Intravascular donor monocytes play a central role in lung transplant ischaemia-reperfusion injury

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ABSTRACT

Rationale Primary graft dysfunction in lung transplant recipients derives from the initial, largely leukocyte-dependent, ischaemia-reperfusion injury. Intravascular lung-marginated monocytes have been shown to play key roles in experimental acute lung injury, but their contribution to lung ischaemia-reperfusion injury post-transplantation is unknown.

Objective To define the role of donor intravascular monocytes in lung transplant-related acute lung injury and primary graft dysfunction.

Methods Isolated perfused C57BL/6 murine lungs were subjected to warm ischaemia (2 hours) and reperfusion (2 hours) under normoxic conditions. Monocyte retention, activation phenotype and the effects of their depletion by intravenous clodronate-liposome treatment on lung inflammation and injury were determined. In human donor lung transplant samples, the presence and activation phenotype of monocytic cells (low side scatter, 27E10+, CD14+, HLA-DR+, CCR2+) were evaluated by flow cytometry and compared with post-implantation lung function.

Results In mouse lungs following ischaemia-reperfusion, substantial numbers of lung-marginated monocytes remained within the pulmonary microvasculature, with reduced L-selectin and increased CD86 expression indicating their activation. Monocyte depletion resulted in reductions in lung wet:dry ratios, bronchoalveolar lavage fluid protein, and perfusate levels of RAGE, MIP-2 and KC, while monocyte repletion resulted in a partial restoration of the injury. In human donor lung transplant samples, the presence and activation phenotype of monocytic cells were evaluated by flow cytometry and compared with post-implantation lung function.

Conclusions These results indicate that lung-marginated intravascular monocytes are retained as a ‘passenger’ leukocyte population during lung transplantation, and play a key role in the development of transplant-associated ischaemia-reperfusion injury.

INTRODUCTION

Lung transplantation is the final treatment option for patients with end-stage lung disease. Primary graft dysfunction (PGD) plays a major role in recipient morbidity and mortality in the days following transplantation, and negatively impacts on long-term graft survival. Ischaemia-reperfusion (I/R) injury is an inevitable feature of the lung transplantation process and the primary pathophysiological mechanism responsible for PGD.

What is the key question?

Resident lung mononuclear phagocytes have been implicated in ischaemia-reperfusion injury and the development of primary graft dysfunction following lung transplantation, but the contribution of lung intravascular monocytes to lung transplant-related injury is not known.

What is the bottom line?

Retention and activation of intravascular monocytes in perfused murine and human donor lungs suggests an important and previously unrecognised role as passenger leukocytes contributing to lung injury and primary graft dysfunction, emphasising their potential as therapeutic targets in lung transplantation.

Why read on?

This study suggests that vascular monocytes play a key role in lung injury following ischaemia-reperfusion.
with a two-step process involving neutrophils as late stage effector cells. Thus, the early resident leukocyte responses are likely to play a significant role in ‘initiating’ I/R lung injury during transplantation, representing potentially important therapeutic targets for reducing PGD.

In addition to alveolar macrophages, other substantial monocytic populations are indeed present within the interstitial and vascular compartments of the donor lungs. Monocytes marginated in the narrow pulmonary capillaries have been shown to play a crucial role in enhancing acute lung injury resulting from microbial or mechanical insults in preclinical models. There is also evidence from human transplant studies that donor lung intravascular monocytes are not completely removed by perfusion and carried over as ‘passenger’ cells, with their presence shown in post-perfusion lung transplant tissue as well as in the circulation of recipients. However, the potential contribution of these intravascular monocytes to the pathogenesis of I/R injury, and thus PGD, has not been studied.

We hypothesised that donor lung-marginated, intravascular monocytes, exposed directly to the I/R-induced vascular stress and in close contact with the pulmonary capillary endothelium, would play a key role in development of transplant-related I/R lung injury, and thus PGD. We investigated this hypothesis using a mouse isolated perfused lung (IPL) model of transplant-related early I/R injury, combined with intravascular monocyte depletion and repletion treatments. We then evaluated numbers and phenotypes of mononuclear phagocytes within pre-implantation lungs and studied their relationships with post-implantation gas exchange and PGD severity in human lung transplantation.

