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RED BLOOD CELLS ELICIT PLATELET-DEPENDENT NEUTROPHIL RECRUITMENT INTO LUNG AIRSPACES

Stephanie Arnold¹, Sajeel A. Shah², Simon J. Cleary³, Blaze G. O’Shaughnessy¹, Richard T. Amison¹, Kate Arkless¹, Clive P. Page¹ and Simon C. Pitchford^{1*}

¹Sackler Institute of Pulmonary Pharmacology, Institute of Pharmaceutical Science, King’s College London, London, UK.

²Institute for Infection and Immunity, St George’s, University of London, 58 Cranmer Terrace, London, UK.

³Department of Medicine, University of California San Francisco, San Francisco, California, USA.

*Author for correspondence: Dr Simon Pitchford: simon.pitchford@kcl.ac.uk

Authorship: S.A. designed and performed the research, analyzed the data, and wrote the paper. S.A.S., S.J.C., B.G.O’S., R.T.A., and K.A contributed to assay development and performed in vivo procedures. S.C.P., and C.P.P proposed the project, designed research and wrote the paper.

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Running header: Respiratory inflammation by trauma cues is platelet-dependent.

Keywords: Platelets, neutrophils, red blood cells, alveolar haemorrhage, ARDS

Abstract

Haemolysis that occurs in intravascular haemolytic disorders, such as sickle cell disease and malaria, is associated with inflammation and platelet activation. Alveolar haemorrhage, for example following primary blast lung injury (PBLI) or acute respiratory distress syndrome (ARDS), results in the escape of erythrocytes (RBCs) into alveolar spaces, where they subsequently lyse and release their intracellular contents. However, the inflammatory effects of RBCs in the airways are not fully understood. We hypothesized that RBCs in the airway induce an inflammatory response, associated with platelet activation. By instilling whole RBCs or lysed RBCs into the airways of mice, we have demonstrated that whole RBCs elicit macrophage accumulation in the lung. However, lysed RBCs induce significant inflammatory cell recruitment, particularly neutrophils and this was associated with a 50% increase in circulating platelet neutrophil complexes (PNCs). Platelet depletion prior to lysed RBC exposure in the lung resulted in reduced neutrophil recruitment, suggesting that the presence of intracellular RBC components in the airways can elicit inflammation that is platelet dependent. To identify specific platelet dependent signalling pathways involved in neutrophil recruitment, anti-P-selectin ligand and anti-PSGL1 blocking antibodies were tested, however neither affected neutrophil recruitment. These findings implicate an involvement for other, as yet unidentified platelet-dependent signalling and adhesion mechanisms. Further understanding of how platelets contribute to lung inflammation induced by the presence of RBCs could offer novel therapeutic approaches to attenuate inflammation that occurs in conditions associated with alveolar haemorrhage.

Introduction

Alveolar haemorrhage occurs when blood escapes from pulmonary capillaries and leaks into the alveolar spaces (1). Alveolar haemorrhage occurs as a consequence of primary blast lung injury (PBLI), acute respiratory distress syndrome (ARDS) and certain types of pulmonary infection (2) (3) (4). PBLI is characterised by haemorrhagic alveolar regions, occurring due to damage of the alveoli and capillaries by the instantaneous blast wave produced from an explosive device (5). The vascular integrity in ARDS and pneumonia also becomes compromised, due to local release of pro-inflammatory mediators (6) (7) allowing proteins and larger blood components to pass across the endothelial and epithelial barriers, to enter and reside in air spaces (8).

Red blood cells (RBCs) are the most abundant blood cell, with evidence suggesting that RBCs that enter air spaces undergo intrapulmonary haemolysis and subsequently release their intracellular components (9), and the process of haemolysis can be induced by trauma, for example blast wave (10). Furthermore, instillation of the RBC intracellular component cell free heme (CFH) into the lung causes neutrophil recruitment and impaired lung function (9). Studies have shown that CFH acts as a damage associated molecular patterns (DAMP), leading to the activation of toll like receptors (TLRs) on inflammatory cells and epithelial cells (11). CFH can also be scavenged by heme-oxygenase-1 (HO-1), attenuating the pulmonary inflammatory response in preclinical models of PBLI and ARDS (12) (13).

