Online Supplement:

Resolution of acute lung injury and inflammation – a translational mouse model

Dr Brijesh V Patel MRCP FRCA, Dr Michael R Wilson PhD, Professor Masao Takata MD PhD

A) Detailed Methods.

Animal model of acid aspiration.

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. We used male C57BL6 mice (Charles River, Margate, UK) aged 10–12 weeks and weighing 25-30g. In total 68 animals were used – 23 for measurements of respiratory mechanics and alveolar inflammation, 25 for assessing alveolar fluid clearance, and 20 for lung wet/dry weight and histology scoring.

Mice were anaesthetised by intraperitoneal injection of xylazine (6mg/kg) and ketamine (60mg/kg), and given an intraperitoneal fluid bolus of 10µl/g 0.9% normal saline as preemptive fluid resuscitation. Mice were suspended vertically on a custom-made mount from their incisors for orotracheal instillation as described previously [1]. A non-thermal light source was used to transilluminate the trachea. Careful laryngoscopy using blunt curved forceps allowed a grade 1-2 view of the vocal cords (figure A1).

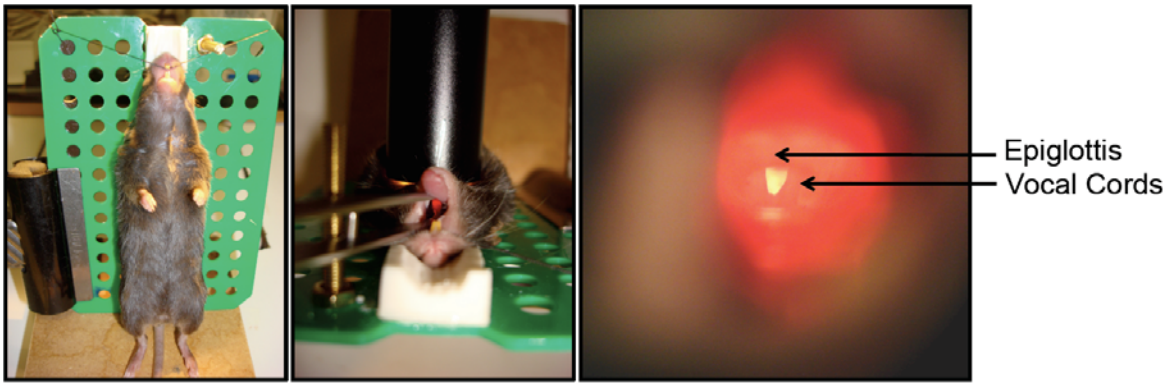

Figure A1 – Acid instillation setup and protocol.

A fine catheter was subsequently guided 1cm below the vocal cords and 75µl of an isoosmolar (to mouse plasma - 322mosmol/L) solution of 0.1M hydrochloric acid (pH 1.0) was instilled. Initial experiments performed with intratracheal instillation of Evans blue dye demonstrated that this methodology produces bilateral distribution of instilled agents. Animals were left suspended for one minute. For the next 4 hours, during which animals exhibited significant respiratory depression/distress as an acute result of acid aspiration-induced ALI, mice were kept in a custom-made transparent recovery box under humidified supplemental oxygen (FiO_2 reduced gradually from 1.0 to 0.21). During this period animals were carefully monitored and body temperature was maintained using external heat sources, after which they were transferred to individually ventilated cages with air and free access to food and water.

Physiological Measurements.

At specified time-points (1, 2, 3, 5 and 10 days) after acid instillation, animals were weighed and underwent surgical preparation for mechanical ventilation, to conduct standardised assessment of pulmonary gas exchange capability and respiratory mechanics. The preparation for mouse mechanical ventilation and pulmonary function tests has been described in detail previously [2, 3]. Anaesthetised mice underwent tracheostomy and were ventilated with 8-9 ml/kg tidal volume (V_T), 2.5cmH_2O positive end-expiratory pressure (PEEP), and respiratory rate (RR) of 120 breaths/minute with FiO_2 of 1.0, using a custom-made mouse ventilator-pulmonary function testing system. Airway pressure and gas flow were continuously monitored throughout the experimental protocol, and cannulation of the left carotid artery allowed monitoring of mean blood pressure. After an initial lung recruitment

manoeuvre (30cmH_2O, 5sec), animals were ventilated for 30 min to standardise the volume history of the lungs. At the end of ventilation, blood gases were measured and respiratory mechanics determined using the end-inspiratory occlusion technique, as described previously [2, 4]. Acid-injured animals were compared to otherwise untreated mice, receiving just the surgical preparation and 30 minute ventilation procedures.