Figure 1: Pulmonary intravascular lung monocytes are retained during lung perfusion. The location and phenotype of monocyte subsets in the lungs were determined by dual compartment, intra-vascular and intra-alveolar leukocyte staining. Mice were injected intravenously (i.v.) with anti-CD45 (PE-CF594) before anaesthetic overdose, followed by intra-tracheal (i.t.) instillation of anti-CD45.2 (APC). To analyse mononuclear leukocytes within the lung cell suspensions, extra-alveolar leukocytes were first identified as CD45.2− and CD11b+ cells (A). In these cell populations, monocytes/macrophages were further identified as F4/80+ and subdivided into Ly6CHigh and Ly6CLow subsets (B). Intravascular monocytes were identified as anti-CD45 PE-CF594+ (C and D). MHCII was used as a marker for interstitial CD11b+, F4/80+ macrophages. To evaluate intravascular monocyte removal during perfusion, lungs were flushed with open circuit perfusion for 15 min after compartmental antibody staining. Only partial reduction in intravascular monocyte numbers was observed following washout (black diamonds) compared with non-surgical controls (white boxes) with both intravascular Ly6CHigh (E) and Ly6CLow (F) populations. Numbers of interstitial Ly6CLow, MHCII+ macrophages (G) were not significantly changed, confirming their extravascular location. These numbers of intravascular monocyte subsets far exceeded those expected in an unlikely situation when residual blood within the pulmonary vasculature (estimated ∼50 μL as maximum) was not washed out, and totally preserved within the lung despite this extended perfusion, and hence can be ascribed to a marginated pool. Data are displayed as mean±SD, and analysed by t test. n=4–6, *p<0.05, ***p<0.001.

Figure 1 P: Pulmonary intravascular lung monocytes are retained during lung perfusion. The location and phenotype of monocyte subsets in the lungs were determined by dual compartment, intra-vascular and intra-alveolar leukocyte staining. Mice were injected intravenously (i.v.) with anti-CD45 (PE-CF594) before anaesthetic overdose, followed by intra-tracheal (i.t.) instillation of anti-CD45.2 (APC). To analyse mononuclear leukocytes within the lung cell suspensions, extra-alveolar leukocytes were first identified as CD45.2− and CD11b+ cells (A). In these cell populations, monocytes/macrophages were further identified as F4/80+ and subdivided into Ly6CHigh and Ly6CLow subsets (B). Intravascular monocytes were identified as anti-CD45 PE-CF594+ (C and D). MHCII was used as a marker for interstitial CD11b+, F4/80+ macrophages. To evaluate intravascular monocyte removal during perfusion, lungs were flushed with open circuit perfusion for 15 min after compartmental antibody staining. Only partial reduction in intravascular monocyte numbers was observed following washout (black diamonds) compared with non-surgical controls (white boxes) with both intravascular Ly6CHigh (E) and Ly6CLow (F) populations. Numbers of interstitial Ly6CLow, MHCII+ macrophages (G) were not significantly changed, confirming their extravascular location. These numbers of intravascular monocyte subsets far exceeded those expected in an unlikely situation when residual blood within the pulmonary vasculature (estimated ∼50 μL as maximum) was not washed out, and totally preserved within the lung despite this extended perfusion, and hence can be ascribed to a marginated pool. Data are displayed as mean±SD, and analysed by t test. n=4–6, *p<0.05, ***p<0.001.

METHODS
Detailed methods are provided in the online supplementary methods.
Figure 2  Activation of retained lung monocytes and neutrophils during ischaemia-reperfusion (I/R). Monocyte and neutrophil activation during I/R was indicated by changes in expression of their respective surface activation markers relative to 2 hours of perfusion only, with values for those from untreated (non-surgical) control mice indicated by a dotted line. Ly6C<sup>High</sup> monocyte activation was indicated by L-selectin shedding and CD86 upregulation (A); Ly6C<sup>Low</sup> monocytes and interstitial macrophages by increased CD86 expression (B and C); and neutrophils by L-selectin shedding and increased surface CD11b expression (D). Data are displayed as mean±SD and analysed by t tests. n=4–8, *p<0.05, **p<0.01, ***p<0.001.