Platelets are small, anucleate, cells that circulate in the blood, originating from megakaryocytes in the bone marrow and, as more recently observed, in the lung (14). In addition to their fundamental function in haemostasis and coagulation, platelets are now recognised as playing a central role in the pathogenesis of various pulmonary inflammatory disease states, demonstrated in animal models of asthma, acute respiratory distress syndrome (ARDS) and pulmonary infection (15). Furthermore, platelets have been strongly associated with the innate immune response and tissue pathology (for example loss of alveolar-capillary integrity) that occurs during traumatic lung injuries (16). Upon

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80 activation during inflammation, platelets upregulate expression of surface proteins such as P-selectin,
81 increasing their ability to undergo selectin dependent binding to neutrophils, forming platelet
82 neutrophil complexes (PNCs) (17). The formation of PNCs leads to integrin mediated binding and
83 increased surface expression of neutrophil adhesion proteins (18). Platelet dependent signalling
84 causes neutrophils to undergo enhanced recruitment towards an inflammatory stimulus, including
85 transmigration across the blood vessel wall from the circulation into tissues (19), where co-localization
86 of neutrophils and platelets is apparent (20).

87 Although alveolar haemorrhage can occur with a pulmonary infection and in ARDS and PBLI, currently
88 little is known about the contribution of RBCs to the airways inflammation resulting from the presence
89 of RBCs in the air spaces. Inflammation caused as a direct consequence of diffuse alveolar
90 haemorrhage can be difficult to assess in models of ARDS and PBLI due to the myriad of inflammatory
91 events that occur. Therefore, we sought to determine whether whole RBCs or lysed RBCs administered
92 to airways can induce an inflammatory response and to assess the importance of platelets in this
93 response.

94 95 **Materials and Methods**

96 *Animals*

97 Animal experiments were approved by King's College London Ethics committee and carried out in
98 accordance with the UK Animals (Scientific Procedures) Act 1986 (United Kingdom) and EU directive
99 2010/63/EU. Animal studies are reported in accordance with the ARRIVE guidelines (21). Female
100 BALB/c mice (18-20g) were used throughout these experiments and were obtained from Charles River
101 (Harlow, U.K.). Mice had access to chow RM1 (E) diet (Special Diets Services) and water *ad libitum*
102 throughout, and were randomly allocated to experimental groups so as not to confer 'cage bias'.
103

Red Blood Cell Preparation

Mice were terminally anaesthetised using 0.25ml urethane (250 mg/ml, i.p) (Sigma, UK). Citrated whole blood was obtained from mice via cardiac puncture as a terminal procedure. Approximately 500µl fresh whole blood was collected per mouse. Blood underwent centrifugation for 1 minute at 1800g to separate components. The platelet rich plasma (PRP) was aspirated and discarded and the remaining RBC pellet was gently mixed with 500µl PBS. RBCs were then further centrifuged for 5 minutes at 1800g and the PBS was aspirated from the RBC pellet. This wash step was repeated twice more. On the final wash, PBS was aspirated to leave a dense RBC pellet. At this stage RBC pellets from multiple donor mice were pooled. A sample of the RBC pellet was diluted in PBS and whole RBCs were quantified using an Improved Neubauer chamber (Hawksley and Sons Ltd) and a brightfield microscope (Leica DM 2000 LED). For each experiment, donor blood was harvested on the same day it was prepared for isolating RBCs and administered to recipient mice. Blood harvested from 1 donor mouse yielded sufficient numbers of RBCs for 4 recipient mice.

