

SUPPLEMENTARY MATERIALS AND METHODS:

Bronchoalveolar lavage collection and processing

Mechanically ventilated preterm infants born <32 weeks gestation who either developed chronic lung disease (CLD) or not were recruited from the Regional Neonatal Unit at University Hospital of Wales in Cardiff, UK as part of a previously published study [1]. Term infants ventilated for non-respiratory reasons and requiring $\leq 28\%$ O_2 were also recruited to the study. Bronchoalveolar lavage fluid (BALF) was obtained by non-bronchoscopic bronchoalveolar lavage (BAL) at times of clinically indicated toileting of the endotracheal tube according to guidelines published by the European Respiratory Society task force on bronchoalveolar lavage in children [2]. Ethical approval was obtained from the local Research Ethics Committee and written informed consent was obtained from the parents. BAL was not performed within the first 12h of an infant receiving surfactant. The procedure was performed with the infant supine and the head to the left. An FG6 catheter was fed down the endotracheal tube until wedged when resistance was felt. BAL was performed using two aliquots of 1ml normal saline per kg and suction was applied immediately after instillation. The median yield from BALs in the original study was 50% (IQR: 38-63%). Samples were collected daily during the first week of life or until 28 days of life or extubation, whichever occurred earlier. Upon collection, samples were transferred on ice to the laboratory for further processing. Samples were subjected to centrifugation (1000g/10min) and the supernatant was aspirated to fresh microcentrifuge tubes in 25 or 50 μ l aliquots. Samples were stored at -80°C within 30 minutes of collection until further analysis was undertaken. Samples used for all experiments were collected between June 2005 and November 2006 and were not subjected to freeze-thaw cycles. Samples were thawed and subjected to micro-centrifugation at 13,000rpm for 1 minute immediately prior to analysis. Due to the limited volume of BALF obtained from the neonatal population, not all samples recruited to the original study were available for analysis here. The sole inclusion criterion for samples in this study was availability of sufficient sample volume over the first week of life in the same patient.

Zymosan and maltose-agarose binding assay: 10 μ l of a Zymosan-A suspension (1% w/v in 154mM NaCl) was added to a 0.5ml microcentrifuge tube and washed twice with 10 volumes of TBS-Ca (20mM Tris-HCl, 154mM NaCl, 20mM $CaCl_2$; pH 7.6) with microcentrifugation at 13,000rpm for 1 minute between washes. The pellet was resuspended with 10 μ l of TBS-Ca and 10 μ l of either freshly thawed BALF or, as a positive control, 10 μ l of native SP-D diluted to 2 μ g/ml in 154mM NaCl with 1.5mg/ml protease free BSA (Sigma, Dorset, UK) to give a final

concentration of 10mM CaCl₂ in all binding assays. Where Ca²⁺ or carbohydrate dependence of the binding assay was tested, native SP-D or BALF was diluted to give a final concentration of 10mM EDTA in TBS or 100mM D-Maltose plus 10mM CaCl₂ in TBS in the binding assay. Where the capacity of BALF to influence native SP-D binding to zymosan was tested, 10µl of native SP-D (2µg/ml) in TBS-Ca was pre-incubated with 10µl of BALF for 30 minutes at 37°C prior to incubation with zymosan. For all assay formats, samples were incubated for 30 minutes at 37°C with occasional gentle mixing to maintain zymosan in suspension over the course of the assay. Post assay, samples were microcentrifuged at 13,000rpm for 1 minute and the supernatant was carefully aspirated to a fresh tube, avoiding disturbance of the zymosan pellet. In some experiments this supernatant fraction was subjected to a further round of binding to a freshly prepared zymosan pellet. In other experiments the supernatant fraction was used as input material for solid phase binding assays as described below. Following aspiration of the supernatant, the zymosan pellet was washed once with 100µl TBS-Ca as described above and the washed pellet was resuspended in 20µl TBS with 20mM EDTA. Where samples were subsequently subjected to SDS-PAGE both pellet and supernatant fractions were immediately treated with SDS sample buffer with or without β-mercaptoethanol as a reducing agent and boiled at 100°C for 2 minutes prior to electrophoretic separation.

