In vivo compartmental analysis of leukocytes in mouse lungs

Brijesh V Patel¹, Kate C Tatham¹, Michael R Wilson¹, Kieran P O’Dea¹, and Masao Takata¹

¹ Section of Anaesthetics, Pain Medicine and Intensive Care, Faculty of Medicine, Imperial College London, Chelsea and Westminster Hospital, London, United Kingdom.

Running Head: Compartmental analysis of mouse lungs

Address correspondence to:

Professor Masao Takata
Anaesthetics, Pain Medicine and Intensive Care
Imperial College London
Chelsea and Westminster Hospital
369 Fulham Road, London, SW10 9NH, UK.
Email: m.takata@imperial.ac.uk
Fax: 44 20 3315 5109, Tel: 44 20 3315 8816
ABSTRACT
The lung has a unique structure consisting of three functionally different compartments (alveolar, interstitial, and vascular) situated in an extreme proximity. Current methods to localize lung leukocytes using bronchoalveolar lavage and/or lung perfusion have significant limitations for determination of location and phenotype of leukocytes. Here we present a novel method using in vivo antibody labelling to enable accurate compartmental localization/quantification and phenotyping of mouse lung leukocytes. Anesthetized C57BL/6 mice received combined in vivo intravenous and intratracheal labelling with fluorophore-conjugated anti-CD45 antibodies, and lung single cell suspensions were analyzed by flow cytometry. The combined in vivo intravenous and intratracheal CD45 labelling enabled robust separation of the alveolar, interstitial, and vascular compartments of the lung. In naive mice, the alveolar compartment consisted predominantly of resident alveolar macrophages. The interstitial compartment, gated by events negative for both intratracheal and intravenous CD45 staining, showed two conventional dendritic cell populations, as well as a Ly6C<sup>lo</sup> monocyte population. Expression levels of MHCII on these interstitial monocytes were much higher than the vascular Ly6C<sup>lo</sup> monocyte populations. In mice exposed to acid-aspiration induced lung injury, this protocol also clearly distinguished the three lung compartments showing the dynamic trafficking of neutrophils and exudative monocytes across the lung compartments during inflammation and resolution. This simple in vivo dual labelling technique substantially increases the accuracy and depth of lung flow cytometric analysis, facilitates a more comprehensive examination of lung leukocyte pools, and enables the investigation of previously poorly defined ‘interstitial’ leukocyte populations during models of inflammatory lung diseases.
KEY WORDS:

Alveolus
Lung interstitium
Pulmonary capillary vasculature
Flow cytometry
Leukocyte trafficking
INTRODUCTION

Resident alveolar and interstitial leukocytes (6, 43) as well as infiltrating blood-derived leukocytes (3, 37, 47) play pivotal roles in the lung during health and disease. However, current experimental techniques in animal models do not allow discrimination between the precise locations of these cells across different lung compartments, thereby significantly limiting our understanding of pathophysiology. For instance, blood leukocytes start to play fundamental roles in the evolution of inflammatory lung diseases when they are margined/sequestered within the lung microvasculature (6, 26, 47), and subsequently show distinct intercellular interactions during transendothelial and transepithelial migration (15, 25, 38, 59). As the majority of investigations do not separate alveolar, interstitial, vascular-margined, and blood leukocyte populations, precise phenotype changes and functions of leukocytes as they migrate through each of the different lung compartments remain unknown.

Many investigations assume lavage of the alveolar space or flushing of the pulmonary vasculature removes almost all alveolar and vascular cells, respectively. However, this is indeed not the case. Considerable portions of resident alveolar macrophages still remain within the lung even with multiple aggressive lung lavages using EDTA (5, 14). Similarly, flushing the vasculature of the lung was found to leave behind a substantive number of cells that are margined and still attached to the endothelium (2, 56), making the differentiation between lung interstitial and intravascular spaces very difficult. In addition, vigorous lung perfusion has been shown to have a negative impact on the preservation of lung architecture (2). Hence, these protocols do not allow a reliable and accurate assessment of cell localization and number. More recently, imaging techniques such as intravital microscopy have been utilized to investigate
interstitial leukocytes, but these techniques only allow the labelling of 2 or 3 cellular epitopes limiting the examination of complex cellular phenotypes (8, 26). Furthermore, their application is limited to the sub-pleural areas, which may not be representative of the whole lung (8, 27, 52). Morphometric analysis (e.g. light and electron microscopy) remains subjective, semi-quantitative and is time consuming.

The aim of this study is to develop and validate an in vivo antibody labelling methodology to enable the flow cytometric separation of leukocyte subsets into the various lung compartments. A number of previous studies have utilized intravenous staining protocols to define vascular versus tissue leukocyte population in various organs (2, 4, 47), but such techniques do not allow clear identification of the interstitial compartment within the lungs. Here we utilize, for the first time, a simultaneous in vivo dual intratracheal and intravenous administration of anti-leukocyte antibodies to positively discriminate the alveolar and vascular cells, and through negative gating define the interstitial compartment. This protocol allows the identification, quantification and classification of leukocytes into three compartments of the mouse lung, in particular, the elusive lung interstitium. This simple yet powerful experimental approach will provide researchers the opportunity to further explore biological and immunological mechanisms important in not only the diverse range of inflammatory lung diseases but also in the maintenance of lung homeostasis during health.
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. Wild-type C57BL/6 mice (Charles River, Margate, UK) aged 10–12 weeks and weighing 25-30g were used for all experiments. We first developed the dual in vivo labeling technique using uninjured mice, and then tested whether this method could be used to chase leukocyte trafficking across the lung compartments in disease states using mice exposed to acid-induced lung injury.

In vivo antibody labelling. Animals were anesthetized by intraperitoneal injection of xylazine (8mg/kg) and ketamine (80mg/kg) and underwent tracheostomy whilst spontaneously breathing on FiO₂ 1.0. The right external jugular vein was cannulated and 2µg of a PE-conjugated anti-CD45 antibody (diluted in 100µl sterile PBS) was administered, together with heparin (10iu/g) for systemic anticoagulation. After five minutes, allowing for the circulation of the antibody, animals were exsanguinated. The thoracic cavity was exposed through a midline sternotomy and the lungs gently teased from their pleural adhesions. Subsequently, 5-0 silk sutures were placed carefully around the right and left main bronchi. One or both lungs underwent instillation (through the tracheostomy) with 0.5ml or 1ml sterile PBS, respectively, containing 2µg/ml of a PE-Cy7 anti-CD45 antibody. Each suture was securely tied at the hilum to avoid spillage of the intratracheal antibody, and lungs removed from the thoracic cavity. These ligated lungs were exposed to the intratracheal antibody for 5 minutes after which they underwent processing for preparation of single cell suspension.
Preparation of single cell suspension. Lungs underwent mechanical disruption using a GentleMacs tissue dissociator (Miltenyi Biotec) for 1 minute in 2mls of intracellular fixative (eBioscience). This technique allows immediate fixation and dissociation enabling the best stability of epitopes (39, 43). The fixation reaction was stopped through the addition of 20mls of ice-cold flow cytometry wash buffer (FWB - PBS with 2% FCS, 0.1% sodium azide, and 5mM EDTA). The suspension was subsequently sieved through a 40µm nylon filter and washed again with ice-cold 20mls FWB. The lung cell suspension was centrifuged at 4°C at 2000rpm. The cell pellet was resuspended in 1ml of FWB and placed on ice.