Preparing Whole and Lysed Red Blood Cells

The concentration of whole RBCs was assessed prior to haemolysis (as described above). For lysed RBC preparations, RBCs underwent hypotonic lysis with the addition of DI H₂O (1-part RBCs: 2-parts DI H₂O) and vortexed for 10 seconds. The solution was returned to isotonic conditions using 10 X PBS, achieving a final solution of lysed RBCs taken from 5.1-7.3x10⁹ RBCs/ml. No un-lysed RBCs remained after the preparation had been made. Animals that were administered whole RBCs, received the equivalent concentration used in the creation of lysed RBCs for each experiment.

Exposure of Murine Lungs to Red Blood Cells

On day 0, mice were exposed to 50µl lysed RBCs (5.1-7.3x10⁹ RBCs/ml), 50µl whole RBCs (5.1-7.3x10⁹ RBCs/ml), or 50µl PBS intranasally under inhaled isoflurane anaesthesia.

129

130 *Experimental Platelet Depletion in Mice*

131 To investigate the contribution of platelets to the inflammatory response induced by whole or lysed
132 RBCs, 1 hour prior to pulmonary RBC exposure some mice were intravenously dosed with 100µl of an
133 anti-GPIb α antibody (R300, Emfret Analytics, DE) (1mg/kg, i.v), or an IgG control antibody (BE0089,
134 BioXcell, USA) (1mg/kg, i.v), via the tail vein.

135

136 *P-Selectin and PSGL-1 Blocking Experiments*

137 To investigate the mechanisms that contribute towards RBC-induced neutrophil recruitment, 1 hour
138 prior to pulmonary RBC exposure some mice were intravenously dosed with 100µl of an anti-CD62P/P-
139 selectin antibody (553741, BD Biosciences, UK) (4mg/kg, i.v), an anti-CD162/PSGL-1 antibody (BE0186,
140 BioXcell, USA) (4mg/kg, i.v) or an IgG control antibody (BE0089, BioXcell, USA) (4mg/kg, i.v).

141

142 *Blood and BALF Sample Collection*

143 To investigate the inflammatory response following instillation of whole or lysed RBCs into the
144 airways, mice were terminally anesthetized using 0.25ml urethane (250mg/ml, i.p) (Sigma, UK) and
145 whole blood was collected via intracardiac puncture. Following exsanguination, the trachea was
146 exposed and cannulated. Bronchoalveolar lavage fluid (BALF) was collected by gently flushing lungs
147 with 3x0.5ml PBS in 1ml syringes. BALF was stored on ice for the remainder of the experiment.

148

149 *Inflammatory Cell Quantification*

150 To quantify inflammatory cells in blood and BALF, samples were diluted in Turks solution (Sigma, UK)
151 and inflammatory cells were quantified using an Improved Neubauer chamber (Hawksley and Sons
152 Ltd), and a bright field microscope (Leica DM 2000 LED). To identify the types of inflammatory cell
153 present in BALF, 5ul of whole blood was smeared onto a microscope slide. BALF cytopsin slides were
154 created by the addition of 100µl BALF to a cytopsin funnel and centrifugation to transfer BALF onto

microscope slides at 1000rpm for 1 minute (Shandon, UK). Slides were stained with Diff-Quick (ThermoFisher Scientific, UK) for differential cell quantification.

Lung Preparation for Histology

To investigate the histopathological consequences of pulmonary RBC exposure, 24h post exposure the trachea was cannulated, and lungs were inflated *in situ* with 0.5ml 4% formalin (Sigma, UK), excised and submerged in 4% formalin (Fisher, UK) for 24 hours. Left lobes underwent paraffin wax embedding (Citadel, UK). Lungs were sectioned to 7 micrometre thickness using a microtome (Microtom, UK) and loaded onto Superfrost Plus slides (Fisher Scientific, UK).

Histological Staining

For routine histological staining, lung sections were cleared in xylene, rehydrated in decreasing concentrations of ethanol and stained with haematoxy and eosin (Sigma Aldrich, UK). Sections were dehydrated in increasing concentrations of ethanol, cleared in xylene and cover-slipped with DPX. Images were captured using a brightfield microscope (DM2000 LED Bright Field Microscope, Leica Microsystems, UK) attached to a camera (DFC295 Bright Field Microscope Camera, Leica Microsystems, UK).