Table 1  Lung leukocyte numbers 24 hours after clodronate-liposome injection

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<th>Control mice</th>
<th>Clodronate treated mice</th>
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<tr>
<td>Vascular Ly6C&lt;sup&gt;High&lt;/sup&gt; monocytes ×10&lt;sup&gt;5&lt;/sup&gt;/lungs</td>
<td>4.51 (3.3–7.3)</td>
<td>1.98 (1.6–2.3)***</td>
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<tr>
<td>Vascular Ly6C&lt;sup&gt;Low&lt;/sup&gt; monocytes ×10&lt;sup&gt;5&lt;/sup&gt;/lungs</td>
<td>3.15 (3.0–4.8)</td>
<td>0.72 (0.3–1.0)***</td>
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<td>Interstitial MHCII&lt;sup&gt;+&lt;/sup&gt; macrophages ×10&lt;sup&gt;5&lt;/sup&gt;/lungs</td>
<td>0.90 (0.7–1.3)</td>
<td>0.70 (0.5–1.6)***</td>
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<td>Alveolar macrophages ×10&lt;sup&gt;5&lt;/sup&gt;/lungs</td>
<td>18.8 (17.0–24.4)</td>
<td>18.98 (18.5–21.3)</td>
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<td>Vascular neutrophils ×10&lt;sup&gt;5&lt;/sup&gt;/lungs</td>
<td>5.42 (4.1–7.8)</td>
<td>10.0 (6.3–18.7)</td>
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Lung leukocyte numbers were quantified via flow cytometry in control (non-surgical) mice, with and without prior clodronate administration. Data were analysed by Mann-Whitney U test (medians±IQR×10<sup>5</sup>/lungs); n=9 and 6, **p<0.01, ***p<0.001 vs control mice.

Animal experimentation

All protocols were approved by the Imperial College Ethical Review Board and UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986, UK, and performed in line with the ARRIVE guidelines (see online supplementary methods). Male C57BL/6 mice aged 8–12 weeks (24–28 g) were used.

Isolated perfused lung

As previously described, anaesthetised mice were mounted on an IPL system, and after exsanguination and thoracotomy, their pulmonary artery and left atrium cannulated. Lung perfusion with supplemented RPMI-1640 and ventilation was commenced (see online supplementary figure E1). After an initial washout period, lungs underwent either 15 min of open perfusion (compartment protocol, see online supplementary figure E2A), 2 hours of ischaemia/2 hours of reperfusion (I/R protocol, see online supplementary figure E2B) or adoptive transfer of blood monocytes prior to I/R (adoptive transfer protocol, see online supplementary figure E2C).

For monocyte depletion experiments, mice received intraperitoneal clodronate liposomes (Formumax Scientific, Palo Alto, California, USA) 24 hours prior to the I/R protocol. For adoptive transfer experiments, monocytes were isolated from blood of donor mice using a negative magnetic bead selection kit (StemCell Technologies, Grenoble, France) and infused into the IPL.
Sample analysis

On completion of the protocols, left lungs were tied off for wet:dry weight ratio determination, and remaining lung lobes underwent BAL and disaggregation for flow cytometric analysis. BAL fluid was analysed for protein content, and peritoneal soluble inflammatory markers (MIP-2, KC, MCP-1, RAGE, TNF-α, IL-β, IL-6) were quantified by ELISA. Lung cell suspensions were prepared by tissue disaggregation in fixative in a gentleMACS dissociator (Miltenyi, Bisley, UK) and samples were passed through a 40 μm strainer and washed.15 16 Cell samples were incubated with the antibodies in online supplementary table E3. Electron micrographs were prepared for, and acquired by, the Transmission Electronic Microscope JEOL 1200EX, detailed in table E1.