In vitro antibody staining. This was performed by incubation of lung single cell suspension (or whole blood for reference of cell identification) with fluorochrome-conjugated antibodies for 30 minutes in the dark at room temperature. The following panel of antibodies for myeloid cell markers was used: CD45 (30-F11; BioLegend); CD11c (N418, eBioscience); CD11b (M1/70; BD Biosciences); F4/80 (Cl:A3-1; Biolegend); CD103 (2E7; Biolegend); MHC II (M5/114.15.2; Biolegend); Ly6G (RB6-8C5; BioLegend); Ly6C (AL-21, Becton Dickinson Biosciences). A CD4 antibody (GK1.5, BioLegend) was used for T-cell identification. After incubation 4mls of FWB was added to lung cell suspension or 4mls BD LyseFix solution to whole blood and samples centrifuged at 2000rpm at 4°C for 5 minutes. Cells were resuspended in FWB and analyzed using a 7-channel CyAn ADP flow cytometer (Beckman Coulter). Prior to analysis the addition of AccuCheck counting beads (Invitrogen) enabled cell counts to be calculated. Data were analyzed using FlowJo software (Treestar).

Isolated perfused lung (IPL). The efficacy of lung perfusion for the recovery of vascular
leukocytes was evaluated using the mouse IPL model previously established in our laboratory
(18, 40, 56). In brief, anesthetized animals were injected with intravenous anti-CD45 antibody
(with heparin to prevent intrapulmonary coagulation), exsanguinated and placed on the IPL
apparatus (Isolated Perfused Lung Size 1 Type 839; Hugo-Sachs Elektronik, March-Hugstetten,
Germany). After tracheostomy and thoracotomy, the pulmonary artery and left atrium were
cannulated, and lungs were ventilated (tidal volume 7ml/kg, PEEP 5 cmH2O, respiratory rate
80/min, with 21% O2 and 5% CO2 in N2) and perfused by RPMI 1640 without phenol red with
4% BSA in a non-recirculating manner, at a rate of 25ml/kg/min with an left atrial pressure of
2.5 mmHg. Experiments were terminated at 1, 15 or 60 min, washed-out cells during this
perfusion period were collected from the total IPL perfusate, and lung single cell suspensions
were also prepared from the post-perfusion lungs. Both samples were analyzed by flow
cytometry, and the numbers of vascular neutrophils labeled with in vivo administered anti-CD45
antibody were quantified.

Acid-induced lung injury. The in vivo labelling procedure underwent testing in our recently
published mouse model of acid-induced lung injury (44). In brief, anesthetized animals were
vertically suspended on a custom made mount from their incisors using a 2/0 suture. A non-
thermal light source was used to transilluminate the trachea. Careful retraction of the tongue and
laryngoscopy using blunt curved forceps allowed a grade 1-2 view of the vocal cords. A fine
catheter was subsequently guided 1cm below the vocal cords and 75µl of an isoosmolar 0.1M
hydrochloric acid (pH 1.0) solution was instilled. Animals were left suspended for one minute,
and then placed in a custom-made mouse high dependency recovery area. During this recovery
period they were actively warmed and given humidified supplemental oxygen, with FiO2 initially
at 1.0 and then gradually reduced to 0.21 over 4 hours. To ensure even distribution of the instillate, mice were rotated every 5 minutes until spontaneously moving. After this recovery period they were placed in isolated ventilated cages with air and free access to food and water for up to 4 days.

Statistical analysis. Data are expressed as means±SD. The model assumption of normality of residuals was assessed by QQ plot and the Shapiro–Wilk test. Statistical analyses of data were made using either a two-tailed Student t test or one-way ANOVA with Bonferroni tests for intracompartmental comparisons between multiple time-points (if parametric) and Mann–Whitney/Kruskal–Wallis tests (if nonparametric). Analyses of normal distribution were performed using SPSS version 20 (IBM) and statistical comparisons used GraphPad Prism (version 6.0f). A p-value of less than 0.05 was considered significant.
RESULTS

Validation of compartmental staining protocol

To investigate the compartmental localization of leukocytes, lungs were labeled ‘in vivo’ through administration of an intravenous (PE-conjugated) followed by intratracheal (PE-Cy7 conjugated) anti-CD45 antibody in a step-by-step fashion (figure 1). Firstly, in the absence of intravenous and intratracheal labelling of the lung, events (after exclusion of debris using low forward scatter) of lung single cell suspensions analyzed by flow cytometry are all negative in the PE and PE-Cy7 channels (figure 1A). The in vivo intravenous administration of a PE-conjugated anti-CD45 antibody produces a clearly separate population of CD45-positive cells (figure 1B – R1), which we define as cells within the ‘vascular’ compartment, leaving a population of unlabeled non-vascular events (figure 1B – R2). Subsequently, this lung cell suspension (from animals given intravenous PE-conjugated CD45 antibody) was incubated in vitro with a PE-Cy7-conjugated CD45 antibody (figure 1C). As expected, the vascular population (R1) also stained positive for in vitro CD45 (R3). This also led to a population of CD45 positive cells (R4) emerging from the non-vascular gate (R2) leaving behind an unstained non-leukocyte CD45 negative population (R5). Finally, in vivo instillation (instead of in vitro incubation) of the PE-Cy7 conjugated CD45 antibody into the airways, following in vivo intravenous PE-conjugated antibody, stained a population of cells positive for intratracheal CD45 and negative for the intravenous CD45 (figure 1D – R6), which are highly likely to be resident alveolar macrophages. The intravascular population remains clearly defined (figure 1D – R7). The population that are dual ‘negative’ and not accessible to either the intratracheal or intravenous administered CD45 antibodies (figure 1D – R8), are likely to contain the interstitial leukocytes (as well as all other lung parenchymal cells). Importantly, there were virtually no dual
positive events (figure 1D – R9) suggesting minimal bidirectional leak of in vivo antibody staining between compartments. Importantly, the use of bi-exponential scaling (22, 42) provides a clear and distinct separation between alveolar (R6), vascular (R7) and interstitial (R8) populations.