Maltose-agarose pull down assays were similar to the zymosan binding assays with the following differences. 10µl of maltose agarose (Sigma, Dorset, UK) was washed three times in 1ml TBS-Ca prior to commencement of the binding assay. Assays were carried out in a final volume of 30µl, with only 20µl of the supernatant fraction aspirated for SDS-PAGE to avoid disturbing the agarose pellet; once washed, the pellet was resuspended in 30µl of TBS-EDTA, 20µl of which was used for SDS-PAGE.

SDS-PAGE and Western Blot: Appropriately prepared samples were loaded onto 10% SDS polyacrylamide gels with a 4% stacking gel. Electrophoresis with Tris-Glycine SDS buffer proceeded at 40mA/gel for 1 hour. Proteins were subsequently transferred to nitrocellulose in Tris-Glycine buffer with 20% (v/v) methanol for 1 hour at 100V. Post transfer, blots were blocked for 30 minutes with 5% (w/v) skimmed milk powder in PBS (PBS-milk). Blots were incubated with 0.1µg/ml affinity purified goat anti-SP-D in PBS-milk overnight at 4°C, or occasionally for 1 hour at room temperature. Blots were washed (three times with PBS/0.05% (v/v) Tween 20 and three times with PBS) prior to incubation with HRPO conjugated donkey anti-goat IgG in PBS-milk for 1 hour at room temperature. Blots were washed again and exposed to FujiFilm RX X-ray film following development with ECL Substrate (ThermoFisher Scientific, Cramlington, UK) (Supplementary Figure 1) or ECL prime substrate (GE Healthcare Lifesciences, Bucks., UK).

For semi-quantitative densitometry of developed western blots, blots were scanned, brightness and contrast were adjusted appropriately and densitometry was undertaken on inverted images using ImageJ v1.46. Images presented display all visible bands from developed blots.

Solid phase binding assays: Mannan (Sigma, Dorset, UK) at 50µg/ml in PBS was coated overnight at 37°C in Nunc Maxisorp 96-well plates (50µl/well). Wells coated with PBS alone were also included. Plates were washed three times with 100µl TBS-Tween (10mM Tris-HCl, 154mM NaCl; pH 7.6 with 0.05% (w/v) Tween) and blocked for 1 hour at room temperature with 100µl TBS-Tween. Input SP-D to the assay was either 1) freshly thawed BALF diluted in TBS-Tween/10mM CaCl₂ to achieve 1µg/ml SP-D with zymosan binding activity or 2) the supernatant fraction from BALF following a zymosan binding assay diluted to achieve an SP-D concentration of 1µg/ml (SP-D concentrations were calculated based on data from ELISA and functional assays). Native SP-D diluted to 1µg/ml in TBS-Tween/10mM CaCl₂ with 0.75mg/ml protease free BSA was also used as a positive control. For competition assays, the above binding assays were carried out in the presence of 100mM D-maltose, without calcium but in the presence of 10mM EDTA or in the presence of 100µg/ml Poractant Alfa (Chiesi Ltd., Cheadle, UK). Samples were incubated for 90 minutes at room temperature. Wells were washed three times with 100µl TBS-Tween/10mM CaCl₂ (or TBS-Tween/10mM EDTA where appropriate). Wells were incubated for 1 hour at room temperature with 50µl goat anti-SP-D (1µg/ml) in TBS-Tween/10mM CaCl₂ or, in the case of assays with Poractant Alfa, 50µl rabbit anti-SP-D (5µg/ml) in TBS-Tween/10mM CaCl₂. Wells were washed three times with TBS-Tween/10mM CaCl₂ prior to incubation with 50µl HRPO conjugated donkey anti-goat IgG or donkey anti-rabbit IgG in TBS-Tween/10mM CaCl₂ for 30 minutes at room temperature. Wells were washed three times and developed with TMB substrate (eBioscience, Hatfield, UK). Development was quenched with 1M H₂SO₄ and the plate was read at 450nm on a MRX TC Revelation plate reader (Dynex Technologies, West Sussex, UK).

REFERENCES

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2. de Blic J, Midulla F, Barbato A, et al. Bronchoalveolar lavage in children. ERS Task Force on bronchoalveolar lavage in children. *European Respiratory Society. Eur Respir J* 2000;**15**(1):217-31