Human samples

All patients provided informed consent to participate in the ethically approved POPSTAR study (Peri-OPerative Study of lung Transplantation and Acute Respiratory failure; reference 13/LO/ 0152), investigating surgical and technological aspects of transplantation. Human samples and patient data were stored in accordance with the study protocol at the Royal Brompton and Harefield NHS Foundation Trust, UK. Lower lobe biopsies (~3 cm×1 cm×1 cm) were obtained from a total of 13 donor lungs (already flushed at the time of retrieval), immediately prior to implantation, and stored on ice. Lung tissue was minced and disaggregated with Liberase/DNAse for 20 min at 37°C, passed through a 40 μm strainer and washed. Lung cell suspensions and blood samples from healthy volunteers were incubated with the antibodies in online supplementary table E3. Electron micrographs were prepared for, and acquired by, the Transmission Electronic Microscope JEOL 1200EX, detailed in the online supplementary methods.

Flow cytometry

Antibody-stained cell samples were analysed using a Cyan flow cytometer (Beckton Coulter, High Wycombe, UK) with FlowJo software (V10.0.8, Ashland, Oregon, USA).

Statistics

Normality was determined using QQ plots and Shapiro-Wilk tests. Group comparisons were made by Student’s t tests or Mann–Whitney U tests, or by ANOVA with Bonferroni tests or Kruskal–Wallis with Dunn’s tests for more than two groups. Correlation analysis was performed with Spearman rank test. Data are presented as mean±SD (parametric) or median±IQR (non-parametric). Statistical significance was defined as p<0.05.

RESULTS

Pulmonary intravascular monocytes are retained during lung perfusion

To investigate the potential for retention of intravascular monocytes within the lungs during ex vivo perfusion, we first determined the size of the lung-marginated monocyte pool (figure 1), using flow cytometry and a dual-compartment (vascular and alveolar) antibody staining technique developed in our laboratory.16 Intra-vascular leukocytes were identified by intravenous injection of mice with an anti-CD45 antibody (Clone 30-F11, PE-CF594) and intra-alveolar leukocytes by intra-tracheal installation of anti-CD45.2 (Clone 104, APC). Cells double-negative for intravascular CD45 and intra-tracheal CD45.2 included interstitial leukocytes, while double-positive cells were rarely observed (<1%), indicating compartment specificity of the method. Intravascular Ly6C<sup>High</sup> and Ly6C<sup>Low</sup> monocytes were clearly identified using their cell surface marker characteristics (CD11b<sup>+</sup>, F4/80<sup>+</sup>, MHCII<sup>+</sup>) combined with this compartmental staining technique. The lung Ly6C<sup>High</sup> monocytes were largely situated in the intravascular space (figure 1C:R2), with a small interstitial subpopulation (figure 1C:R4) that may be intravascular but not fully stained by injected intravenous antibody. Similarly, the lung Ly6C<sup>Low</sup> monocytes were largely intravascular (figure 1D:R6). There was a population of mononuclear phagocytes in the interstitial space, which was CD11b<sup>+</sup>, F4/80<sup>+</sup>, Ly6C<sup>Low</sup> but MHCII<sup>+</sup> (figure 1D:R7), and their number was comparable to that of intravascular Ly6C<sup>Low</sup> monocytes. These cells are equivalent to the interstitial macrophage and/or dendritic cell populations previously described by others17 and ourselves.16 The alveolar compartment was comprised predominantly (>99%) of alveolar macrophages (CD11b<sup>+</sup>, F4/80<sup>High</sup>, CD11C<sup>High</sup>).

Monocyte retention during extended ex vivo perfusion was then determined following dual compartment labelling (figure 1E–G). Following open-circuit non-recirculating perfusion (RPMI-1640, human albumin 4%, 25 mL/kg/min) for 15 min, numbers of intravascular Ly6C<sup>High</sup> monocytes were...
reduced to only approximately one-half (54.1±12.3%) compared with control non-perfused lungs, and an even larger proportion (62.5±10.3%) of Ly6C\text{Low} monocytes were retained. As expected, numbers of ‘interstitial’ (Ly6C\text{Low}, CD11b+, F4/80\text{+}, MHCII\text{+}) cells were not altered significantly by this perfusion procedure. The total number of monocytes (Ly6C\text{High}+Ly6C\text{Low}) collected in the effluent from the perfused lungs (‘perfusate’) approximated to the difference between the (non-perfused) controls and remaining (retained) monocytes in the perfused lungs.