Pulmonary inflammation. At the end of the above procedure, mice were sacrificed by anaesthetic overdose. Bronchoalveolar lavage (BAL) was performed with 750µl of saline as described previously [2] and the samples were centrifuged at 1500rpm. Protein levels in BAL fluid (BALF) were quantified (Bio-Rad Laboratories, Hemel Hempstead, UK) and BALF levels of IL-6, TNF-α, keratinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2), and soluble Receptor for Advanced Glycation End-products (RAGE) were determined using ELISA assay kits (R&D Systems, Abingdon, UK). BALF cell counts were obtained by haemocytometer, with differential cytology performed on Diff-Quik-stained samples prepared by cytospin (Shandon, Runcorn, UK).

Alveolar Fluid Clearance. In a separate series of experiments, alveolar fluid clearance (AFC) was determined at days 1, 2, 3, 5 and 10 after acid instillation (and in untreated animals), using a modification of previously described in situ models [5]. Mice were anaesthetised, surgically prepared for tracheostomy and carotid artery cannulation, and ventilated as described above. After intravenous heparinisation (20iu), animals were sacrificed and exsanguinated through transection of the carotid artery, internal and external jugular veins, inferior vena cava and abdominal aorta, to drain pulmonary vessels and minimise any potential impact of pulmonary vascular

pressure on AFC. Each animal then received 3 lung recruitment manoeuvres, to standardise volume history of the lungs and further empty pulmonary compliant vessels. The entire procedure took no more than 15 minutes.

Mice then underwent intratracheal instillation with 700µl of an isoosmolar medium containing 5% low endotoxin bovine serum albumin (BSA, Sigma-Aldrich Ltd, Dorset, UK) and 50µg/ml fluorescent AlexaFluor 594-conjugated BSA (Invitrogen, Paisley, UK). Without disconnecting the instillation syringe, 400µl of instillate was immediately withdrawn and re-instilled, followed by removal of a first aliquot of 200µl as a t=0 reference sample. This process ensured homogeneous distribution of the instillate, and that the reference sample was as representative of alveolar contents as possible. Mice were placed on a continuous positive airway pressure system delivering 100% oxygen at 8cmH_2O. All animals were maintained at a temperature of 36.5-38^oC (measured by an oesophageal probe) throughout the protocol. Thirty minutes after instillation, a surgical pneumothorax was induced through blunt dissection of the diaphragm to maximise the recovery of the remaining instillate from the lungs. Percentage AFC over 30 minutes was determined by the equation: [1-(F0/F30)] x100, where F0 is the fluorescence of the t=0 reference sample and F30 is the fluorescence of the t=30 sample. Experiments using isoproteronol (2mM) and amiloride (2mM) to respectively enhance and inhibit water movement confirmed that measurements accurately reflected AFC (figure A2).

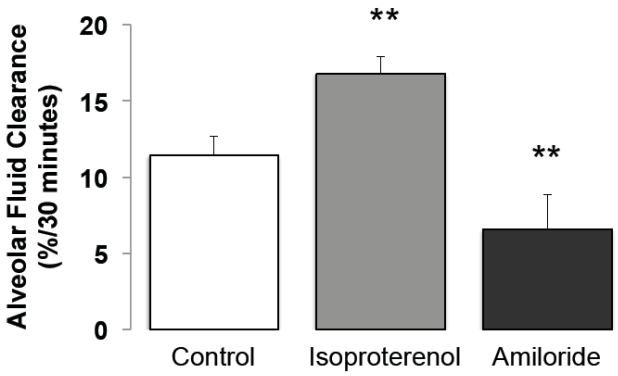

Figure A2 – Validation of alveolar fluid clearance (AFC) measurement. Basal AFC in our system was 11.5% over 30 minutes. Isoproterenol significantly enhanced AFC by 46.4% and amiloride significantly inhibited AFC by 42.5%. ** P<0.01 *vs control; N=4-5.*