**Distinguishing between lung alveolar and interstitial compartments**

Analysis of the alveolar (PE-Cy7 CD45pos) compartment (i.e. intratracheal antibody positive and intravenous antibody negative) revealed that a CD11cposCD11bneg population accounted for over 95% of total alveolar events (Figure 2B – gate A1). This population was also highly autofluorescent, MHCIIneg and CD103neg, and F4/80pos and SiglecFpos, identifying them as resident alveolar macrophages, consistent with previous literature (6, 23, 32, 33). The remaining 5% of leukocytes within the alveolar compartment consisted of CD11cposCD11bnegMHCIIposCD103pos (2%) and CD11cposCD11bposMHCIIposCD103neg (2%) cells which are likely to be dendritic cell populations as described below, and CD11bposLy6Gpos neutrophils (1%).

Within the interstitial compartment (i.e. double negative for intratracheal PE-Cy7 and intravenous PE antibodies), there were two CD11cpos leukocyte populations that can be clearly distinguished by CD11b expression (figure 2C - B1 and B2). These are both conventional dendritic cell (cDC) populations as they have very high expression of MHCII. The two cDC populations can be further characterized by differences in CD103 expression and this has previously been shown to determine their specific location within the lung – intra-epithelial versus lamina propria (28, 55). Population B1 is CD11bnegCD103pos (intra-epithelial cDC)
whereas population B2 is CD11b\textsuperscript{pos}CD103\textsuperscript{neg} (lamina propria located cDC). Furthermore, in contrast to resident alveolar macrophages, these DC populations have low or negligible F4/80 and SiglecF expression. The intra-epithelial type cDC also shows slightly greater CD86 expression. In summary, the intratracheal labelling strategy enables us to clearly separate and distinguish between ‘alveolar’ resident macrophages and ‘interstitial’ resident dendritic cell populations. Table 1 describes an overview of basic phenotypic characteristics of myeloid cells within the three compartments of the mouse lung.

Distinguishing between lung interstitial and vascular compartments

The in vivo labelling technique showed that virtually all CD11c\textsuperscript{neg}CD11b\textsuperscript{pos} events are situated within the interstitial or vascular compartments, with minimal CD11c\textsuperscript{neg}CD11b\textsuperscript{pos} events (<1% of alveolar events) within the alveolar space in healthy uninjured mouse lungs (figure 3A). The CD11c\textsuperscript{neg}CD11b\textsuperscript{pos} leukocytes, within the interstitial compartment (figure 3B), consisted of a predominant population of Ly6C\textsuperscript{lo} monocytes (CD11c\textsuperscript{neg}CD11b\textsuperscript{pos}Ly6G\textsuperscript{lo}Ly6C\textsuperscript{lo} cells, >95%; B2) with the suggestion of a minor neutrophil presence (CD11c\textsuperscript{neg}CD11b\textsuperscript{pos}Ly6G\textsuperscript{hi}Ly6C\textsuperscript{hi} cells, less than 5%; B1). In contrast, the vascular compartment (figure 3C) showed greater proportion of neutrophils (C1; 35%) as well as a more varied proportion of monocyte subsets: Ly6C\textsuperscript{lo} (C3) – 25%; Ly6C\textsuperscript{inter} (C4) – 10%; and Ly6C\textsuperscript{hi} (C5) – 25%. Given the limited number of flow cytometry channels a complete exclusion of NK cells and eosinophils was not possible, but eosinophils (occupying approximately 5% of events) are likely to be represented in gate C2 (CD11c\textsuperscript{neg}CD11b\textsuperscript{pos}Ly6G\textsuperscript{inter}Ly6C\textsuperscript{inter} cells) (48).

Finally, interstitial Ly6C\textsuperscript{lo} monocyte populations were found to have a significantly higher
expression of MHCII compared to their vascular Ly6C\textsuperscript{lo} monocyte counterparts (figure 3D). This clear separation in MHCII phenotype between interstitial and vascular Ly6C\textsuperscript{lo} monocytes confirms minimal leak of intravenous antibody into the interstitial space in the uninjured lung, further supporting the case that this in vivo labelling protocol allowed robust differentiation between cells within the vascular and interstitial compartments of the lung.

Compartmental analysis increases accuracy of anatomical location.

Current methods to analyze the anatomical location of leukocytes rely on both lung lavage (to remove alveolar cells) and pulmonary vascular perfusion (to remove vascular cells) with subsequent analysis of the lung homogenate (for interstitial cells) (5, 51). Thus, a separate set of experiments was performed to assess the efficacy of lung lavage for retrieval of alveolar macrophages (figure 4A). Uninjured mouse lungs underwent five repetitive lavages via the endotracheal tube using one syringe containing 750\mu l normal saline. The recovered lavage fluid and lung single cell suspensions prepared from the remaining post-lavage lungs were analyzed by flow cytometry. The numbers of resident alveolar macrophages (CD45\textsuperscript{pos}CD11c\textsuperscript{pos}CD11b\textsuperscript{neg}F4/80\textsuperscript{pos}) were 4.5±2.4x10\textsuperscript{4} in the recovered lavage fluid versus 9.4±1.5x10\textsuperscript{5} in the lung homogenate. Hence, the overall retrieval of resident alveolar macrophages by this standard lavage procedure was found to be only less than 5%.

In another series of experiments, the efficacy of lung vascular perfusion for recovery of vascular leukocytes was evaluated using the IPL model. Mice underwent injection of intravenous anti-CD45 antibody for in vivo labelling of vascular leukocytes, and subsequently underwent lung perfusion for 1, 15, and 60 minutes. The numbers of intravenously labeled CD45 positive
neutrophils were counted in the washed-out cell populations in the IPL perfusate as well as in the remaining lung single cell suspensions prepared from the post-perfusion lungs (figure 4B). The results showed that up to 50% of vascular neutrophils labeled with the intravenous CD45 antibody still remain within the lung tissue even after 60 minutes of lung perfusion (figure 4B).

Application of compartmental analysis to experimental acid-induced lung injury

Analyses of the flux of leukocytes through the various compartments of lungs at various stages of lung injury are crucial to gain better understanding into pathophysiology. The methods described above were applied to our recently published acid aspiration model to gain a preliminary insight into the effectiveness of the compartmental strategy during injury. Animals underwent in vivo labelling at days 1, 2 and 4 after acid aspiration, with day 0 representing uninjured animals. These time-points were predicted to best show influx and efflux/loss of leukocytes during inflammation and its resolution during lung injury (44). Figure 5 shows representative flow cytometry plots for the compartmental gating strategy as discussed previously in the uninjured mouse. The proportions of dual positive events (figure 5 - D1) i.e. $\text{PE}^{\text{pos}}\text{PE-Cy7}^{\text{pos}}$, representing the extent of bidirectional leak of antibodies across the alveolar capillary barrier, remains unchanged (at <0.5%) between uninjured and injured animals even during significant alveolar edema at days 1 and 2. Indeed, we have previously shown that this acid induced model produces a greater than 60% increase in lung water content with significantly increased lavage protein concentrations at days 1 and 2 (44). Hence, this protocol allows consistent separation and gating of the three compartments in a model with severe disruption of the alveolar-capillary barrier.
Figure 6 shows the CD11c and CD11b characteristics of leukocytes after compartmental gating of the lungs as injury progresses. In the uninjured mouse lung, there are minimal numbers of alveolar CD11b^{pos} events and vascular CD11c^{pos} events. The alveolar compartment contains alveolar macrophages and some dendritic cells populations with no monocytes and negligible numbers of neutrophils. The interstitial compartment contains the two conventional dendritic cell populations and the CD11c^{neg}CD11b^{pos}Ly6C^{lo}MHCII^{pos} subset of interstitial monocytes (as in figure 2). Immediately after acid aspiration, small numbers of CD11b^{pos} events are seen to enter the alveolar space as early as 3 hours (not shown) and at day 1. However, the predominant CD11b^{pos} infiltration into the alveolar and interstitial compartments occurs on day 2.

