lungs after 2-3 hours of high stretch ventilation was comparable to that of recruited neutrophils, although the Gr-1\textsuperscript{high} monocyte:neutrophil proportion in the lungs was smaller than that observed in response to systemic endotoxin, where the number of Gr-1\textsuperscript{high} monocytes equals or even exceeds the number of neutrophils (13). The mechanisms by which Gr-1\textsuperscript{high} monocytes are recruited to the lung by stretch are not addressed in this study, although high stretch ventilation has been shown to increase lavage fluid concentrations of the chemokine CCL2/monocyte chemoattractant protein-1 (MCP-1) (26). The receptor for CCL2 (CCR2) is preferentially expressed on Gr-1\textsuperscript{high} (and not Gr-1\textsuperscript{low}) monocytes (15), and CCL2 is considered to be central to the recruitment of monocytes to inflammatory sites (17, 27). Importantly, Gr-1\textsuperscript{high} monocytes were recruited to the lungs within the same time frame as were neutrophils, although...
the current data do not allow us to determine which, if either, cell is recruited into the lung first, and precisely when the process begins. As only Gr-1\textsuperscript{high} monocytes were recruited by high stretch ventilation we chose to focus our attention on this subset. However, it has recently been proposed that Gr-1\textsuperscript{low} ‘resident’ monocytes may play a patrolling role in certain microvascular beds such as mesenteric post-capillary venules (28), so their potential involvement within the lung cannot be entirely discounted.

The activation status of lung marginated Gr-1\textsuperscript{high} monocytes was assessed through the surface expression levels of the adhesion molecules CD11b and L-selectin, both of which are expressed on Gr-1\textsuperscript{high} monocytes as well as neutrophils. CD11b levels were significantly increased with high stretch ventilation versus non-ventilated animals, while L-selectin levels were significantly reduced with high stretch versus low stretch ventilation, consistent with increased proteolytic shedding of L-selectin, i.e. increased activation following high stretch ventilation. Interestingly, Gr-1\textsuperscript{high} monocytes from the lungs of both high and low stretch ventilated animals had higher levels of surface L-selectin than untreated controls. High levels of surface L-selectin are considered to be a marker of immature monocytes (29), suggesting that both ventilation strategies recruit newly mobilised monocytes (as opposed to cells demarginated from other organs) to the lungs. Indeed, we have shown that a major proportion of lung-margined Gr-1\textsuperscript{high} monocytes during systemic endotoxemia come directly from bone marrow, rather than from the circulating pool (13). Overall, the data indicate that both high and low stretch ventilation (or some other associated factor, such as anesthesia or surgery) may induce some degree of Gr-1\textsuperscript{high} monocyte margination to the lungs, but that high stretch ventilation is associated with both greater recruitment, and greater monocyte activation.

Having found that high stretch mechanical ventilation promotes both the recruitment and activation of Gr-1\textsuperscript{high} monocytes within the lung, we investigated whether these lung-margined
monocytes may play any role in the development of high stretch induced pulmonary edema. To investigate this we used two complementary approaches, firstly promoting the recruitment of additional monocytes to the lungs using systemic LPS administration, and secondly depleting circulating and lung-marginated monocytes using intravenous clodronate-loaded liposomes. Consistent with our previous study with intravenous LPS (13), the use of a low, sub-clinical dose of intraperitoneal LPS caused substantial margination of both Gr-1\(^{\text{high}}\) monocytes and neutrophils to the lungs, without producing any manifestations of sickness or changes in lung function prior to the start of ventilation. Subsequent low stretch ventilation did not induce any change in respiratory parameters, indicating that LPS treatment per se did not induce lung dysfunction. However, high stretch ventilation in LPS-challenged mice induced a substantial worsening of VILI compared to animals that did not receive LPS, indicating that systemic inflammation sensitises the lungs to the effects of high stretch ventilation. It is well known that pre-injured or pre-stimulated lungs are more sensitive to the effects of mechanical ventilation (30-35). Such sensitisation has previously been ascribed to synergistic increases in soluble mediators (26, 31-33), and/or increased neutrophil load within the lungs (30, 32, 35), but the involvement of monocytes has never been described.

