

The Development of an Inhalable Platelet-Based Therapy for Lung Injury

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Summary

Acute lung injury (ALI) and related inflammatory lung diseases remain clinical challenges as there are limited therapeutic options. This urgent unmet need highlights the importance of developing novel regenerative therapies to directly promote lung repair or resolution of injury. Apart from their role in haemostasis, platelets possess anti-inflammatory, pro-resolving, pro-regenerative properties. This study explores the potential for delivering platelet-based therapies *in vivo* via the inhaled route and investigates the impact of nebulisation on platelet function using *in vitro* and *ex vivo* tools.

Utilising a previously characterised *in vivo* model of ALI, our studies have shown that multiple dose administration of platelets following Lipopolysaccharide (LPS) administration significantly improved lung injury. When platelets were re-administered following platelet depletion and LPS inhalation the levels of haemorrhage in the mouse lung were significantly decreased. *In vitro*, when exploring platelet function in different preparations before and after nebulisation, it was found that nebulisation significantly reduced platelet aggregation, but chemotactic responses remained intact. *Ex vivo*, nebulised platelet-based preparations did not impair the viability of precision-cut lung slices (PCLS) following initial evaluation of their safety and efficacy.

Platelet-based therapies show promise in mitigating lung injury and retain key functions post-nebulisation. These findings support further development of inhaled platelet formulations as a novel regenerative strategy for treating lung diseases such as ALI.

Key Message

As proof of concept, instillation of platelets showed *in vivo* efficacy for treating ALI in mice. Towards developing an inhaled therapy, we have shown key platelet functions are preserved after nebulisation and an *ex vivo* safety and efficacy screen has been established to support the development of a nebulised platelet formulation.

Introduction

Platelets play critical roles in lung biology, particularly in maintaining the integrity of the alveolocapillary barrier. Beyond their well-established role in haemostasis, platelets also exhibit anti-inflammatory, immunomodulatory and reparative properties, making them promising candidates for regenerative therapies [1] [2]. Clinically, platelet-rich plasma (PRP) has already been applied in settings such as sports medicine and dental surgery for its regenerative potential.

In previous *in vivo* studies conducted in our laboratory, it was demonstrated that platelet-based products have efficacy in treating ALI. More specifically, administration of platelets following LPS-induced lung injury significantly improved pathological outcomes (such as an improved lung injury score and a reduction in the level of haemorrhage in their lungs) supporting their potential as a novel treatment modality for inflammatory lung conditions.

Given these promising results, we are now investigating the feasibility of delivering platelet-based therapeutics directly to the lungs via inhalation. Nebulisation represents a non-invasive route for targeted delivery to the pulmonary system; however, it is essential to determine whether the therapeutic functions of platelets are preserved following the nebulisation process. This work aims to evaluate the stability and functional integrity of platelet-based products post-nebulisation as a critical step toward developing an inhaled platelet therapy for lung disease.

Experimental Methods

Animals

Male and female C57BL/6 mice (8-10 weeks of age) used at King's College London were obtained from Charles River. Animal experimentation and care were compliant with the UK Home Office regulations (Animal Scientific Procedures Act 1986) after local ethics approval.

Following brief anaesthetisation by inhaled isoflurane, LPS-induced lung inflammation was achieved by administering an intranasal dose of 5mg/kg LPS (from E.coli, O55:B5 stereotype, Sigma-Aldrich).

Blood platelets were depleted by the intravenous administration of 1 mg/kg anti-GP1b α antibodies (R300, Emfret). Control mice received 1 mg/kg control antibody (anti-IgG).

For platelet administration, platelets were isolated from donor mice via centrifugation and administered at a dose of 3×10^9 platelets/ml to mice in the experimental groups

A minimum of 4 mice were used per experimental group. BAL fluid, blood samples and the lungs of all mice were collected for further analysis such as ELISA testing and histological analysis..

Human blood collection

Citrated venous blood was collected from healthy volunteers that had not taken anti-inflammatory medication in the past 10 days. Blood collection was carried out in accordance with the regulations of the Human Tissue Act 2004 and granted local ethical approval by King's College London.

