ORIGINAL ARTICLE

Increased prevalence of low oligomeric state surfactant protein D with restricted lectin activity in bronchoalveolar lavage fluid from preterm infants

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ABSTRACT

Background Surfactant protein D (SP-D) is a soluble oligomeric C-type lectin known to protect against lipopolysaccharide and ventilator-induced lung injury in preterm lambs. Here we assess the expression and functional status of SP-D in bronchoalveolar lavage fluid (BALF) from preterm infants at risk of chronic lung disease (CLD) of prematurity and term controls. This is the first systematic evaluation of SP-D function in any clinical cohort.
Methods SP-D was quantified in BALF from 28 ventilated preterm infants and five ventilated term infants. SP-D lectin activity was tested in a zymosan binding assay followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot in BALF from the same infants. SP-D lectin activity was also tested towards maltose-agarose and mannan for selected BALF samples.
Results SP-D expression was lower on day 1 in those preterm infants who subsequently developed CLD but increased over the first 5 days of life in term and preterm neonates. The percentage of neonatal SP-D capable of binding zymosan rarely exceeded 50% in any BALF sample and was 3.5 times lower in preterm infants than term infants on day 1 of life. Similar binding defects were observed towards maltose-agarose and mannan. SDS-PAGE analysis revealed that zymosan-bound SP-D was more highly oligomerised (≥12-mers) than unbound SP-D, which was composed primarily of trimers and lower oligomeric forms.
Conclusions Substantial and functionally relevant variation in the expression and oligomeric distribution of SP-D exists between preterm and term neonatal lung secretions. This has implications for proposed SP-D replacement therapy in this population.

Key messages

What is the key question?

- Is there a quantitative or functional deficiency of the antimicrobial and inflammatory regulator, surfactant protein-D (SP-D), in lung fluid from preterm infants at risk of chronic lung disease of prematurity?

What is the bottom line?

- Substantial and significant variation in the expression, oligomeric state and lectin activity of SP-D is evident in preterm infants compared with term infants and also in preterm infants at risk of developing chronic lung disease.

Why read on?

- This is the first systematic analysis of SP-D functionality in any clinical population and highlights a novel functional deficiency in preterm infants of a molecule known to protect against inflammation and ventilator-induced injury in models of prematurity.

INTRODUCTION

Infectious and inflammatory disease is a particular problem for premature infants due to recognised deficiencies of innate and adaptive immunity[1 2] Many very premature infants develop respiratory distress syndrome (RDS) due to pulmonary immaturity and this, combined with infection and ventilator-induced injury, contributes to the development of chronic lung disease (CLD) of prematurity or bronchopulmonary dysplasia.[3]

Surfactant protein D (SP-D) is a soluble C-type lectin best described in the lung but also expressed at other mucosal sites. Like other soluble C-type lectins including mannose binding lectin and surfactant protein A (SP-A), the lectin domain of SP-D recognises carbohydrate structures on a wide spectrum of bacteria, fungi and viruses.[4] Additionally, SP-D plays a critical role regulating inflammatory cell activity,[5] and deficiency of this molecule results in a spontaneous and progressive inflammatory lung disease.[6] SP-D exists in a variety of oligomeric states. Dodecamers, consisting of four trimeric subunits, form by covalent and non-covalent interactions between amino-terminal peptide sequences and these, along with trimers, are the most commonly isolated oligomers in humans.[7] Larger oligomers, sometimes referred to as 'fuzzy balls' or 'astral bodies', are observed less frequently but have greater opsonic and antiviral activity.[8] By contrast, naturally occurring trimers exhibit poor affinity for traditional carbohydrate ligands otherwise bound by corresponding dodecamers[7 9]

SP-D functional activity is influenced by a number of factors. One common polymorphism (Thr[11]) substantially limits SP-D oligomerisation to trimers with restricted lectin activity.[7] SP-D is also subject to structural and functional modification by elements of the innate inflammatory response, including neutrophil-derived serine proteases,[10] and oxidative mediators such as myeloperoxidase and peroxynitrite[11 12]