Detecting Platelets and Neutrophils in Lung Tissue

For immunohistochemistry (IHC), lung sections were cleared in xylene and rehydrated in decreasing concentrations of ethanol before undergoing blocking in 4% hydrogen peroxide (Sigma, UK). Sections underwent antigen retrieval in sodium citrate buffer (pH6.0) for 5 minutes at 100 degrees Celsius. Slides were blocked in 1% BSA before the addition of anti-elastase antibody (ab68672, Abcam, UK) for neutrophil IHC. This was followed by biotinylated anti-rabbit antibody (BA2100, Vector Laboratories, DE) and detected using the ABC detection kit (Vector Laboratories, DE). Sections underwent DAB

development (Sigma, UK) and a haematoxylin counterstain. Sections were dehydrated in increasing concentrations of ethanol, cleared in xylene and cover slipped with DPX (Fisher, UK).

182

183 *Histological Quantification*

To quantify neutrophils, 6 non-overlapping brightfield images were captured per lung section using a 20x objective on a Leica DM upright microscope. Images underwent colour deconvolution and conversion to a binary image using Fiji (ImageJ) using an intensity threshold. Positive events were quantified using size discrimination criteria.

188

189 *Circulating Platelet Neutrophil Complexes*

To measure the number of circulating platelet neutrophil complexes (PNCs), 50µl citrated whole blood was incubated with anti-mouse Ly-6G PE (551461, BD Biosciences, UK) and anti-mouse CD41 FITC (11-0411-82, eBioscience, USA). Blood samples were incubated in the dark for 30 minutes and run on a flow cytometer (Beckman-Coulter FC500, UK) to detect Ly-6G+/CD41+ double positive events on the gated Ly-6G+ population. A CD41+ Ly-6G+ double positive event was considered a PNC.

195

196 *Statistics*

All results are expressed as mean $\pm$ SEM. Group sizes were determined following guidance (22) and predicted power analysis curves generated using inVivoStat software where the number of animals required could be estimated to reach a biological relevant effect at >90% power. Normality was determined for each data set using a Shapiro-Wilk normality test using SPSS (Version 23) or GraphPad Prism (Version 7). Where appropriate, a log or square root transformation was performed for normalisation. Outliers were identified via the ROUT test using Graphpad Prism (Version 7). Significance was determined using either a paired parametric student's t-test, a Kruskal-Wallis test with a Dunn's post hoc test, a one-way ANOVA and a Dunnett's multiple comparisons test, or a two-

way ANOVA with a Sidak's post-hoc test. Data was plotted using GraphPad Prism (Version 7). A P value of <0.05 was considered significant.

Results

Whole or haemolysed RBCs into the airways induce different inflammatory responses

To investigate the inflammatory response following administration of whole or lysed RBCs in the airways, lysed and whole RBC preparations were prepared and instilled into the lungs of mice (**Figure 1A**). Both preparations of RBCs caused acute inflammatory cell recruitment to the airways (whole RBCs $P<0.01$ and lysed RBCs $P<0.05$, **Figure 1B**). In mice exposed to lysed RBCs, neutrophils were the predominant cell type recruited ($P<0.0001$, **Figure 1C**), whereas whole RBC exposure caused macrophage accumulation in the lungs ($P<0.001$, **Figure 1D**). Lysed RBCs in the airways caused weight loss, an effect that was not observed with whole RBC exposure ($P<0.01$, **Figure 1E**). H&E staining of the lungs 6 hours post-dosing reveals that intranasal administration caused whole RBCs and lysates to reside in the larger airways, as well as in distal alveolar spaces (**Figures 1F**). Immunohistochemistry of lungs showed an increase in pro-inflammatory proteinase neutrophil elastase (NE) following lysed RBC exposure, demonstrating neutrophil activation in lung tissue ($P<0.05$, **Figure 1G**).