Figures 7 shows a more focused analysis of CD11b^{pos} subpopulations within each compartment using Ly6G and Ly6C staining, while Figure 8 shows a quantification of the dynamics of neutrophil and monocyte influx and efflux within the alveolar and interstitial lung compartments. There is an increase of Ly6G^{pos} neutrophil populations at 24 hours of acid-induced lung injury in both alveolar and interstitial compartments. This neutrophil infiltration peaks at day 2, and at the same time we also see a large infiltration of CD11c^{neg}CD11b^{pos}Ly6G^{lo} monocyte-like cells, with a phenotype consistent with so-called ‘monocyte-derived exudative macrophages’ (19), which we term ‘mono-mac’ (figure 8A and B). At day 2, this mono-mac infiltrate consists initially of Ly6C^{inter/hi} populations within the alveolar and interstitial spaces. In contrast, by day 4, the Ly6C^{inter/lo} mono-mac events remain constant in number whereas the alveolar and interstitial Ly6C^{hi} populations disappear. Of note there are initial reductions in CD11c^{pos}CD11b^{neg}F4/80^{pos} alveolar macrophage populations at 24 hours (figure 8C). Interestingly, the number of these CD11c^{pos}CD11b^{neg}F4/80^{pos} cells increases three-fold (as compared to uninjured numbers) on day
2 during the insurgence of these exudative mono-macs. As previously described, it is from day 2 onwards that injury resolves within this model (44), and coincident with this are reductions in lung neutrophils (likely through apoptosis).

To further characterize changes in leukocyte populations within the alveolar and interstitial compartment during resolution, we focused on CD11b<sup>neg</sup>CD103<sup>pos</sup> dendritic and CD4<sup>pos</sup> T-cell populations (figure 9). As expected, in the uninjured lung there are significant proportions of interstitial CD4<sup>pos</sup> T-cells. During acid-induced lung injury there were significant reductions in CD103<sup>pos</sup> dendritic cell populations from day 1 of injury, which persisted throughout the injury period studied. Additionally, there were significant increases in alveolar CD4<sup>pos</sup> T-cell populations on day 2 during mono-mac migration into the lung.
In this study we developed and validated a novel in vivo labelling method that enables an in-depth analysis of leukocyte location and phenotypes within the alveolar, interstitial, and vascular compartments of the mouse lung by flow cytometry. The protocols described here enable the positive identification of leukocytes within the lung vascular and alveolar compartments, and through a negative gating strategy the identification of the interstitial compartment. Combining this in vivo labelling strategy with in vitro use of a panel of multiple leukocyte surface markers, we achieved robust differentiation and identification of various leukocyte populations across three lung compartments in both the uninjured and injured mice. We showed that CD11c and CD11b are very useful global discriminators of myeloid cell populations in various compartments of the mouse lung. We confirmed clear separation between the alveolar and interstitial compartments through establishing distinct locations of resident alveolar macrophages (CD11c\textsuperscript{pos}CD11b\textsuperscript{neg}MHCII\textsuperscript{neg}CD103\textsuperscript{neg} F4/80\textsuperscript{pos}SiglecF\textsuperscript{pos} events) and two interstitial dendritic cells (CD11c\textsuperscript{pos}CD11b\textsuperscript{neg} MHCII\textsuperscript{pos}CD103\textsuperscript{pos} and CD11c\textsuperscript{pos}CD11b\textsuperscript{pos}MHCII\textsuperscript{pos}CD103\textsuperscript{neg} events). An important distinction was found between the interstitial and vascular CD11c\textsuperscript{neg}CD11b\textsuperscript{pos}Ly6G\textsuperscript{lo}Ly6C\textsuperscript{lo} monocyte populations, with the former showing significantly higher MHCII expression, similar to dendritic cells. On application to a clinically-relevant mouse model of lung injury with substantive alveolar-capillary disruption, we presented the validity of this protocol and described, for the first time, the dynamics of leukocyte populations within the various lung compartments during inflammatory and resolution phases of acute lung injury.

Current methods for compartmental analyses of lungs have critical limitations for determination of location and phenotype of leukocytes. Despite recent advances in lung biology research, there
has been no study to accurately and reliably address the precise numbers and phenotypes of leukocytes within each lung compartment (5, 24, 33, 58), mainly due to the unique morphology of the lung with very thin alveolar-capillary structures, which significantly limits the application of traditional histological techniques. In particular, investigation into the interstitial compartment of the lung has long proven elusive. Johnston et al performed cardiac perfusion and lavage procedures to determine which cells were present within the lung interstitium (24). However, we have demonstrated that standard lung lavage does not retrieve the majority of cells from the alveolar space. More aggressive lavage procedures (e.g. using EDTA) would damage the lung parenchyma, leading to contamination of cells from the interstitial and vascular spaces.

Importantly, after the onset of lung injury the recoverability of alveolar macrophages would substantively worsen as a consequence of increased activation and adherence, a concept called ‘the macrophage disappearance reaction’ (7, 20, 34). Our results have also shown that lung perfusion does not remove all of neutrophils from the pulmonary vasculature, consistent with our previous IPL study on monocytes (56), and those marginated leukocytes adhered relatively firmly to the lung endothelium would play a substantial role in the pathophysiology of lung injury (2, 39-41, 56, 57). In addition, lung perfusion following a period of zero pulmonary flow (during surgical preparation) may produce activation of endothelial cells (9, 10), stimulating production of reactive oxygen species (50) and promoting neutrophil adherence/recruitment to the lung (36), which may further reduce the recovery of vascular leukocytes.

Recent studies have also provided panels of markers to distinguish between various myeloid cells within the lung (1, 5, 24, 33, 58). However, such panels do not distinguish the anatomical localization of leukocytes and does not account for the potential changes in phenotypic markers.
during various stages of lung injury. In particular, macrophages show significant plasticity in their cell surface markers during injury and resolution (1). Our in vivo labelling method should supplement these myeloid marker panels but importantly, is not restrictive to myeloid cells, and has the added benefit to also enable the localization of other immune cells e.g. T-cells. Indeed, it is also feasible to perform in vivo labelling with alternative antibodies, or antibody cocktails.