In order to dissect out the contribution of enhanced margination of Gr-1\(^{\text{high}}\) monocytes versus neutrophils in this sensitisation, we carried out monocyte depletion experiments using clodronate-liposomes. This substantially depleted Gr-1\(^{\text{high}}\) monocytes from the lungs after 24 hours, and substantially attenuated the development of VILI, in terms of lower PIP after 2 hours of high stretch, and smaller changes in both resistance and compliance. Importantly, this treatment had no impact on the numbers of neutrophils or alveolar macrophages within the lungs, depletion of either of which would be expected to impact on VILI progression. To exclude the possibility that these effects may be related to ‘off-target’ effects of clodronate-liposomes (e.g.
effects on splenic macrophages or Kupffer cells, which would also be depleted at this 24 hour time point (36, 37)), a separate group of mice were left for 48 hours after clodronate-liposome pretreatment before experimentation. At this time point the number of Gr-1\textsuperscript{high} monocytes within the lung returned to normal (i.e. levels without clodronate-liposome pretreatment), whereas liver and splenic macrophages are not expected to start to reappear until 5-6 days post depletion (36, 37). In contrast to the findings at 24 hours, there was no attenuation of VILI at 48 hours after clodronate-liposome pretreatment. Together these data strongly indicate a role for lung-marginated monocytes in VILI progression.

It is possible in this two-hit model that circulating monocytes may be activated following the LPS injection, thus producing various cytokines and exacerbating the development of VILI. Although we are not able to completely exclude this possibility, we speculate that this is of lesser importance than lung-marginated cells, because i) mice treated with LPS and low stretch ventilation did not develop lung dysfunction, and ii) monocyte depletion tended to reduce injury in ‘pure VILI’ experiments, in the absence of systemic activation. In these ‘pure VILI’ experiments the high stretch-induced change in PIP was marginally attenuated by monocyte depletion, and this was associated with a reduction in the deterioration of Rrs but not Crs. We suggest that the more obvious attenuation of injury in the two-hit versus the one-hit model of VILI relates to the degree of pre-margination of monocytes caused by LPS challenge, such that monocytes are already present (and perhaps primed) when injurious ventilation begins. The two-hit scenario is more likely to reflect the clinical situations, wherein many patients who require ventilator support would already have underlying inflammation and pre-existing monocyte margination within the lung. The precise mechanisms by which monocytes play a role in the progression of lung injury induced by high stretch ventilation are not yet clear. We have previously demonstrated that monocytes recruited to the lung during systemic endotoxemia
express increased levels of membrane-associated TNF, and that these monocytes activate pulmonary endothelial cells in a cell-contact dependent, TNF-mediated manner (12), although whether such a mechanism occurs in VILI is not known.

Historically, the involvement of recruited leukocytes in VILI has focused on neutrophils for a number of reasons: i) neutrophils are easily identifiable and accumulate within a readily accessible compartment (alveolar space) during clinical ALI/ARDS and experimental VILI (7, 38), and ii) attenuation of lung neutrophil recruitment, either by depletion of circulating neutrophils (39), interference with leukocyte adhesion processes (40), or by inhibition of leukocyte chemoattractants (8) can reduce injury. However, the supposedly critical role of neutrophils in ALI progression may need to be re-evaluated in light of emerging evidence. For example, it is known that simple recruitment of neutrophils into the lungs does not necessarily induce edema (41), and that ALI can develop in neutropenic patients (42). In addition, it has been reported that Gfi-deficient mice (lacking a zinc finger protein which acts as a transcriptional repressor) are highly susceptible to LPS-induced pulmonary inflammation (43). These animals are neutropenic but display increased cytokine production and substantial monocyte infiltration to the lungs, which could play a substantial role in the enhanced sensitivity of these mice to inflammatory stimuli. Further, we have recently demonstrated in a mouse model of VILI that pulmonary edema formation is modulated by TNF signaling, in a manner that is independent of the degree of pulmonary neutrophil recruitment (44). The current data strongly support a role for recruited monocytes in the development of VILI, although whether this is independent of, or intertwined with, neutrophil biology is unclear. The role of monocytes in all forms of ALI may have been substantially underestimated (and potentially, the role of neutrophils overestimated) in the past, because a number of the methods used to quantify and interfere with neutrophil biology, such as anti Gr-1 antibodies, myelosuppressing pharmacological agents and adhesion molecule
antagonists are not neutrophil specific, and are known (or likely) to affect Gr-1\textsuperscript{high} monocytes also (13, 45, 46). Even blockade of ‘neutrophil chemoattractants’ such as CXCL2 (MIP-2/KC) is likely to alter monocyte biology, as monocyte trafficking is substantially influenced by CXCL2 (47). Overall, while the involvement of neutrophils is not heavily disputed, the important roles of other inflammatory cell types in ALI is becoming more greatly appreciated.