Lysed RBCs elicit an acute, localised inflammatory response in the lungs.

Evidence suggests that in the disease setting, whole RBCs lyse once in the airway to induce an inflammatory response (9). To fully characterise acute inflammation following airway exposure to lysed RBCs, temporal analyses were performed (**Figure 2A**). Studies revealed that total inflammatory cell numbers were elevated in the lung 24 hours post exposure and returned to baseline at 48 hours ($P<0.001$, **Figure 2B**). Inflammatory cell accumulation was associated with pulmonary neutrophil accumulation at 24 hours ($P<0.0001$, **Figure 2C**) and whilst this remained elevated at 48 hours, had declined in magnitude ($P<0.001$, **Figure 2C**). Neutrophil recruitment correlated with a significant

increase in BALF neutrophil chemoattractant KC/CXCL1 (murine homologue of IL-8) at 6 and 24 hours (P<0.001 and P<0.01 respectively, **Figure 2D**). TNF alpha and IL-6 in BALF was below the limit of detection (LOD) at all time points investigated (data not shown) and there was no change in pulmonary macrophage recruitment at any time point (**Figure 2E**). Furthermore, there was a significant increase in circulating leukocytes, 24 hours after lysed RBC exposure (P<0.01, **Figure 2F**), but no change in circulating platelet numbers was evident (**Figure 2G**).

In other lung inflammation models, neutrophil accumulation is dependent on platelet neutrophil interactions and platelet-dependent processes, and intravascular platelet activation can be quantified using flow cytometry as detection of PNCs. Thus, platelet (CD41) events were gated on neutrophils (Ly-6G) in whole blood (**Figure 3A**). At 24 hours post airway exposure to lysed RBCs, a 50% increase in circulating PNC was detected above basal levels (PBS Control: 22.0±2.5% vs lysed RBC: 34.0±4.0% P<0.05, **Figure 3B**), Whilst there were more PNCs, the average number of platelets (CD41+ events) adhered to each neutrophil (Ly-6G+ events) remained unaltered (**Figure 3C**).

Experimental platelet depletion inhibits neutrophil recruitment following exposure of the lungs to lysed red blood cells

To understand whether the increased incidence of circulating PNC after RBC instillation was indicative of platelet activation contributing to neutrophil recruitment, mice were experimentally depleted of >99% of circulating platelets using an anti-GP-1bα antibody, prior to lysed RBC exposure (**Figures 4A & 4B**, P<0.0001). Blood platelets were quantified 30 minutes after mice received the platelet depletion antibody to ensure thrombocytopenia, and mice remained thrombocytopenic for the remainder of the experiment. Mice depleted of platelets had a significant reduction in inflammatory cells recruited to the lung (P<0.01, **Figure 4C**), coupled with significantly reduced neutrophil recruitment (P<0.05, **Figure 4D**). Despite this, platelet depleted mice still had elevated levels of KC (IL-8) in the lung, comparable to the control group, as evidence that neutrophil chemokines are still prevalent and not a reason for the decrease in neutrophil recruitment (**Figure 4E**). Furthermore, platelet depletion had

no effect on the number of circulating inflammatory cells (**Figure 4F**), and animal health (as assessed by weight) was maintained (**Figure 4G**).

P-selectin or PSGL-1 blockade had no effect on pulmonary neutrophil recruitment

To identify the platelet-dependent mechanisms required for neutrophil recruitment, prior to lysed RBC exposure, some mice were dosed with blocking antibodies to either P-Selectin or PSGL1 (**Figure 5A**). Despite efficacy in controlling neutrophil and inflammatory cell recruitment in other lung inflammation models (18) (20) (23), neither anti-P-selectin nor an anti-PSGL-1 blocking antibody affected recruitment of total inflammatory cells, neutrophils or macrophages into the airway (**Figure 5B, 5C, 5D**).