**Activation of intravascular monocytes during lung I/R**

In situ mouse IPLs were subjected to a protocol of warm normoxic ischaemia (2 hours) and reperfusion (2 hours in a recirculating manner), incorporating three 5 min open-circuit washout periods pre ischaemia, post ischaemia and post reperfusion (see online supplementary figure E2B). Intravascular monocytes were identified as Ly6C\text{High} or Ly6C\text{Low}, CD11b+, F4/80+ and MHCII\text{−} cells in these experiments, based on the compartmental analysis of lung leukocytes in figure 1. Substantial proportions of the intravascular Ly6C\text{High} and Ly6C\text{Low} monocyte populations were retained at the end of I/R, comparable to, or somewhat higher than, the 15 min washout (Ly6C\text{High} 79.8±20.7%; Ly6C\text{Low} 69.7±19.3%), presumably due to I/R-induced cell activation. Such retention of intravascular monocytes, exposed to the ischaemic insult and present subsequently during the reperfusion period, could play a significant role in the progression of lung injury in this ex vivo I/R model.

To evaluate the role of these retained intravascular monocytes in the development of I/R injury, we first determined whether they became activated during the IPL-I/R procedure (figure 2A–D). On Ly6C\text{High} monocytes, L-selectin expression, a classical ‘endpoint’ marker of non-transcriptional cell activation,\textsuperscript{18} was substantially reduced, while CD86, an inflammation-induced T-lymphocyte costimulatory molecule,\textsuperscript{19} was upregulated at the end of the I/R protocol. On intra-vascular Ly6C\text{Low}/MHCII\text{−} monocytes, which

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**Figure 4** Intravascular monocyte depletion modifies soluble mediator release during lung ischaemia-reperfusion (I/R). Lungs from normal (black squares) or clodronate-liposome pretreated (white circles) mice were subjected to I/R, with perfusate samples obtained pre ischaemia, post ischaemia and post reperfusion. Levels of soluble KC (A), MIP-2 (B) RAGE (C), MCP-1 (D) and IL-6 (E) were determined by ELISA. Increases in KC, MIP-2 and RAGE post ischaemia and post reperfusion were reduced by clodronate-induced monocyte depletion, whereas this treatment resulted in higher MCP-1 levels at all sampling points. Data are displayed as mean±SD, and analysed by two-way ANOVA with t tests with Bonferroni correction. n=4–6, **p<0.01, ***p<0.001, ****p<0.0001.
do not classically express appreciable amounts of L-selectin, I/R-induced activation was indicated by CD86 upregulation. This response was also observed on the interstitial MHCII+/Ly6Clow macrophage population, indicating cross-compartmental activation as a result of I/R. I/R-dependent neutrophil activation was also observed with reduction in L-selectin expression and increased cell surface CD11b expression.

Depletion of monocytes attenuates I/R-induced lung injury and inflammation

To further determine the role of pulmonary intravascular monocytes in I/R-induced lung injury, we pretreated mice by intravenous injection with clodronate liposomes to produce intravascular compartment-specific monocyte depletion. This treatment was effective in significantly reducing the lung intravascular monocyte population whilst leaving the neutrophils, interstitial (CD11b+, F4/80+, MHCII+) and alveolar macrophage populations intact (table 1).

I/R-induced lung injury, as determined by increased lung wet: dry weight ratio and BAL fluid protein levels, was substantially reduced in monocyte pre-depleted lungs, with both indices attenuated to the levels seen in perfusion-only controls (figure 3).