Bedoret et al characterized resident alveolar macrophages, interstitial macrophages, and dendritic cells using immunohistochemistry and extensive lung lavage (6), and showed that alveolar macrophages were $\text{CD}11c^{\text{pos}}F4/80^{\text{pos}}\text{MHCII}^{\text{neg}}$, interstitial dendritic cells were $\text{CD}11c^{\text{pos}}F4/80^{\text{neg}}\text{MHCII}^{\text{pos}}$, and interstitial macrophages were $\text{CD}11c^{\text{neg}}F4/80^{\text{pos}}\text{MHCII}^{\text{pos}}$. The nomenclature of monocytes versus macrophages remains very confusing within the literature. In contrast to the paper by Bedoret, here we have defined mature macrophages as those cells that exhibit clear $\text{CD}11c$ expression within the tissues. Such blood-borne Ly6C$^{\text{hi}}$ monocyte-derived cells can differentiate either into macrophages (or mono-mac) or dendritic cells (mono-DC) (54, 60). Interestingly, these resident interstitial monocytes, unlike vascular monocytes, show a significant increase in CD86 expression within a model of lung ischemia-reperfusion (unpublished results). Taken together with the high expression of MHCII, this suggests a potential important immune modulatory role for these previously ‘invisible’ cells, in particular, a link to adaptive T-cell responses.

It is important to consider the potential confounding factors that would impede the ideal separation of lung compartments in our methodology. The use of negative selection would theoretically more or less ‘overestimate’ the interstitial compartment as 100% labelling by
intratracheal and intravascular antibody would not be possible, e.g. due to some non-
perfused/occluded vessels in the pulmonary vasculature. However, Reutershan et al
intravenously administered a monoclonal antibody (against TER-119 on erythrocytes) and
showed near total labelling of all erythrocytes in the pulmonary microvasculature of C57BL/6
mice (47). Similarly, our intratracheal antibody showed labelling of over 98% of resident
alveolar macrophages within the alveolar space with less than 2% unstained.

On the other hand, if intravenous or intratracheal antibodies inadvertently label cells in other
compartments either in vivo or during sample processing, e.g. unbound excess antibodies left
within the lung samples bind to cells in other compartments, it would ‘underestimate’ the
interstitial compartment. However, such ‘leaks’ of in vivo administered antibodies to other lung
compartments appeared minimal in this study, because double positive events for intratracheal
and intravascular antibodies (expected to occur if significant bidirectional leaks take place) were
always less than 0.5% of total events, and this proportion remained unchanged even in acid-
injured mice with significant alveolar-capillary barrier disruption. Furthermore, the majority of
these double positive events were secondary to autofluorescence of resident alveolar
macrophages.

Several precautions incorporated into our in vivo labeling protocol may have helped to minimize
such leak. Firstly, intravenous CD45 antibody was injected and allowed to circulate for only 5
minutes. In our previous study using the same acid model, when fluorescence-labeled albumin
(with a molecular weight of 66.5kDa, as compared to 160kDa for anti-CD45 IgG) was injected
intravenously to measure permeability index, we found that at least 30 minutes is required to
detect measurable amounts of albumin in lung lavage fluid (43). Thus, within the time frame used in this study, it is unlikely that sufficient amounts of intravenous antibody are translocated into other lung compartments despite a substantively disrupted alveolar capillary barrier. Secondly, mice were exsanguinated (bled from inferior vena cava under systemic anticoagulation) and their lungs were inflated for 5 minutes with a positive alveolar pressure (with the intratracheal anti-CD45 antibody solution), which enables maximal drainage of pulmonary blood (and excess intravenous antibody). Further removal of excess intravascular antibody can be obtained by flushing the pulmonary vasculature, although this was not performed in this study to maintain lung architecture as intact as possible. Thirdly, to minimize the binding of any remaining unbound intratracheal antibody (left within the alveoli and bronchial tree), steps were implemented, including homogenization of the lung in the largest possible fluid volume followed by immediate dilution into 20mls of ice-cold flow cytometry wash buffer. Drainage of excess antibody out of the tracheobronchial tree was not performed, given the potential for significant loss of cells from the alveolar compartment. An alternative method would be to homogenize the lung in excess non-conjugated anti-CD45 antibody to saturate the CD45 epitope, preventing further conjugated antibody binding.

The compartmental analysis enables a simple yet dynamic insight into the kinetics of leukocyte migration within the lung during inflammatory pathology. Acid-induced lung injury induced gradual increases in CD11b positive events mainly on day 2, consistent with our previous work (44). In addition to neutrophils, we found a dual neutrophil and monocyte recruitment into the lung interstitium at this time-point, as has been suggested in an endotoxin model of lung injury (17). There are also significant increases in Ly6c\textsuperscript{hi} monocytes within blood at this stage (data not
shown) coinciding with their substantial increase in the interstitial and alveolar compartments. Interestingly on day 4, this infiltrating Ly6c\(^{hi}\) monocyte population adopts a Ly6c\(^{lo}\) phenotype within the interstitium whereas it adopts a Ly6c\(^{inter}\) phenotype within the alveolar space. Hence, these data are consistent with the notion that exudative Ly6c\(^{hi}\) mono-macs could serve to replenish resident populations (dendritic cells and resident alveolar macrophages are respectively Ly6c\(^{lo}\) and Ly6c\(^{inter}\)) within the lung after injury (29, 32). This is also suggested by the increase in numbers of resident alveolar macrophages on day 2 after their initial reduction on day 1. Landsmann et al showed that Ly6c\(^{hi}\) monocytes could give rise to alveolar macrophages after having adopted a Ly6c\(^{lo}\) phenotype, potentially explaining the sustained alveolar presence of Ly6c\(^{lo}\) monocytes (29).