There are a number of potential limitations regarding the clinical relevance of the methods used in the current study which warrant some discussion. Firstly, the V\textsubscript{T} used to produce VILI was greater than would be used in the clinical setting in humans. However, the results should still give important insights into the pathophysiology of VILI, as the lung stretch induced in healthy lungs by such V\textsubscript{T} may not be dissimilar from that experienced clinically (48), due to the loss of aerated lung capacity (‘Baby Lung’) with ARDS (49). In addition, directly comparing the absolute values of either V\textsubscript{T} or inspiratory pressure between mice and humans is likely to be misleading, as the mouse respiratory system mechanics are very different to other species, such that intact mouse lungs can be temporarily inflated to pressures above 60 cmH\textsubscript{2}O (relating to a V\textsubscript{T} of 60-70 ml/kg) without reaching a traditionally defined total lung capacity or producing morphological damage (50). Secondly, while we have not assessed the role of monocytes on indices of lung injury (e.g. morphology, wet:dry weight) in addition to respiratory mechanics, we (21, 44) and others (51) have previously demonstrated that in the case of VILI, changes in respiratory mechanics correlate very closely with these other lung injury markers. Finally, the flow cytometry-based quantification of lung-marginated monocytes used in this study may underestimate the precise number of cells within the tissue, as the recovery of cells may not be complete during the preparation of lung cell suspensions due to their tendency to adhere to the glassware. However, the observation that the number of Gr-1\textsuperscript{high} monocytes increases during VILI whereas that of Gr-1\textsuperscript{low} monocytes does not, indicates that the ‘relative’ changes in
marginated Gr-1$^{\text{high}}$ monocytes during the different ventilation strategies are likely to be realistic. In addition, we have previously shown that for neutrophils, flow cytometric quantification agrees very closely with quantification by myeloperoxidase activity assay (11). Currently there is no established alternative to flow cytometry for the detection and quantification of monocyte subsets within tissue samples, although we have demonstrated using immunohistochemistry that the density of Gr-1 positive cells within the lungs (Gr-1$^{\text{high}}$ monocytes plus neutrophils) is increased in response to systemic LPS challenge, consistent with changes determined by flow cytometry (13).

In conclusion, we have shown for the first time that high stretch mechanical ventilation promotes the margination of activated Gr-1$^{\text{high}}$ ‘inflammatory’ monocytes within the lung, and that these monocytes are involved in the development of stretch-induced pulmonary edema. The current data support an important, novel role for lung marginated Gr-1$^{\text{high}}$ monocytes in the pathophysiology of VILI.
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REFERENCES


FIGURE LEGENDS

Figure 1. Design of experimental groups for study of involvement of monocytes in pulmonary edema formation during VILI. **A**, ‘two-hit’ VILI model. Mice were treated with/without clodronate-loaded liposomes (200µl, tail vein) 24 or 48 hours before challenge with/without LPS (20ng, intraperitoneal). 2 hours post LPS, some animals were culled to determine lung leukocyte margination, while other mice were then instrumented and ventilated for 2 hours using high/low stretch ventilation. A slightly lower $V_T$ was used for this two-hit model with LPS, than the one-hit model. **B**, ‘one-hit, pure VILI model’. Mice were treated with/without clodronate-loaded liposomes 24 hours before either culling to determine lung leukocyte margination, or 2 hours of high stretch ventilation.

Figure 2. Numbers of Gr-1$^{\text{high}}$ (A) and Gr-1$^{\text{low}}$ (B) monocytes, Gr-1$^{\text{high}}$ : Gr-1$^{\text{low}}$ ratio (C), and number of neutrophils (D) within the lung tissue of non-ventilated mice, or mice ventilated with low or high stretch ventilation. N=6-9. Cell counts were square-root transformed to ensure Gaussian distribution before analysis by ANOVA with Bonferroni tests. *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Expression of adhesion molecules CD11b (A) and L-selectin (B) on the surface of lung-marginated Gr-1$^{\text{high}}$ monocytes. Data are expressed as mean fluorescence intensity (MFI). N=7-14 for CD11b and 5-10 for L-selectin. **p<0.01, ***p<0.001, by ANOVA with Bonferroni tests.