Lung perfusates sampled pre and post ischaemia and at the end of the reperfusion period showed increasing levels of KC, MIP-2 and RAGE during the protocol (figure 4A–C). Depletion of monocytes by intravascular clodronate-liposome treatment produced a considerable reduction in the levels of these markers, which was clearly evident in the case of RAGE at the immediate post-ischaemia time point, indicating an early role for monocytes in development of I/R injury. However, MCP-1 levels were higher in clodronate-treated mouse lungs than normal lungs throughout the I/R protocol (figure 4D), consistent with reduced MCP-1 absorption by monocytes, as previously described and confirming monocyte-specific effects of the depletion method. TNF and IL-1β levels, which have previously been reported to be elevated in lung I/R models, were negligible in the vascular compartment, while IL-6 was increased, but unaffected by monocyte depletion (figure 4E), indicating alternative cell sources or mechanism of induction. We also demonstrated a reduction in I/R-induced upregulation of CD11b on lung neutrophils in monocyte-depleted mice (I/R: 1506±373MFI; clodronate-liposome + I/R: 540±176MFI, p<0.0001), consistent with the observed lower release of neutrophil-activating chemokines KC and MIP-2.

Adoptive transfer of blood-derived monocytes partially restores I/R-induced acute lung injury

To confirm the role of intravascular lung-marginated monocytes in I/R-induced injury response, monocytes from normal mice were adoptively transferred into isolated lungs of monocyte-depleted mice (figure 5). Monocytes were enriched by negative selection from the blood of donor mice and injected via the pulmonary artery into pre-flushed lungs prior to the ischaemic period. This method restored monocytes to the levels approaching those in normal I/R lungs seen in table 1, albeit with a larger variance that may reflect variable margination rates for ex vivo isolated monocytes and/or their potential loss within the perfusion circuit (eg, adherence to tubing). Both lung injury parameters were increased by adoptive transfer of monocytes, although this did not reach statistical significance in the case of BAL protein.

Analysis of monocytes in pre-implantation human lungs

To assess the relationship between donor monocytes sequestered within human transplant lungs and their potential contribution to development of PGD in recipients, a pilot study was performed in which a total of 13 lungs were analysed (seen online supplementary table E2 for demographic, physiological and outcome data). Donor monocytes in lung cell suspensions, prepared from biopsy samples taken immediately before implantation, were identified as low side scatter/27E10+/CD14+ events, distinguished from 27E10+ granulocytes by absence of CD66b expression (figure 6). The flow cytometry profile of these lung-associated monocytes was identical to that of the CD14+/CD16– phenotype of classical subset monocytes in healthy volunteer blood, with further confirmation based on their comparable HLA-DR and CCR2 expression. We limited

Figure 5 Adoptive transfer of monocytes restores ischaemia-reperfusion (I/R)-induced lung injury in monocyte-depleted lungs. Isolated lungs from monocyte-depleted mice were infused slowly (1 min) with normal blood-derived monocytes and recirculated for 10 min prior to initiation of the ischaemic period. At the end of I/R, Ly6CHigh monocyte numbers were found to be elevated in infused lungs (A). Lung wet: dry weight ratios (B) and bronchoalveolar lavage (BAL) protein levels (C) were increased in monocyte-infused lungs, indicating monocyte-dependent I/R injury. Post-I/R treatment values obtained in previous experiments in normal (non-monocyte-depleted) I/R mice are indicated by a dotted line. Data are analysed by t tests (A and B; mean ±SD) or by Mann–Whitney U tests (C; median±IQR). n=4, *p<0.05. clod-lipo, clodronate-liposome.
our analysis to these classical CD14+/CD16− subset monocytes in lungs, omitting CD14+/CD16+ cells due to potential overlap with lung macrophages expressing CD16.25

Large numbers of donor monocytes (3.8±2.0×10^6 cells/g dry weight) and granulocytes (8.1±6.7×10^6 cells/g) were still found in tissue from these lungs before implantation, despite the fact that they had been subjected to the standard anterograde and retrograde (5L perfusate) perfusion protocols to remove blood elements prior to implantation. Consistent with this, H&E and immunohistochemistry microscopy showed significant presence of leukocytes in the pre-implantation lung biopsies (see online supplementary figure E3). Electron microscopy (EM) provided further clear evidence of monocyte margination within pulmonary capillaries, where they were found in close apposition with endothelial cells (figure 7).