Janssen et al compared dynamics of resident and recruited macrophages in model of endotoxin-induced lung injury (23). To place this into context with our study, which shows an increase in the CD45\(^{pos}\)CD11c\(^{pos}\)CD11b\(^{neg}\)F4/80\(^{pos}\) alveolar macrophage pool, the Janssen study also showed a small increase in resident alveolar macrophages after a dramatic increase in recruited macrophages. However, their study only analyzed cells within bronchoalveolar lavage and therefore, should have substantially underestimated the precise numbers of resident and recruited macrophages within the alveolar compartment. Furthermore, it is important to consider the relative efficacies of retrieval by lung lavage between various immune cell types (e.g. resident versus monocyte-derived exudative macrophages; newly recruited versus apoptotic exudative macrophages) and between various phases of injury (e.g. inflammatory versus resolving phases). Our protocol of in vivo labelling removes many of these potential sources of error intrinsic to methods relying on lung lavage.
On day 4, our data show reductions in Ly6<sup>hi</sup> monocytes with concomitant increases in Ly6<sup>lo</sup> monocytes, consistent with evidence in other models of inflammatory resolution of a phenotypic switch from Ly6C<sup>hi</sup> to Ly6C<sup>lo</sup> (11, 46). During sterile injury, this switch from inflammatory Ly6C<sup>hi</sup> to resolving Ly6C<sup>lo</sup> mono-mac phenotypes would imply an increased phagocytosis potential and hence, could explain the reductions in neutrophils (through apoptosis and efferocytosis) within the alveolar space (11). Plantinga et al (45) showed that monocytes rapidly downregulate Ly6C on differentiation to mono-DCs within the lung and suggest this to be a highly specific effector of T-cell responses. Simultaneously with this transition in mono-mac Ly6C phenotype, we also found increases in CD4<sup>pos</sup> T-cells within the interstitium at day 2. Furthermore, circulating Ly6C<sup>hi</sup> monocytes have been shown to differentiate into fibrocytes under the influence of CD4<sup>pos</sup> T-cells (35), and Ly6C<sup>hi</sup> monocytes have been implicated in driving fibrosis within a bleomycin-induced lung injury model (21). Interestingly, our model of acid aspiration produces significant fibrosis and collagen deposition from day 2 (44). Whether the highly immunomodulatory subset of T-cells (CD4<sup>pos</sup>CD25<sup>pos</sup>FoxP3<sup>pos</sup>) affect such changes in mono-mac Ly6C phenotype and ultimately resolution of inflammation (12, 13) has yet to be determined.

The complex interactions between innate and adaptive immune responses facilitated by dendritic cells have been of considerable interest in numerous mucosal surfaces including the gut and skin (30, 54). Indeed, CD11b<sup>neg</sup>CD103<sup>pos</sup> intra-epithelial cDCs bind to epithelial cells using the α<sub>e</sub>β<sub>7</sub> integrin CD103, the only known ligand for the epithelial integrin e-cadherin (31, 53). Their early loss during acid-induced lung injury may reflect the significant epithelial injury induced within
this model (44). Indeed, these CD103$^{\text{pos}}$ cDCs have been shown in the gut epithelium to initiate
adaptive immune responses in local lymph nodes (49) and within the lung to transport apoptotic
cell associated antigens to draining lymph nodes (16). A limitation of our study was the
restriction to a 7-channel flow cytometer, and only 5-channels after dual intravenous and
intratracheal labelling. Additional channels to differentiate CD11b$^{\text{pos}}$CD103$^{\text{neg}}$ dendritic cell
populations from infiltrating CD11b$^{\text{pos}}$ mono-DCs using antibodies against CD68 or MAR-1
would have further increased the depth of our analysis (45). More detailed compartmental
analysis of the lungs could potentially yield invaluable insights into the roles of such leukocyte
interactions and transitions during inflammation and its resolution.

In conclusion, we present an in vivo antibody labelling methodology to analyze the subsets of
myeloid cells within the three compartments of the lung. This protocol takes full advantage of
the multi-parameter analysis capabilities of flow cytometry and enables robust discrimination of
the location and phenotype of leukocytes within healthy and injured mouse lungs. Combining
this protocol with more recent myeloid cell panels and technologies such as conditional/inducible
gene modification would substantively facilitate investigations into trans-compartmental
trafficking of leukocytes within the lungs. We propose application of this simple in vivo lung
compartmental analysis to other lung biology research to discover novel insights into the roles of
leukocytes within the lung.
ACKNOWLEDGEMENTS

Supported by the Wellcome Trust, UK (#092851), National Institute of Academic Anaesthesia, UK (BJA/RCoA project grant) and an Academy of Medical Sciences (UK) Starter Grant for Clinical Lecturers.
REFERENCES


Figure 1. (A) The absence of antibodies shows a clear negative population of cells when lung single cell suspension is analyzed with the PE (x-axis) and PE-Cy7 (y-axis) channels. (B) The in vivo intravenous (IV) injection of a PE conjugated CD45 antibody leads to a clear shift rightward of an intravascular population (R1) leaving a non-vascular region (R2). (C) Subsequent labeling of whole lung single cell suspension in vitro with a PE-Cy7 conjugated CD45 antibody leads to an upward shift of all leukocyte populations. Of note, all intravenously CD45 labeled leukocytes also stain positive for exogenous CD45 antibody (R3). Additionally, a population of cells emerges from the intravascular CD45 negative group which also express CD45 (R4). These are likely to be lung alveolar and interstitial leukocyte populations. (D) When the same PE-Cy7 conjugated CD45 antibody is instilled intratracheally (IT), one population is labeled strongly positive (R6) and is likely to contain alveolar macrophages. The vascular compartment (R7) remains negative for the intratracheal antibody. The events that are dual negative for both intravenous and intratracheal CD45 antibodies is likely to contain interstitial leukocyte populations (R8). The number of events found in R9, representing the extent of bidirectional leak of antibody, is less than 0.5% of the total population. This confirms that the in vivo labeling enables separation of the alveolar, interstitial, and vascular compartments.

Figure 2. Distinguishing between the alveolar and interstitial compartments of the lung. (A) In vivo labeling enables separation of the alveolar and vascular compartments from the rest of the lung homogenate. The interstitial compartment is dual negative to both intravenous and intratracheal CD45 antibodies. (B) Gating on the alveolar compartment shows that it predominantly contains a resident alveolar macrophage population (A1: red gate) which are
relatively MHCII<sup>neg</sup>, CD103<sup>neg</sup>, F4/80<sup>pos</sup> and SiglecF<sup>pos</sup>. (C) The interstitial compartment (defined by those events not labeled by the intratracheal (PE-Cy7 CD45) and intravenous (PE CD45) antibodies) contains two populations of dendritic cells which are CD11c<sup>pos</sup> and MHCII<sup>pos</sup>. One population is the intra-epithelial type conventional dendritic cells (cDC): CD11b<sup>neg</sup>CD103<sup>pos</sup> (B1: blue gate) and the second are those cDCs resident in the lamina propria: CD11b<sup>pos</sup>CD103<sup>neg</sup> (B2: green gate). (D) The vascular compartment does not contain CD11c<sup>pos</sup> events and only contains expected CD11b<sup>pos</sup> events which are described in subsequent panels. E) The relative expression of cell surface markers in resident alveolar macrophages (red line); intra-epithelial type cDC (blue line); and lamina propria type cDC (green line) using compartmental analysis.