Figure 4. Numbers of Gr-1$^{\text{high}}$ monocytes (A) and neutrophils (B) in lung tissue of non-
ventilated mice. Animals were either untreated or challenged with LPS for 2 hours. Of the LPS-challenged animals, a number were pretreated for 24 or 48 hours with intravenous clodronate-liposomes (clod) prior to LPS. N=3-10. Cell counts were square-root transformed to ensure Gaussian distribution before analysis by ANOVA with Bonferroni tests. *p<0.05, **p<0.01, ***p<0.001.

**Figure 5.** Peak inspiratory pressure changes during high stretch mechanical ventilation of either untreated animals, LPS challenged animals, or animals pretreated with clodronate-liposomes (clod) for 24 or 48 hours and then challenged with LPS. Pressure changes over time during ventilation were different between the treatment groups as indicated by a significant interaction p-value by 2-way ANOVA (p<0.001).

**Figure 6.** Final peak inspiratory pressure (PIP) (A), and change in respiratory system compliance (B) and resistance (C) during high stretch ventilation of either untreated animals, LPS challenged animals, or animals pretreated with clodronate-liposomes (clod) for 24 or 48 hours and then challenged with LPS. Mechanics changes are expressed as % increase in Crs or Rrs between 30 minutes after start of protocol and the end of protocol. N=4-6. *p<0.05, **p<0.01, by ANOVA with Bonferroni tests.

**Figure 7.** Numbers of Gr-1\textsuperscript{high} monocytes (A) and neutrophils (B) in lung tissue of non-ventilated mice. Animals were either untreated or pretreated for 24 hours with intravenous clodronate-liposomes (clod) prior to culling. N=3-4. Cell counts were square-root transformed to ensure Gaussian distribution before analysis by unpaired t-tests. 0000**p<0.01.
**Figure 8.** A, peak inspiratory pressure changes during high stretch mechanical ventilation of either untreated animals, or animals pretreated with clodronate-liposomes for 24 hours before ventilation. Pressure changes over time during ventilation were different between the treatment groups as indicated by a significant interaction p-value by 2-way ANOVA (p<0.05). B, change in respiratory system compliance and C, resistance, during high stretch ventilation of either untreated animals, or animals pretreated with clodronate-liposomes for 24 hours before ventilation. Mechanics changes are expressed as % increase in Crs or Rrs between 30 minutes after start of protocol and the end of protocol. N=5. *p<0.05, by unpaired t-tests.
Table 1. Physiological variables of animals ventilated with low or high stretch ventilation.

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<tr>
<td>PIP (cmH$_2$O)</td>
<td>11.2±0.9</td>
<td>11.5±0.8</td>
<td>29.2±1.0</td>
<td>43.0±1.0*</td>
</tr>
<tr>
<td>∆Crs (%)</td>
<td>-</td>
<td>-2.0±4.4</td>
<td>-</td>
<td>-30.4±3.6 †</td>
</tr>
<tr>
<td>∆Rrs (%)</td>
<td>-</td>
<td>-6.7±8.5</td>
<td>-</td>
<td>175±30.7 †</td>
</tr>
<tr>
<td>pO$_2$ (mmHg)</td>
<td>105±7.3</td>
<td>111±6.8</td>
<td>139±6.9</td>
<td>54.8±15.6 *</td>
</tr>
<tr>
<td>pCO$_2$ (mmHg)</td>
<td>40.0±3.9</td>
<td>35.0±3.0</td>
<td>41.1±3.9</td>
<td>46.9±4.6*</td>
</tr>
</tbody>
</table>

Initial measurements were taken after 30 minutes of ventilation. PIP – peak inspiratory pressure; Crs – respiratory system compliance; Rrs - respiratory system resistance. Change in mechanics (∆Crs, ∆Rrs) was defined as the % increase/decrease in Crs or Rrs at the end of protocol versus the 30 minute value. Note that air containing 4% CO$_2$ was used for animals ventilated with high stretch to avoid excessive hypocapnia. N=6-8. *p<0.01 vs 30 minute value (paired t-test). † p<0.01 vs low stretch (unpaired t-test).
Table 2. Baseline physiological variables of high stretch ventilated animals with/without treatments