Comparison of donor monocyte numbers with P:F ratios at 48 and 72 hours post implantation (figure 8A) revealed a high level of negative correlation at 72 hours (r=−0.70, p=0.016), although there was only a weak non-significant correlation at 48 hours. In contrast, there was no apparent relationship between donor granulocytes and P:F ratios at either time point. Examination of donor monocyte CD86 and TREM-1 expression also indicated a relationship between the CD86 levels and lung dysfunction, showing a high inverse correlation with P:F ratios at 48 hours (r=−0.78, p=0.004), while no clear relationship was found between neutrophil CD11b expression and P:F ratios (figure 8B). The significance of the donor monocyte activation status and severity of PGD was further assessed by analysis of CD86/TREM-1 expression and PGD scores at 48 and 72 hours (figure 9). CD86 expression was higher in transplant recipients with PGD grade III at 48 hours, and remained higher in those recipients at 72 hours. Levels of TREM-1 expression were also higher at 48 hours, but the inverse correlation with P:F levels did not reach significance (r=−0.57, p=0.078).

**DISCUSSION**

A role for pulmonary intravascular monocytes as passenger leukocytes, contributing to I/R injury and PGD following lung transplantation, is not widely recognised.26 We demonstrate, for the first time, that intravascular monocytes retained in an IPL model become activated and contribute significantly to the development of I/R lung injury. The clinical significance of these observations was supported by our pilot study with human donor lungs, where monocyte numbers and their expression of surface activation markers were associated with reduced postoperative gas exchange and development of PGD in recipients. As a substantial intravascular population with immediate exposure to and interactions with donor and recipient environments, donor-derived passenger monocytes could represent a novel therapeutic target to improve the quality and function of lung allografts.

We found that substantial numbers of intravascular monocytes were still present after prolonged non-recirculating perfusion in murine lungs. A similar degree of monocyte retention was found after lung I/R, where non-recirculating perfusion was repeated three times. Sequestration of leukocytes within the pulmonary microvasculature, in particular monocytes, during ex vivo perfusion of rodent lungs has previously been reported,20 27 and the traditional method to define lung tissue (interstitial) leukocytes as cells remaining after lung perfusion is now recognised...
as a flawed approach. Near-complete removal of these marginated pools would require draconian perfusion methods (eg, using EDTA or enzyme-containing perfusate). Crucially, intravascular monocytes have also been observed in transplant lungs by histological examination. This is consistent with our novel EM data, which clearly confirm that monocytes are located in the pulmonary microvasculature, and indicate a likely interaction with the endothelium. The ability of marginated monocytes to withstand perfusion was attributed to inflammation within moribund donors, leading to upregulated expression of leukocyte adhesion molecules. Viable donor monocytes have also been found in the circulation of lung recipients in numbers that were disproportionately higher than other leukocyte subpopulations. In addition, the presence of significant numbers of blood monocytes had been confirmed in human lung tissue, using a combination of flow cytometry, cell sorting and histological analysis. Thus, although not an ‘anchored’ resident cell population such as alveolar macrophages, lung-marginated intravascular monocytes seem to be a bona fide passenger leukocyte population.

Despite their presence in mouse and human lungs, the potential contribution of lung-marginated monocytes to lung I/R injury has largely been unrecognised. In models of lung I/R, injury has been attributed to lung macrophages, with a delayed secondary role of vascular neutrophils. However, we and others have previously demonstrated that lung margination of monocytes can play a significant role in both direct and extra-pulmonary models of acute lung injury. Moreover, unlike I/R in extra-pulmonary organs where anoxia and re-oxygenation can result in a diffuse injury, I/R injury during lung transplantation develops initially under normoxic conditions, with inflammation propagated locally by endothelial cell sensing of flow cessation. In spite of this localised ‘vascular’ origin of lung I/R injury, the role of marginated monocytes in direct contact with endothelial cells has been overlooked until now.