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Recent gene association studies implicate variants of SP-D in the incidence of spontaneous preterm birth[13] and the development of RDS in preterm infants.[14] Low levels of SP-D are associated with infection in models of preterm ventilation[15] and inflammation and hyperoxic stress in the mouse.[16 17] Exogenous SP-D therapy reduces endotoxic shock and ventilator-induced inflammation in preterm lambs.[18 19] Despite this, only one study has investigated SP-D expression in ventilated preterm infants at risk of CLD, describing lower levels in those who develop CLD.[20] No studies have yet described the functional status of SP-D in this or any other clinical cohort. Here we examine the expression and lectin activity of SP-D in bronchoalveolar lavage fluid (BALF) from a cohort of ventilated preterm and term infants.

MATERIALS AND METHODS

Patient recruitment and sample processing

BALF was obtained from ventilated preterm and term infants recruited as part of a previously published study.[21] Full details of sample collection and processing are provided in the online supplementary materials and methods. Ethical approval was obtained from the local Research Ethics Committee and written informed consent was obtained from the parents. Cell-free supernatants were stored at −80°C within 30 min of collection. Prior to analysis, samples were thawed and subjected to microcentrifugation at 13 000 rpm for 1 min. Not all samples from the original study were available for analysis, and for a small number of samples, sufficient volume was not available to enable analysis in ELISA and zymosan binding assays (see table 1; figures 1, 3 and 4, panels C,D). Data were excluded from two preterm infants with large sacrococcygeal teratomas.

Reagents

All reagents were from Fisher Scientific (Loughborough, UK) unless otherwise stated. Goat anti-SP-D was from R&D systems (Abingdon, UK). Minimally cross-reactive horseradish peroxidase (HRPO)-conjugated donkey anti-goat IgG and donkey anti-rabbit IgG was from Jackson ImmunoResearch (Suffolk, UK). Native SP-D was purified as previously described[22] and stored at −80°C.

ELISA

SP-D was quantified using a two monoclonal antibody sandwich ELISA from Hycult Biotech (Uden, The Netherlands) according to the manufacturer's instructions (measurable range 6.3–400 ng/ml). BALF samples were diluted between 1 : 10 and 1 : 160 and measured in duplicate.

Zymosan and maltose-agarose binding assay

Full methodology is detailed in the online supplementary materials and methods. Briefly, native SP-D (2 μg/ml in 154 mM NaCl) or neonatal BALF was diluted 1 : 1 in tris-buffered saline (TBS), pH 7.6 with 10 mM $CaCl_2$ or 10 mM ethylenediaminetetraacetic acid (EDTA) or 100 mM D-maltose. This was incubated with a washed pellet from 10 μl of a 1% w/v suspension of Zymosan-A or 10 μl maltose-agarose. If indicated, native SP-D was preincubated with an equal volume of BALF prior to substrate binding, which proceeded for 30 min at 37°C. Following microcentrifugation, supernatants were carefully aspirated, the pellet was washed once and SP-D eluted with TBS-EDTA. In some experiments, supernatants were subjected to a further round of zymosan binding. In other experiments supernatants were subsequently assessed for mannan binding activity as described below. Samples for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were immediately boiled for 2 min with SDS sample buffer with or without β mercaptoethanol.

SDS-PAGE and western blot

Full methodology is detailed in the online supplementary information. Briefly, samples were separated on 10% SDS-PAGE gels, transferred to nitrocellulose and sequentially incubated with 0.1 μg/ml goat anti-human SP-D and HRPO-conjugated donkey anti-goat IgG in phosphate buffered saline (PBS) milk. Blots developed with ECL prime (GE Healthcare LifeSciences, Bucks, UK) were subjected to densitometry as previously described using ImageJ V.1.46.[23] Images presented display all visible bands from developed blots.