Platelet-rich plasma preparations and nebulisation

Whole blood was centrifuged to produce PRP which was isolated. Leukocyte-rich PRP was collected (which includes the plasma layer and the rich buffy coat layer). Leukocyte-poor PRP was collected by only collecting the plasma layer. Washed platelets were collected after a second centrifugation of the PRP, producing a platelet pellet, which was resuspended in RPMI.

All preparations were nebulised using the Aerogen Pro vibrating mesh nebuliser and collected over ice.

Platelet aggregation assay

The PRP preparations before and after nebulisation were added to a 96-well plate with 10 μ m or 100 μ m adenosine diphosphate (ADP) as the aggregatory stimulus. Platelet aggregation was measured using light transmission aggregometry with a shaking plate reader (Spectramax Plus 384, Molecular Devices) at 595nm and 37°C.

Platelet chemotaxis assay

Blocking buffer (1% BSA IN RPMI) was added to block the wells of the bottom chamber of a HTS Transwell 96-well plate (3 μ m membrane pore size, Fischer Scientific) and left for 1 hour. The wells were then washed and 30nm N-Formylmethionyl-leucyl-phenylalanine (fMLP) was added as the chemotactic stimulus. All platelet preparations before and after nebulisation were added to the top chamber of the plates at a concentration of 5×10^7 platelets/ml. The plate was incubated at 37°C for 90 minutes before counting the number of platelets in the bottom compartment of the plates. The chemotactic index was calculated by dividing the platelets counted in the wells of the plates that contained fMLP and ADP by the platelets counted in the negative control wells that contained no fMLP and no ADP.

Precision cut lung slices (PCLS)

PCLS were generated by inflating the lungs of the culled mice with 2% agarose solution and sliced at a thickness of 300 μ m using an automated vibratome (Compresstome VF-300-OZ, Precisionary Instruments LLC). Slices were cultured at 37°C in 5% CO₂ for up to 72 hours in media containing 1% penicillin-streptomycin.

PRP preparations at different concentrations were added to the PCLS and allowed to incubate overnight. Following overnight incubation, the PCLS were washed and left to incubate overnight in fresh media. The following day CCK8 was added to the wells in a 1:10 dilution and left to incubate for 1 hour before the optical density was measured at 450nm as a measure of viability.

Results and Discussion

Multiple doses of platelet administration *in vivo* in a mouse model of ALI significantly reduced the lung injury score (Figure 1A) while the concentration of Annexin A1 significantly increased after this dosing regime (Figure 2B). Following platelet depletion and a subsequent worsened LPS-induced lung injury, platelet re-administration of platelets significantly reduced the levels of BAL haemorrhage in the lungs of the mice (Figure 1C). These data indicate that repeated doses of platelets significantly improved lung injury outcomes. Mice that received platelets as treatment following LPS injury showed significant reductions in lung injury scores and an increase in the levels of Annexin A1 in the BAL (a specialised

pro-resolving mediator involved in the resolution of inflammation). Additionally, in platelet-depleted mice, re-administration of platelets following LPS significantly reduced BAL haemorrhage. These *in vivo* studies provide preclinical proof-of-concept for the efficacy of platelet-based therapies.