**Figure 3.** Distinguishing between the interstitial and vascular compartments of the lung. (A) There are no CD11c<sup>neg</sup>CD11b<sup>pos</sup> events in the alveolar space. (B) The interstitium contains a CD11c<sup>neg</sup>CD11b<sup>pos</sup> population which consists of some Ly6G<sup>hi</sup> neutrophils (B1 – <5%) but mainly Ly6C<sup>lo</sup> monocytes (B2 - 95%). (C) The vascular space consists of a much larger population of CD11c<sup>neg</sup>CD11b<sup>pos</sup> events. These are separated into neutrophils (C1 – 35%), eosinophils (also Ly6G<sup>int</sup>Ly6C<sup>int</sup> (C2) – <5%) and monocytes (Ly6C<sup>lo</sup> (C3) – 25%, Ly6C<sup>int</sup> (C4) – 10%, and Ly6C<sup>hi</sup> (C5) – 25%). (D) The interstitial Ly6C<sup>lo</sup> monocytes (B2 – green gate) have a significantly higher MHCII expression compared to the vascular Ly6C<sup>lo</sup> monocyte (V3 - red gate) population. This supports our notion that the compartmental strategy robustly distinguishes between interstitial and vascular compartments of the mouse lung. Data are mean±SD with N=6; **P<0.01 versus vascular subset.

**Figure 4.** (A) The proportions of resident alveolar macrophages retrieved after 5 repetitive lung
lavages using one syringe containing 750µl normal saline. (N=4) (B) The proportions of
neutrophils (labeled in vivo with intravenous anti-CD45 antibody) that are present in the lung
perfusate and remaining post-perfusion lung homogenate after varying periods of lung vascular
perfusion (N=4-6).

Figure 5. Changes in intravenous and intratracheal labelling during acid-induced lung injury.
The compartmental approach maintains a clear separation between the three lung compartments
despite significant barrier disruption during days 1 and 2 of injury. The extent of bidirectional
leak of in vivo applied antibody, as determined by dual positive staining (D1), remains
unchanged (and less than 0.5% of all events) during the course of injury.

Figure 6. Compartmental gating showing changes in CD11b and CD11c events during injury
(between days 0 and 2) and resolution (between days 2 and 4) of acid aspiration. There are
increases in CD11b positive events at day 2 within the alveolar and interstitial spaces. As injury
progresses, resident CD11c^{pos}CD11b^{neg} alveolar macrophages in the alveolar compartment gain
CD11b expression whereas recruited CD11c^{neg}CD11b^{pos} cells gain CD11c.

Figure 7. Changes to CD11b^{pos} subpopulations within each compartment during acid-induced
lung injury. The alveolar and interstitial compartments show an increase in Ly6G^{hi} neutrophils
on day 1 (red arrows) as well as the beginnings of an infiltration of Ly6C^{hi} monocytes into the
interstitial space (orange arrow). This is followed by a marked increase in Ly6C^{hi/inter} exudative
mono-mac populations within the interstitial and alveolar compartment on day 2 (green arrow).
This population changes to a Ly6C^{lo/inter} phenotype on day 4 (blue arrow). There are associated
reductions in neutrophils within the alveolar and interstitial space consistent with the resolution phase of lung injury. The vascular compartment shows a reduction in monocyte numbers on day 1, which normalizes on day 4.

Figure 8. (A) Neutrophil numbers significantly increase on day 2 after acid aspiration. Nearly 50% of all neutrophils are found within the interstitial space at this time point. Interstitial neutrophils may have been newly recruited. (B) There is also a significant increase in monocyte migration into the interstitial and alveolar spaces on day 2. On day 4, whilst the numbers of Ly6C^{lo/inter} monocytes are maintained within the compartments, the numbers of Ly6C^{hi} monocytes reduce significantly in the alveolar compartment. (C) There is a non-significant reduction in resident alveolar macrophage (CD11c^{pos}CD11b^{neg}F4/80^{pos}) numbers on day 1 of injury followed by a three-fold increase in cells (on day 2) that have adopted a similar phenotype to resident alveolar macrophages. This replenishment of CD11c^{pos}CD11b^{neg}F4/80^{pos} cells coincides with the infiltration of exudative monocytes on day 2. The vascular space data has been omitted as this may be subject to variability as a result of differences in exsanguination between each animal. Data are mean±SD with N=3-5/time-point; day 2 versus day 0: *P<0.05 **P<0.01; day 4 versus day 2: #P<0.05 (analysis performed within compartments).

Figure 9. Changes in compartmental (A) CD11b^{neg}CD103^{pos} conventional dendritic cells and (B) CD4^{pos} T-cells during lung injury. There are reductions in dendritic cells possibly suggesting emigration to nearby lymph nodes. There is a significant increase in alveolar CD4^{pos} T-cells on day 2. Data are mean±SD with N=3-5/time-point; *P<0.05 ***P<0.001 versus day 0 (analysis performed within compartments).
Table 1. The phenotypic characteristics of myeloid cell populations in the three compartments of the naïve mouse lung
The absence of antibodies shows a clear negative population of cells when lung single cell suspension is analyzed with the PE (x-axis) and PE-Cy7 (y-axis) channels. The in vivo intravenous (IV) injection of a PE conjugated CD45 antibody leads to a clear shift rightward of an intravascular population (R1) leaving a non-vascular region (R2). Subsequent labeling of whole lung single cell suspension in vitro with a PE-Cy7 conjugated CD45 antibody leads to an upward shift of all leukocyte populations. Of note, all intravenously CD45 labeled leukocytes also stain positive for exogenous CD45 antibody (R3). Additionally, a population of cells emerges from the intravascular CD45 negative group which also express CD45 (R4). These are likely to be lung alveolar and interstitial leukocyte populations. When the same PE-Cy7 conjugated CD45 antibody is instilled intratracheally (IT), one population is labeled strongly positive (R6) and is likely to contain alveolar macrophages. The vascular compartment (R7) remains negative for the intratracheal antibody. The events that are dual negative for both intravenous and intratracheal CD45 antibodies is likely to contain interstitial leukocyte populations (R8). The number of events found in R8, representing the extent of bidirectional leak of antibody, is less than 0.5% of the total population. This confirms that the in vivo labeling enables separation of the alveolar, interstitial, and vascular compartments.
Figure 2

Distinguishing between the alveolar and interstitial compartments of the lung. (A) In vivo labeling enables separation of the alveolar and vascular compartments from the rest of the lung homogenate. The interstitial compartment is dual negative to both intravenous and intratracheal CD45 antibodies. (B) Gating on the alveolar compartment shows that it predominantly contains a resident alveolar macrophage population (A1: red gate) which are relatively MHCIIneg, CD103neg, F4/80pos and SiglecFpos. (C) The interstitial compartment (defined by those events not labeled by the intratracheal (PE-Cy7 CD45) and intravenous (PE CD45) antibodies) contains two populations of dendritic cells which are CD11cpos and MHCIIpos. One population is the intra-epithelial type conventional dendritic cells (cDC): CD11b-CD103pos (B1: blue gate) and the second are those cDCs resident in the lamina propria: CD11bposCD103neg (B2: green gate). (D) The vascular compartment does not contain CD11cpos events and only contains expected CD11bpos events which are described in subsequent panels. E) The relative expression of cell surface markers in resident alveolar macrophages (red line); intra-epithelial type cDC (blue line); and lamina propria type cDC (green line) using compartmental analysis.
Figure 3

A) Alveolar Compartment:

B) Interstitial Compartment:

C) Vascular Compartment:

D) The interstitial Ly6C<sub>lo</sub> monocytes (B2 – green gate) have a significantly higher MHCII expression compared to the vascular Ly6C<sub>lo</sub> monocyte (V3 - red gate) population. This supports our notion that the compartmental strategy robustly distinguishes between interstitial and vascular compartments of the mouse lung. Data are mean±SD with N=6; **P<0.01 versus vascular subset.