We found that monocytes present during I/R became activated, indicating their potential to enhance pulmonary vascular inflammation in mouse lungs. Using clodronate-liposome treatment, we observed reversal of the I/R-induced injury. When combined with an isolated organ-perfusion system, intravenous clodronate-liposome depletion provides an optimal method to evaluate the localised role of intravascular mononuclear phagocytic cells in isolation. Alternative monocyte depletion methods available were not suitable for this purpose: in the case of mAb anti-CCR2 treatment monocyte depletion is limited to the Ly6CHigh subset, while monocyte-macrophage depletion in the CD11b diphtheria toxin transgenic mouse model is not compartment specific, potentially producing accumulation of apoptotic interstitial macrophages with resultant modulation of local inflammation.

To further validate our clodronate-liposome-based findings, we performed monocyte transfer experiments in monocyte-depleted lungs and found a restoration of injury based on an increase in wet:dry ratios.

In human lung tissue, we identified monocytes as low granularity (side scatter)/CD14+/27E10 High cells, expressing high levels of HLA-DR and CCR2, mirroring the phenotype of blood monocytes. This combination of markers alone does not directly define their location, but their close resemblance to classical subset monocytes in blood, as well as our EM data and the
Figure 8  Correlation of donor lung monocyte numbers and activation state with P:F ratios following transplantation. Numbers of monocytes retained in donor lungs post harvest were found to negatively correlate with P:F ratios at 72 hours post implantation (p<0.05) (Aii). Donor lung granulocyte numbers did not correlate with P:F ratios at either 48 or 72 hours (A; iii–iv). Donor lung monocyte activation (CD86 expression) correlated negatively with P:F ratio at 48 hours (Bi). No correlation was seen with granulocyte activation (CD11b), at either time point (B; iii–iv). Data are analysed by Spearman’s rank test (showing r values *p<0.05, **p<0.01), n=11–13.
facilitators of neutrophil migration following intra-alveolar insults.\textsuperscript{36} and imaging-based studies suggest that intravascular monocytes are vital in orchestrating neutrophil behaviour during transplant-mediated I/R injury.\textsuperscript{37} ‘Patrolling’ Ly6CLow monocytes that coordinate neutrophil interaction with injured endothelial cells in extra-pulmonary vascular beds,\textsuperscript{38} may also be applicable to the pulmonary vasculature.\textsuperscript{39}

Our findings provide new evidence on how subclinical donor injury contributes to I/R injury and PGD. There are now substantial data that PGD is at least in part determined prior to implantation,\textsuperscript{40} and our results highlight a role for lung-marginalized passenger monocytes in this process. Our concepts are also in keeping with a recent clinical study investigating lung monocyte/dendritic cell populations during the ex vivo lung perfusion.\textsuperscript{5} Although focused on the role of a different monocyte subset (non-classical monocytes) in organ rejection, they advocated the use of ex vivo lung perfusion as a potential mechanism of removing the passenger monocytes. With the emerging use of perfusion systems (eg, Organ Care System) for lung preservation and reconditioning,\textsuperscript{5} there is now an opportunity to remove/modify donor passenger leukocytes, in particular intravascular populations, before transplantation. Such ex vivo perfusion platforms may also allow advanced pharmacological or biological interventions to attenuate monocyte margination and/or activation to reduce PGD. This study provides important insights when considering such novel therapeutic possibilities.

Figure 9  Donor monocyte activation is associated with primary graft dysfunction (PGD) severity. Expression of CD68 and TREM-1 on donor lung monocytes pre implantation was higher in lungs that developed grade 2 or 3 PGD at 48 hours post transplantation (Ai and Bi). Data are displayed as median±IQR and analysed by Mann–Whitney U tests. n=11, *p<0.05. (NB 2 samples were lost from the donor monocyte CD68/TREM-1 analysis due to technical difficulty).

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Contributors Conception and design of the study: KCT, KPO, NM and MT. Human sample acquisition: RR, LT, ARS and POPSTAR investigators. Data acquisition, analysis and interpretation: KCT, KPO, RR, HED, KW, PS, BVP, NM and MT. Writing and revising the article: KCT, KPO, NM and MT.

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