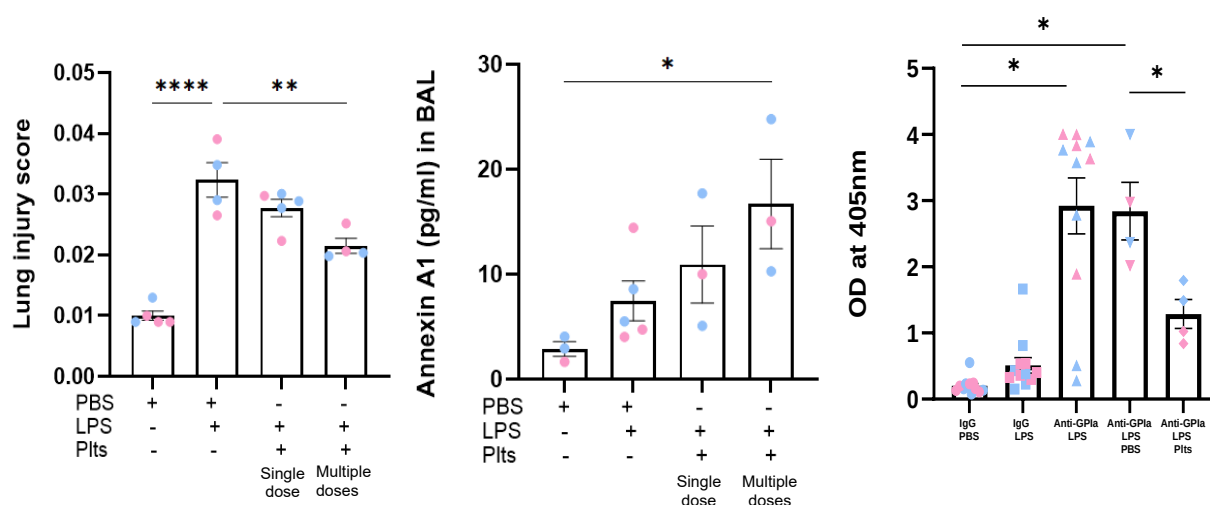
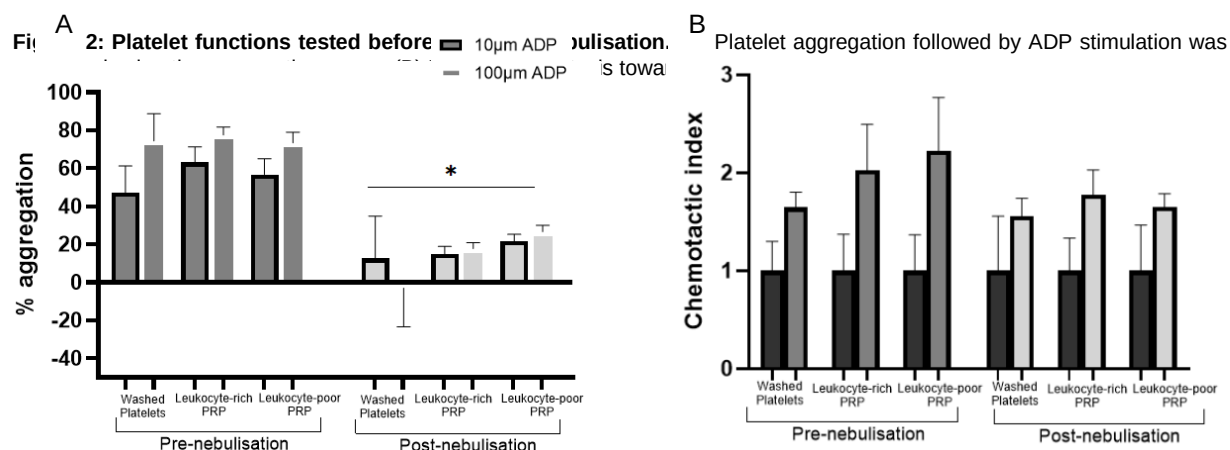


Figure 1: Multiple doses of platelet administration reduce LPS-induced lung injury. Mice received 5mg/kg of LPS via intranasal administration, then treated with either PBS or platelets (Plts) and samples were collected 72 hours later. Mice in the negative control group received PBS only. Mice in treatment group 1 received a single dose of platelets 24 hours post-LPS inhalation. Mice in treatment group 2 received multiple doses of platelets at 4- 24- and 48- hours post-LPS inhalation. The lung injury score (A) was calculated as lung sections were stained with haematoxylin and eosin and images were captured. (B) The levels of Annexin A1 were measured in the BAL via the use of an ELISA. (C) Mice received 5mg/kg of LPS or 30ul PBS via intranasal administration, followed by either platelet depleting antibody (anti-GPIa) or control antibody (anti-IgG) then treated with either PBS or Plts and samples were collected 72 hours later. The level of haemorrhage was measured in the BAL via an OD reading at 405nm. Data shown as mean $\pm$ SEM. Each data point represents findings from either a male (blue) or female (pink) mouse.