Figure 3

Distinguishing between the interstitial and vascular compartments of the lung. (A) There are no CD11c<sup>neg</sup>CD11b<sup>pos</sup> events in the alveolar space. (B) The interstitium contains a CD11c<sup>neg</sup>CD11b<sup>pos</sup> population which consists of some Ly6G<sup>hi</sup> neutrophils (B1 – <5%) but mainly Ly6C<sup>lo</sup> monocytes (B2 - 95%). (C) The vascular space consists of a much larger population of CD11c<sup>neg</sup>CD11b<sup>pos</sup> events. These are separated into neutrophils (C1 – 35%), eosinophils (also Ly6G<sup>inter</sup>Ly6C<sup>inter</sup> (C2) – <5%) and monocytes (Ly6C<sup>lo</sup> (C3) – 25%, Ly6C<sup>high</sup> (C4) – 10%, and Ly6C<sup>hi</sup> (C5) – 25%). (D) The interstitial Ly6C<sup>lo</sup> monocytes (B2 – green gate) have a significantly higher MHCII expression compared to the vascular Ly6C<sup>lo</sup> monocyte (V3 - red gate) population. This supports our notion that the compartmental strategy robustly distinguishes between interstitial and vascular compartments of the mouse lung. Data are mean±SD with N=6; **P<0.01 versus vascular subset.
Figure 4

(A) The proportions of resident alveolar macrophages retrieved after 5 repetitive lung lavages using one syringe containing 750µl normal saline. (N=4) (B) The proportions of neutrophils (labeled in vivo with intravenous anti-CD45 antibody) that are present in the lung perfusate and remaining post-perfusion lung homogenate after varying periods of lung vascular perfusion (N=4-6).
Changes in intravenous and intratracheal labelling during acid-induced lung injury. The compartmental approach maintains a clear separation between the three lung compartments despite significant barrier disruption during days 1 and 2 of injury. The extent of bidirectional leak of in vivo applied antibody, as determined by dual positive staining (D1), remains unchanged (and less than 0.5% of all events) during the course of injury.
Figure 6

Compartmental gating showing changes in CD11b and CD11c events during injury (between days 0 and 2) and resolution (between days 2 and 4) of acid aspiration. There are increases in CD11b positive events at day 2 within the alveolar and interstitial spaces. As injury progresses, resident CD11c\(^{pos}\)CD11b\(^{neg}\) alveolar macrophages in the alveolar compartment gain CD11b expression whereas recruited CD11c\(^{neg}\)CD11b\(^{pos}\) cells gain CD11c.
Changes to CD11b^{+} subpopulations within each compartment during acid-induced lung injury. The alveolar and interstitial compartments show an increase in Ly6G^{hi} neutrophils on day 1 (red arrows) as well as the beginnings of an infiltration of Ly6C^{hi} monocytes into the interstitial space (orange arrow). This is followed by a marked increase in Ly6C^{hi} exudative mono-mac populations within the interstitial and alveolar compartment on day 2 (green arrow). This population changes to a Ly6C^{lo} phenotype on day 4 (blue arrow). There are associated reductions in neutrophils within the alveolar and interstitial space consistent with the resolution phase of lung injury. The vascular compartment shows a reduction in monocyte numbers on day 1, which normalizes on day 4.
Figure 8

(A) Neutrophil numbers significantly increase on day 2 after acid aspiration. Nearly 50% of all neutrophils are found within the interstitial space at this time point. Interstitial neutrophils may have been newly recruited. (B) There is also a significant increase in monocyte migration into the interstitial and alveolar spaces on day 2. On day 4, whilst the numbers of Ly6C\textsuperscript{hi} monocytes are maintained within the compartments, the numbers of Ly6C\textsuperscript{lo} monocytes reduce significantly in the alveolar compartment. (C) There is a non-significant reduction in resident alveolar macrophage (CD11c\textsuperscript{pos}CD11b\textsuperscript{neg}F4/80\textsuperscript{pos}) numbers on day 1 of injury followed by a three-fold increase in cells (on day 2) that have adopted a similar phenotype to resident alveolar macrophages. This replenishment of CD11c\textsuperscript{pos}CD11b\textsuperscript{neg}F4/80\textsuperscript{pos} cells coincides with the infiltration of exudative monocytes on day 2. The vascular space data has been omitted as this may be subject to variability as a result of differences in exsanguination between each animal. Data are mean ±SD with N=3-5/time-point; day 2 versus day 0: *P<0.05 **P<0.01; day 4 versus day 2: ♯P<0.05 (analysis performed within compartments).
Figure 9

Changes in compartmental (A) CD11b^{−/−}CD103^{+} conventional dendritic cells and (B) CD4^{+} T-cells during lung injury. There are reductions in dendritic cells possibly suggesting emigration to nearby lymph nodes. There is a significant increase in alveolar CD4^{+} T-cells on day 2. Data are mean±SD with N=3-5/time-point; *P<0.05 ***P<0.001 versus day 0 (analysis performed within compartments).
<table>
<thead>
<tr>
<th>Cell Type</th>
<th>CD11c</th>
<th>CD11b</th>
<th>MHCII</th>
<th>CD103</th>
<th>F4/80</th>
<th>SiglecF</th>
<th>Ly6C</th>
<th>Ly6G</th>
<th>CD86</th>
<th>AF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resident Alveolar Mac</strong></td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td><strong>Interstitial Compartment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-epithelial cDC</td>
<td>+++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lamina Propria cDC</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Interstitial monocyte</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td><strong>Vascular Compartment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classical 'inflammatory' monocyte</td>
<td>-</td>
<td>++</td>
<td>+/−</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Non-classical 'resident' monocyte</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>?</td>
<td>++</td>
<td>+</td>
<td>?</td>
<td>+++</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+++</td>
<td>?</td>
<td>+</td>
</tr>
</tbody>
</table>

**KEY**

- +++/++/+ high/intermediate/low expression
- no expression
- not tested
- auto fluorescence
- conventional dendritic cell

Table 1
The phenotypic characteristics of myeloid cell populations in the three compartments of the naïve mouse lung