Discussion

To our knowledge, this is the first side by side comparison study investigating the effects of administering whole or lysed RBCs into the airways. We have demonstrated that airway exposure to these different RBC preparations induces two distinct inflammatory responses.

We have provided evidence that the administration of whole RBCs into the lung induces pulmonary macrophage recruitment, whilst lysed RBCs resulted in a pulmonary neutrophil infiltration. Previous literature indicates that instilling whole blood into mouse airways causes acute neutrophil recruitment (24) (25). Whilst we acknowledge that all the blood components will spill into the airway during alveolar haemorrhage, to determine the specific inflammatory effects of RBCs in the lung we have investigated the contribution RBCs make to the inflammatory effects of alveolar haemorrhage by studying these cells and their lysates separately from other blood components.

Once RBCs have entered the airway lumen following acute lung injury (ALI) or in ARDS, they undergo haemolysis (9). Our research suggests that lysis of RBCs is required to initiate pulmonary neutrophil recruitment. This is likely because once outside of the protective RBC compartment, haemoglobin breaks down into its fractional constituent cell free heme (CFH), which becomes readily oxidised to a

Fe³⁺, pro-inflammatory state (26). Interestingly, following intravascular haemolysis, CFH becomes scavenged by heme-oxygenase-1 (HO-1) and haptoglobin, where the haemoglobin - haptoglobin complex is cleared via CD163 receptors on macrophages. Therefore, it is interesting that within the extravascular compartment of the airway, RBC lysate does not alter macrophage numbers and instead causes neutrophil recruitment, suggestive of a neutrophil-dependent clearance mechanism.

In another in murine model, CFH isolate instilled into mouse lungs resulted in a comparable inflammatory profile, dominated by acute neutrophil recruitment (9). Since CFH activates alveolar epithelial cells in vitro, we speculate that this also occurs in both settings (11). CFH isolate in the airways has been associated with increased CXCL1 levels in the lung (9), although curiously we did not observe changes similar changes in IL-6 or TNF alpha. In contrast, Shaver et al. used a preparation of commercially available CFH, considered by the manufacturer to comprise of methaemoglobin (MetHb). We took advantage of the osmotic sensitivity of RBCs and haemolysed RBCs in a hypotonic solution, immediately prior to administration.

To our knowledge, this is the first time that the inflammatory contribution of platelets has been assessed following airway exposure to lysed RBCs. As with other pulmonary inflammation models, we show that neutrophil recruitment is platelet-dependant (18) (23) (27). Furthermore, in models of sterile lung injury, platelet depletion of just 40% circulating platelets was sufficient to cause a significant reduction in pulmonary neutrophil accumulation in both acid induced and LPS induced inflammation (28). Interestingly, CXCL1 was elevated in the lungs of mice experimentally depleted of platelets, indicating that despite the chemoattractant stimulus still being present, both with the direct instillation of lysed RBC to which neutrophils migrate avidly towards in vitro (data not shown), and endogenously synthesized chemokines, fewer neutrophils were recruited to the airway. This suggests that platelets might be responsible for 1) neutrophil activation and adherence to the endothelium in the circulation and/or 2) neutrophil transmigration into the airway. Further analyses of the production of neutrophil chemokines would be revealing, for example platelet-released platelet factor 4 (PF4:

CXCL4) and β -thromboglobulin (β -TG: CXCL7), or non-protein chemo-attractants, (for example leukotriene B₄ or N-formylated peptides). These analyses could be extended to comparing with the production or absence of macrophage chemokines (e.g. monocyte chemoattractant protein 1, MCP1: CCL2). Increased PNC formation in the blood following RBC lysate exposure suggests that platelets have a fundamental role in intravascular neutrophil activation (17). To reveal the mechanisms that underpin platelet leukocyte interactions, it is possible to modulate selectin dependant signalling pathways. However, in the current experiments, we show that blocking P-Selectin and PSGL-1 did not inhibit the number of neutrophils recruited into lungs. In contrast, in acid induced and LPS- induced lung injury, pulmonary neutrophil recruitment were significantly decreased following platelet specific P-selectin or PSGL-1 inhibition (23, 28) or following pharmacological intervention to suppress purinergic nucleotide activation of platelets (29), or administration of non-anticoagulant heparin that affected P-selectin-dependent platelet-neutrophil interactions (30). Therefore, whilst the lack of inhibition of neutrophil recruitment via blockade of P-selectin and PSGL-1 in this study reflects data published in an LPS model of ARDS (20), we note that other methods of adhesion molecule suppression (e.g. use of transgenic mice deficient in these molecules) could provide an alternative methodology with different characteristics (15). Nevertheless, recruitment patterns and influence of adhesion molecules appear to be dependent on the type of stimulus used to induce lung injury or trauma.

Given the recent observations that patients with COVID-19 have alveolar haemorrhage, 33 out of 38 cases in a publication of pathological findings, and platelet activation associated with neutrophil recruitment, it would also be interesting to investigate the role of platelets in this disease with a unique pattern of ARDS (31) (32). However, platelet activation driving pulmonary inflammation in the model presented here, and the thrombo-inflammatory response observed during SARS CoV-2 infection is difficult to compare. It is interesting to note that haemorrhage induced inflammation, and the roles of platelets in this response draw the apparent contradiction for future clinical considerations that normal haemostasis would need to be promoted, and inhibition of platelet activity (to suppress inflammation) might inadvertently affect haemostasis. Platelet activation in models of

respiratory inflammation has been reported to be distinct to pathways of platelet activation during aggregation and haemostasis (23) (29) (33) (34), emphasizing that a dichotomy in platelet activation exists (35), and future interventions may be based on exploiting this concept.

The pro-inflammatory effects of haemolysis also have implications in the context of whole blood stored for transfusion since stored RBCs lyse to release intracellular components that can cause adverse inflammatory effects to the receiving patient. Mice transfused with stored RBCs in a mouse model of traumatic haemorrhage rendered the animals less able to clear a lung infection than those transfused with fresh RBCs, decreasing their survival (36).

In conclusion, we show that airway exposure to whole or lysed RBCs elicits different inflammatory responses and our observations may have relevance to mechanisms contributing to PBLI, ARDS and possibly COVID-19. Lysed RBCs induce neutrophil recruitment and transmigration into the lung, which is dependent upon platelet activation. Our results suggest that modulating platelet neutrophil signalling pathways may dampen neutrophil recruitment, however the mechanism by which platelets affect neutrophil recruitment in this scenario remain to be resolved. Our studies suggest that methods to inhibit RBC lysis in alveolar haemorrhage may prove therapeutically beneficial in a range of conditions.

Figure Legends

Figure 1. Airway exposure to whole RBCs causes pulmonary macrophage recruitment whilst exposure to lysed RBCs causes pulmonary neutrophil recruitment and activation. (A) Lysed RBCs or whole RBCs were instilled into the airways of mice and 24 hours later BALF and blood was harvested. (B) Total inflammatory cell counts were performed on BALF. (C) Differential cell counts were performed on BALF to quantify the number of neutrophils and (D) macrophages. (E) Mice were weighed on Day 0 and Day 1 to determine percentage weight change. (F) H&E images reveal lysed and whole RBCs in the alveoli (arrows) and bronchioles (asterisk). Lungs were stained with rabbit anti-mouse neutrophil elastase antibody (1:300) (ab68672, Abcam UK). (G) Neutrophil elastase (NE) events appear brown against a haematoxylin counterstain; NE was quantified using ImageJ. Data expressed as mean $\pm$ SEM. Significance was determined using a Kruskal-Wallis Test and a Dunn's Multiple Comparisons post-hoc test or using a one-way ANOVA with Tukey's post-hoc test (G only). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; $n = 7-8$, or $6-7$ (G only)