The effect of nebulisation on three platelet preparations (leukocyte-rich PRP, leukocyte-poor PRP and washed platelets) was investigated by comparing vital platelet functions before and after nebulisation (Figure 2). Platelet aggregation was significantly reduced following nebulisation of all preparations (Figure 2A). In antithesis, the ability of platelets to move towards a chemoattractant was not significantly impaired following nebulisation (Figure 2B).

While nebulisation reduced the aggregation capacity of platelets in all three preparations the chemotactic ability of platelets was preserved which suggests that although mechanical stress during nebulisation may alter some functional characteristics (ie aggregation) other critical reparative and immunomodulatory functions remain intact.



As an *ex vivo* screen for safety, the effects of PRP and washed platelets on PCLS before and after nebulisation were screened and no significant detrimental effect on tissue viability was observed (Figure 3) supporting the safety of these preparations for pulmonary application. The next step is to investigate efficacy in a PCLS model of lung injury.

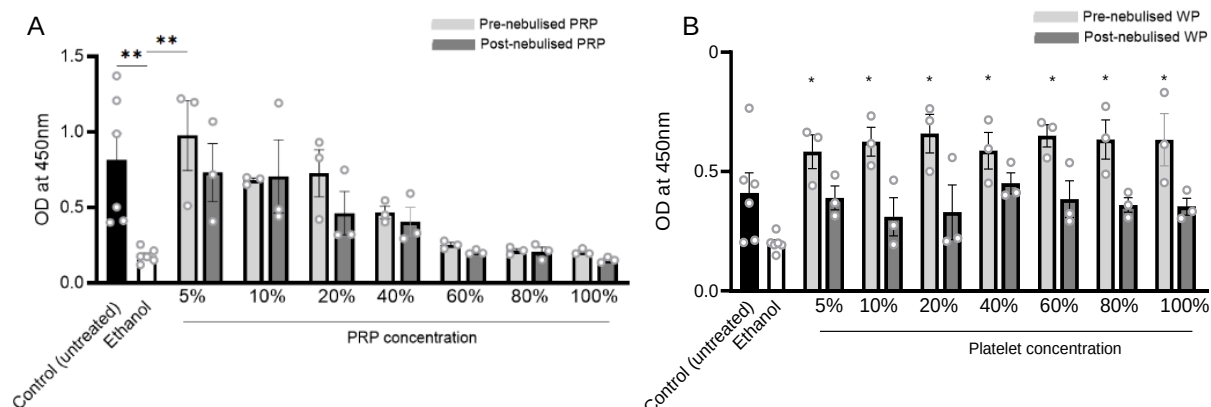


Figure 3: (A) (B) Precision cut lung slice viability was measured using the CCK-8 assay. Data shown as mean $\pm$ SEM.

Together, these findings suggest that platelet-based therapies retain key functional properties post-nebulisation and support their further development as an inhaled regenerative treatment for lung injury. Future studies will expand the investigation of platelet function before and after nebulisation and validate the efficacy of platelet-based formulations in a model of ALI utilising *in vivo* and *ex vivo* tools.

Conclusion

This study demonstrates the therapeutic potential of platelet-based productions for the treatment of ALI and evaluates the feasibility of delivering these therapies via inhalation using nebulisation.

While nebulisation led to a reduction in platelet aggregation, this may in fact be advantageous, as it suggests that platelets are less likely to aggregate within the pulmonary circulation following administration – thereby reducing the risk of unwanted thrombosis or microvascular obstruction during treatment. Importantly, key reparative and chemotactic functions were maintained post-nebulisation, supporting the continued therapeutic potential of these formulations.

Together, these findings lay the groundwork for developing a safe and effective inhaled platelet-based therapy, advancing the field of regenerative medicine for respiratory diseases. Future work should further optimise formulation and delivery parameters and to maximise functional preservation while ensuring pulmonary safety.

References

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