Measurement of AFC is a methodology with multiple potential pitfalls, which are enhanced when measurements are made on injured lungs, as in the current study. In particular, the diluting effect of pre-existing oedema fluid within the lungs would tend to decrease apparent AFC. We therefore took a number of precautions to ensure that our measurements indeed provided accurate reflections of AFC. Most importantly, we used, as our reference sample, an aliquot of the instillate that had been delivered into the lungs and removed, rather than naive instillate per se. Thus any preexisting oedema would dilute both the reference and final samples. We also evaluated the potential for any blood/plasma remaining in the pulmonary vasculature to leak into the alveolar space during the 30-minute protocol (and again decrease apparent AFC by dilution), by intravenously injecting a different fluorescent marker, AlexaFluor 488-labelled BSA (Invitrogen), 5 minutes before exsanguination. The presence of this marker in the alveolar space was less than 1% of the plasma concentration, and we concluded that any confounding effect on our AFC measurements was minimal.

Lung injury scoring. In a separate series of experiments, mice were sacrificed at each time point after acid (and untreated animals), and their left lungs instilled and inflated at 15cmH_2O with 4% paraformaldehyde and removed for paraffin embedding. Slices at 10μm thickness were subsequently stained with haematoxylin and eosin (Sigma-Aldrich Ltd, Dorset, UK), or Masson's Trichrome stain (Sigma-Aldrich Ltd, Dorset, UK) for fibrosis. Lung injury scores were quantified by an investigator blinded to the treatment groups using recently published criteria (table 1), which gives an overall score of between 0 and 1 [6]. The final injury score was derived from the following calculation: Score = [20*(i) + 14*(ii) + 7*(iii) + 7*(iv) + 2*(v)] / (number of fields * 100).

Parameter	**Score per field**		
	0	**1**	**2**
i. Neutrophils in the alveolar space	None	1-5	>5
ii. Neutrophils in the interstitial space	None	1-5	>5
iii. Hyaline membranes	None	1	>1
iv. Proteinaceous debris filling the airspaces	None	1	>1
v. Alveolar septal thickening	None	2x-4x	>4x

Table A1 – Lung injury scoring system (adapted from Matute-bello *et al* [6])

Lung wet/dry weight ratio.

The right lungs from animals used for lung injury scoring were tied off prior to paraformaldehyde instillation and removed, weighed and dried at 60^oC for 24 hours for determination of lung wet:dry weight ratio.

Statistics.

Data are expressed as means±SD. Statistical analyses of physiological parameters were carried out by one-way ANOVA with Bonferroni tests for pairwise comparisons between each time-point and uninjured controls (represented as Day 0). We used Spearman's correlation coefficients to test the correlations between RAGE levels and other continuous variables. Statistical significance was defined as $p<0.05$.

References

1. Wilson MR, O'Dea KP, Dorr AD, Yamamoto H, Goddard ME, Takata M. Efficacy and safety of inhaled carbon monoxide during pulmonary inflammation in mice. *PLoS ONE* 2010; 5: e11565.

2. Wilson MR, Choudhury S, Goddard ME, O'Dea KP, Nicholson AG, Takata M. High tidal volume upregulates intrapulmonary cytokines in an in vivo mouse model of ventilator-induced lung injury. *J Appl Physiol* 2003; 95: 1385-1393.

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6. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM. An Official American Thoracic Society Workshop Report: Features and Measurements of Experimental Acute Lung Injury in Animals. *Am J Respir Cell Mol Biol* 2011; 44: 725-738.

B) Supplementary Figures.