<table>
<thead>
<tr>
<th></th>
<th>No pre-treatment</th>
<th>LPS</th>
<th>LPS + clod 24h</th>
<th>LPS + clod 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP (mmHg)</td>
<td>83±14</td>
<td>80±10</td>
<td>85±14</td>
<td>91±21</td>
</tr>
<tr>
<td>Crs (ml/kg/cmH$_2$O)</td>
<td>1.66±0.09</td>
<td>1.57±0.15</td>
<td>1.56±0.11</td>
<td>1.62±0.19</td>
</tr>
<tr>
<td>Rrs (cmH$_2$O/ml/s)</td>
<td>1.62±0.10</td>
<td>1.62±0.16</td>
<td>1.74±0.31</td>
<td>1.70±0.20</td>
</tr>
<tr>
<td>pO$_2$ (mmHg)</td>
<td>125±10</td>
<td>128±8.5</td>
<td>125±11</td>
<td>119±11</td>
</tr>
<tr>
<td>pCO$_2$ (mmHg)</td>
<td>36.3±2.2</td>
<td>34.3±2.5</td>
<td>33.0±4.0</td>
<td>33.6±2.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.372±0.04</td>
<td>7.377±0.04</td>
<td>7.437±0.02*</td>
<td>7.385±0.04</td>
</tr>
</tbody>
</table>

Baseline physiological variables (i.e. before change to high stretch protocol) of either untreated animals, LPS challenged animals, or animals treated with clodronate-liposomes (clod) for 24 or 48 hours and then challenged with LPS. Note that air containing 4% CO$_2$ was used for animals ventilated with high stretch to avoid excessive hypocapnia. BP – mean arterial blood pressure; Crs – respiratory system compliance; Rrs - respiratory system resistance. N=3-6. *p<0.05 vs LPS challenged mice by ANOVA with Bonferroni tests.
Fig. 1.

A

+/- clod i.v.  +/ LPS i.p.  Cull for leukocyte counts

start  \hline\hline end

-48h/-24h  -2h  0h  2h

B

+/- clod i.v.  Cull for leukocyte counts

start  \hline\hline end

-24h  0h  2h
Fig. 2

A. Gr-1\textsuperscript{high}

B. Gr-1\textsuperscript{low}

C. Gr-1\textsuperscript{high} : Gr-1\textsuperscript{low} ratio

D. Neutrophils
Fig. 3

A CD11b

B L-selectin

MFI

non-ventilated low stretch high stretch

MFI

non-ventilated low stretch high stretch

**

***

*
Fig. 4

A) Gr-1\textsuperscript{high} monocytes

B) Neutrophils

**NS**
Fig. 5

- LPS-/clod-/clod-
- LPS+/clod-
- LPS+/clod 24h
- LPS+/clod 48h

Peak inspiratory pressure (cmH₂O)

Ventilation time (min)
Fig. 7.

A  
Gr-1\textsuperscript{high} monocytes

B  
neutrophils

![Bar charts showing cell number comparison between Untreated and 24h clod for Gr-1\textsuperscript{high} monocytes and neutrophils.](chart.png)
Fig. 8.

A

![Graph showing the relationship between peak inspiratory pressure (cmH₂O) and ventilation time (min). The graph compares VILI and VILI/clod 24h conditions.]

B

![Bar graph showing the change in Crs (%) for VILI and VILI/clod 24h conditions.

C

![Bar graph showing the change in Rrs (%) for VILI and VILI/clod 24h conditions. An asterisk (*) indicates a significant difference.]

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Role of Lung-Marginated Monocytes in an In Vivo Mouse Model of Ventilator-Induced Lung Injury

Michael R Wilson, Kieran P O'Dea, Da Zhang, Alexander D Shearman, Nico van Rooijen, Masao Takata

ONLINE DATA SUPPLEMENT
METHODS

Monocyte recruitment/activation during mechanical ventilation

All protocols were approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK. The in vivo mouse model of VILI used has been described in detail previously (E1, E2). In brief, mice were anesthetized by intraperitoneal injection of ketamine 60mg/kg and xylazine 6mg/kg. Mice were tracheostomized and ventilated via endotracheal tube using a custom-made mouse ventilator-pulmonary function testing system (E2, E3). The left carotid artery was cannulated for monitoring arterial blood pressure (BP) and blood gases, and for saline infusion (0.4ml/hour). During instrumentation, animals were ventilated with low stretch, i.e. tidal volume ($V_T$) of 7-8ml/kg, 2.5cmH$_2$O positive end expiratory pressure (PEEP) and respiratory rate (RR) of 120 breaths/minute, using air. Upon completion of instrumentation, sustained inflation of 30cmH$_2$O for 5 seconds was given two times to standardise volume history of the lungs, and baseline physiological measurements were performed to ensure stability of the preparation.