Figure 2. Airway exposure to lysed RBCs causes acute neutrophil recruitment associated with a significant increase in BALF CXCL1/KC. (A) Temporal analysis was performed on mice exposed to airway whole RBCs or lysed RBCs at 6, 24 and 48 hours post exposure. BALF was harvested for (B) total inflammatory cell counts and, (C) neutrophils. (D) Cytokine CXCL1/KC was detected in BALF supernatant via ELISA. (E) BALF macrophages were quantified. Tail snips were conducted to allow blood microsampling and enumeration of (F) leukocytes, and (G) platelets. Data expressed as the mean value per sample $\pm$ SEM. Significance was determined using a one-way ANOVA and a Dunnetts's post-hoc test, except for graph C which was determined using a Kruskal Wallis test with Dunn's multiple comparisons test. The outlier in graph D at 24 hours was determined via the ROUT method and excluded from statistical analysis. **, $P<0.01$; ****, $P<0.0001$; Inflammatory Cell Quantification (B, C, D) $n=12-14$, CXCL1/KC Quantification (E) $n=7-8$.

Figure 3. Lysed RBCs in the airway cause increased circulating platelet neutrophil complexes. PNCs were defined as a Ly-6G+ CD41+ double positive event. (A) Control scatter plots show single stained CD41+ population with IgG PE isotype control, Ly-6G+ population with IgG FITC isotype control, and Ly-6G+ CD41+ events in whole blood stimulated with 0.1 units (U) thrombin (used as a positive control). (B) % of Ly-6G+ events that are CD41+ (PNCs). (C) Mean fluorescence intensity (MFI) of CD41+ events on Ly-6G+ events. Data expressed as mean $\pm$ SEM. Significance was determined using a Kruskal-Wallis Test and a Dunn's Multiple Comparisons post-hoc test. *, $P<0.05$; $n=6-8$.

Figure 4. Neutrophil recruitment to the lung is attenuated in mice experimentally depleted of platelets. (A) 2 hours prior to RBC exposure mice received either anti-CD42b antibody (anti-GPIb α) or IgG control antibody (1mg/kg, i.v). 50 μ l lysed RBCs ($2.6-3.6 \times 10^7$ RBCs/mouse) or 50 μ l PBS were instilled into the airways of mice and 24 hours post-exposure BALF and blood was harvested. (B) Circulating blood platelet counts. (C) BALF inflammatory cells. (D) Differential cell counts were performed on BALF to quantify the number of neutrophils. (E) Cytokine CXCL1/KC was detected in BALF supernatant via ELISA. (F) Blood inflammatory cell counts. (G) Weight change between Day 0 and Day 1. Significance was determined using a 2-way ANOVA and a Sidak's post-hoc test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. $n=7-8$.

Figure 5: P-selectin and P-selectin glycoprotein-1 blockade had no effect on neutrophil recruitment following airway exposure to lysed red blood cells. (A) 2 hours prior to RBC exposure mice received either anti-P-selectin antibody (anti-CD62P), anti-P-selectin glycoprotein-1 (PSGL-1) antibody (anti-CD162) or IgG control antibody (5mg/kg, i.v). 50 μ l lysed RBCs ($2.6-3.6 \times 10^8$ RBCs/mouse) or 50 μ l PBS were instilled into the airways of mice and 24 hours post-exposure BALF and blood were harvested. (B) total number of inflammatory cells recovered in BALF. (C) Number of neutrophils recovered in BALF. (D) Number of macrophages recovered in BALF. **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$ vs. IgG Ctrl. Significance was determined using a 2-way ANOVA with a Tukey's post-hoc test. Outlier in graph B was excluded from statistical analysis. Data in graph C was normalised prior to analysis. $n=7-8$.

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Figure 1

Figure 2**A** *Lysed RBCs: Temporal Analysis*

Figure 3**A** **B** **C**

Figure 4**A** **B** **C** **D** **E** **F** **G**

Figure 5