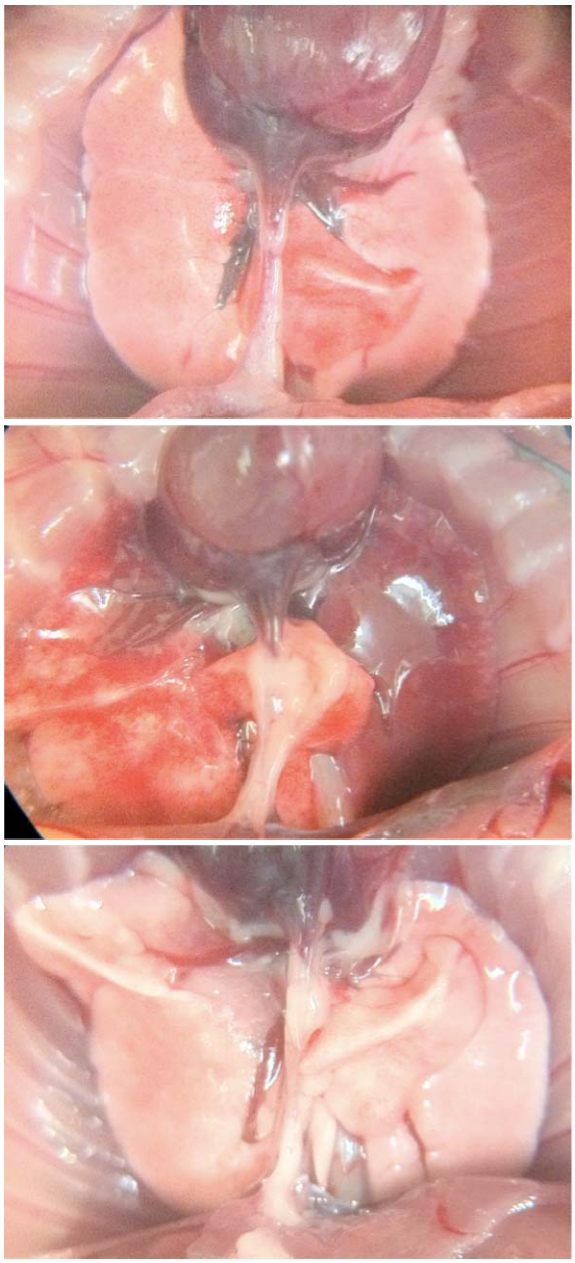

Figure B1 – Gross macroscopic appearance of normal uninjured mouse lungs (top) and lungs of mice after 2 (middle) and 10 days (bottom) after injury (the heart has been moved superiorly). Day 2 injured animals show a bilateral injury with significant lung discolouration as a result of haemorrhage and oedema formation. By day 10 lungs look similar to control lungs.

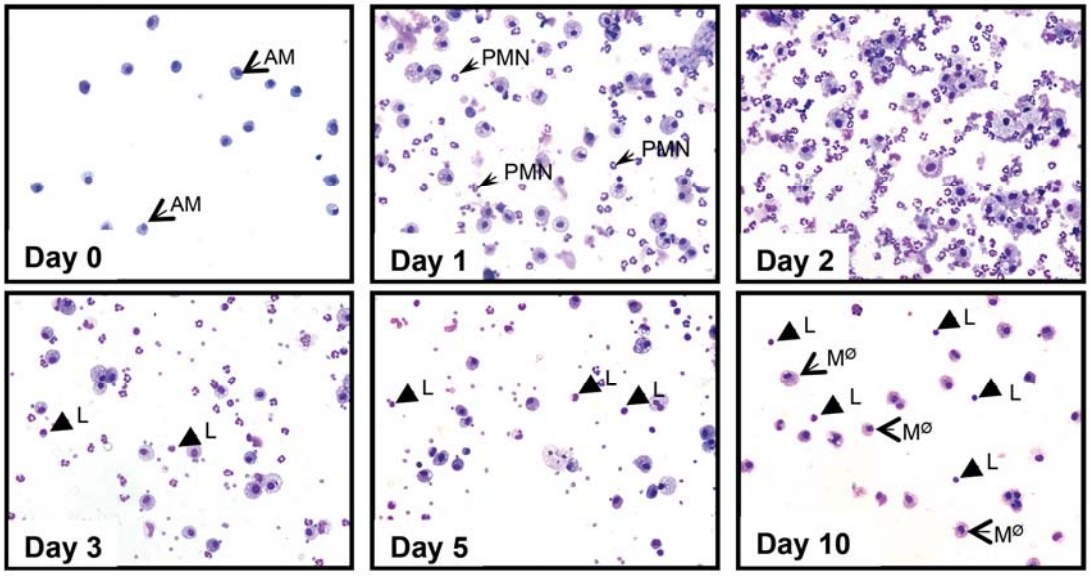

Figure B2 - Representative cytospin prepared slides at different time-points after injury showing significant increases in neutrophils during the first two days of injury and increased proportion of lymphocytic cells at day 10.