Following baseline physiological measurements, animals were randomly allocated to receive one of the following ventilatory protocols –

**High stretch** - $V_T$ 34-36ml/kg, PEEP 0 cmH$_2$O, RR 90 breaths/minute, using air/4% CO$_2$. This protocol produced an initial peak inspiratory pressure (PIP) of ~30 cmH$_2$O, and was continued (with the same constant $V_T$) until PIP increased by ~40%, indicating substantial pulmonary edema. This typically took between 140-200 minutes (mean 159±29 minutes).

**Low stretch** - $V_T$ 7-8ml/kg, PEEP 2.5 cmH$_2$O, RR 120 breaths/minute using air. Sustained inflation of 30cmH$_2$O for 5 seconds was given every 30 minutes to avoid the development of atelectasis, and ventilation was continued for 180 minutes.
Airway pressure, airway flow, and mean BP were monitored continually throughout the experiments. Respiratory mechanics including respiratory system compliance (Crs) and resistance (Rrs) were determined by the end-inflation occlusion technique (E3) at baseline, 30 minutes after the start of the ventilatory protocol, and every 30 minutes thereafter until the end of the protocol. Blood gas analyses were performed at baseline, 30 minutes after the start of the protocol, and at the end of the protocol. In order to prevent hypovolemia, blood sample volume was limited to 70µl, and the volume replaced with saline each time. At the end of the ventilatory protocol, mice were euthanized with anesthetic overdose. A separate group of non-instrumented, non-ventilated mice were also euthanized by anesthetic overdose to act as untreated controls.

**Flow cytometric analysis**

Following termination, lungs were processed to produce a single cell suspension for flow cytometric analysis of monocytes and neutrophils, as described and validated previously (E1, E4, E5). In brief, lung cell suspensions were prepared from the excised lungs of mice by mechanical disruption, followed by forcing through a 40µm filter. Lung samples were stained with fluorophore-conjugated anti-mouse antibodies for CD11b, Gr-1 (Ly6C/G), F4/80, L-selectin or appropriate isotype-matched controls. Cells were quantified using microsphere counting beads (Caltag Medsystems) added to the samples, and activation assessed based on L-selectin and CD11b adhesion molecule expression. Samples were analyzed using a FACSCalibur flow cytometer with CellQuest (Becton Dickinson) and Flowjo (Tree Star) software. In order to differentiate and quantify Gr-1\textsuperscript{high} and Gr-1\textsuperscript{low} monocytes, low side-scatter, high CD11b events were gated (Fig E1A) and these events (G1) analysed in terms of Gr-1 vs F4/80 expression. From within this gated population (Fig E1B), F4/80 positive monocytes were divided into subsets based on either high (R1) or low (R2) expression of Gr-1, and distinguished from
‘contaminating’ neutrophils, which were F4/80 negative with very high Gr-1 expression (R3). L-selectin expression was quantified on these subsets and found only to be present on Gr-1<sup>high</sup> monocytes, as anticipated (E6). CD11b expression was not determined on these events (R1 and R2), due to the use of CD11b in the identification strategy. Neutrophils were identified and quantified using the same Gr-1 vs F4/80 differential staining strategy, but this time within the entire (i.e. no gating) cell population (Fig. E1C), as F4/80 negative, Gr-1 very positive events (R4). A population of Gr-1<sup>high</sup> monocytes could also be identified among the entire lung cell population (R5), and as CD11b was not used in any way to identify these cells, CD11b expression level was determined on them as a marker of activation.

**Involvement of monocytes in pulmonary edema formation during VILI**

The involvement of recruited monocytes in the progression of VILI was assessed using a clinically relevant two-hit model.

**Two-hit model:** LPS challenge (20ng/mouse, Ultrapure LPS, InVivoGen) was given intraperitoneally to induce a sub-clinical inflammation. In some animals, intravascular monocytes and macrophages were depleted by pretreament with intravenous clodronate-loaded liposomes (200µl) given 24 or 48 hours prior to LPS challenge (E6, E7). 2 hours after LPS challenge (with/without clodronate-liposome pretreatment), some animals were sacrificed and neutrophil and Gr-1<sup>high</sup> monocyte numbers within the lungs determined. A separate group of animals (with or without pre-treatments/challenges) were anesthetised, instrumented, and ventilated using either low or high stretch ventilation for 2 hours. In total, five separate groups of animals were studied –

i) High stretch ventilation alone (no challenge/pre-treatment)

ii) High stretch ventilation + LPS (LPS given 2 hours prior to ventilation)
iii) Low stretch ventilation + LPS (to clarify that LPS per se induced no edema formation)

iv) High stretch ventilation + LPS + clodronate 24h (clodronate-liposomes given 24 hours before LPS challenge, to deplete monocytes)

v) High stretch ventilation + LPS + clodronate 48h (clodronate-liposomes given 48 hours before LPS challenge, to allow repletion of monocytes)

Ventilation parameters were similar to those described above, except that the high stretch protocol was designed to induce less injury, so that the expected exacerbation of VILI by pre-treatments/challenges could be more easily detected. A slightly lower $V_T$ (28-30ml/kg) was used, though otherwise the settings were as described previously, i.e. PEEP 0 cmH$_2$O, RR 90 breaths/minute, using air/4% CO$_2$. In addition experiments were all stopped at 2 hours, by which time only minor pulmonary edema formation was present in those animals that had received no pre-treatments/challenges.

**One-hit, pure VILI model:** In order to delineate the potential role of systemic monocyte activation by LPS, depletion experiments were also carried out in the one-hit, pure VILI model. In some animals, intravascular monocytes and macrophages were depleted by pretreatment with intravenous clodronate-loaded liposomes (200µl) given 24 hours prior to either culling (to determine leukocyte numbers within the lungs) or high stretch ventilation. These ventilation experiments used the same parameters described previously for the ‘monocyte recruitment/activation during mechanical ventilation’ experiments (i.e. $V_T$ 34-36ml/kg, PEEP 0 cmH$_2$O, RR 90 breaths/minute, using air/4% CO$_2$), except that experiments were terminated at 2 hours, in order that any attenuation by treatment could be determined.

VILI progression was evaluated by changes in PIP, Crs and Rrs. Clodronate was a gift from Roche Diagnostics GmbH (Mannheim, Germany) and was incorporated into liposomes as described previously (E8). The impact of clodronate-liposome treatment on alveolar
macrophages was assessed by determining the percentage and total number of alveolar macrophages within lung lavage fluid of the different groups of mice. In brief, following termination lungs were lavaged using 750μl saline, and lavage fluids recovered were centrifuged (5 min, 1,500 rpm, 4°C). Cell pellets were used for cell counting by hemocytometer, and differential cell analysis by Diff-Quik staining of samples prepared by Cytospin (Shandon, Runcorn, UK).

**Statistical analysis**

Data are expressed as mean±SD. Statistical comparisons were made by t-tests or ANOVA with Bonferroni tests using Prism software (version 4.0). Statistical significance was defined as p<0.05.
REFERENCES


**FIGURE LEGEND**

**Fig. E1.** Figure showing flow cytometric analysis of monocytes and neutrophils in mouse lungs.

**A.** Dot-plot showing side-scatter (SSC) versus CD11b expression of the total cell population. From this a low side scatter, CD11b positive population (G1) were gated on and further analysed.

**B.** Dot-plot showing Gr-1 versus F4/80 expression of the G1 gated events. Monocytes were identified as being F4/80 positive events and subdivided into Gr-1\textsuperscript{high} (R1) and Gr-1\textsuperscript{low} (R2) populations, and differentiated from neutrophils as being Gr-1 very high, F4/80 negative events (R3).

**C.** Dot-plot showing Gr-1 versus F4/80 expression of the total cell population. Neutrophils within the lung cell population were identified as Gr-1 very high, F4/80 negative events (R4). A population of Gr-1\textsuperscript{high} monocytes (R5) was also identifiable within the total lung cell population (R5).