Solid phase binding assays

Full methodology is detailed in the online supplementary information. Briefly, BALF pre-zymosan and post-zymosan binding, or native SP-D was diluted in TBS with 0.05% (v/v) Tween (TBST), pH 7.6 plus 10 mM $CaCl_2$ (TBST-C) as detailed in the results section. Some assays were carried out in the presence of 100 mM D-maltose or 100 μg/ml Poractant Alfa (Chiesi, Cheadle, UK); others excluded calcium but included 10 mM EDTA (TBST-E). Binding to mannan (50 μg/ml) or PBS-coated 96-well Nunc MaxiSorp plates proceeded for 90 min at room temperature. Wells were washed with TBST-C or TBST-E prior to incubation with goat anti-SP-D (1 μg/ml) in TBST-C or, in

Table 1 Patient characteristics

	Preterm CLD	Preterm No CLD	Term
Number of patients	14	14	5
Number of samples: (ELISA/functional assay)	50 (45/48)	32 (30/32)	16 (15/16)
Gestational age (weeks)*†	26^{+2} (25^{+4}–29^{+1})	28^{+5} (27^{+1}–29^{+3})	Term
Birth weight (g)†	960 (850–1330)	1140 (977–1257)	2710 (2390–2730)
Prolonged rupture of membranes (>24 h)	4/14 (29%)	1/14 (7%)	0/5 (0%)
Antenatal steroids (>24 h)	11/14 (79%)	10/14 (71%)	0/5 (0%)
Surfactant	14/14 (100%)	14/14 (100%)	0/5 (0%)
Caesarean delivery	7/14 (50%)	9/14 (64%)	1/5 (20%)

*Values in superscript refer to days in addition to weeks of gestation.
†Values are reported as median (IQR).
CLD, chronic lung disease.

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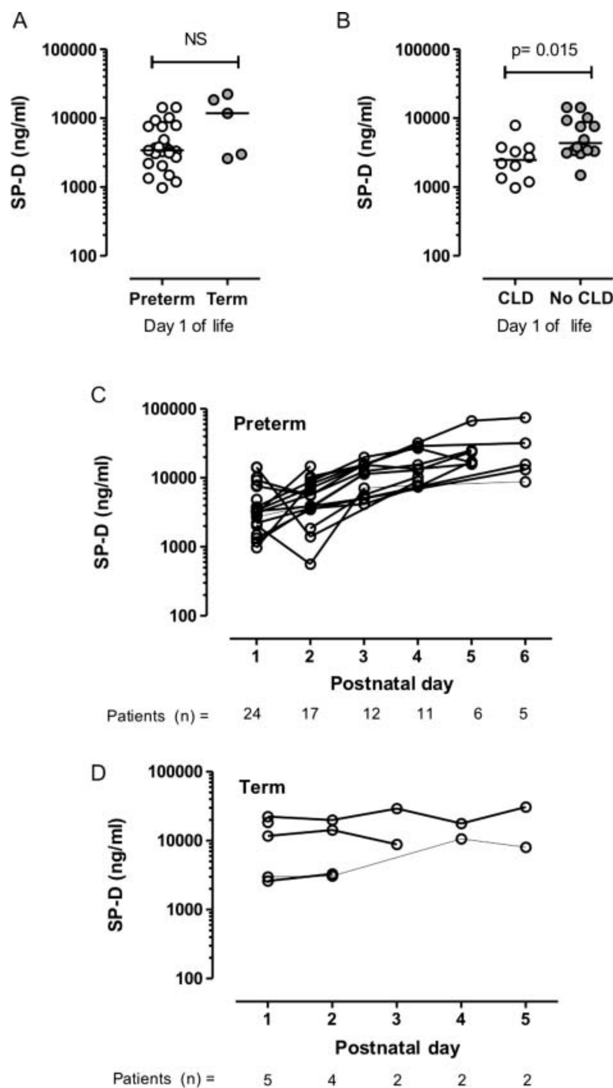

Figure 1 Surfactant protein D (SP-D) expression in neonatal bronchoalveolar lavage fluid (BALF): SP-D was quantified by ELISA in the indicated number of BALF samples from 28 preterm and five term infants. (A) SP-D concentration on day 1 of life in preterm (n=24) and term infants (n=5). (B) SP-D concentration on day 1 of life was lower in preterm infants who developed chronic lung disease (n=10) than those who did not (n=14). (C and D) SP-D concentration was plotted against the day of life for preterm and term infants. In all cases data points for SP-D are the average of duplicate measurements. Note that SP-D concentration is plotted on a logarithmic axis. CLD, chronic lung disease.

the case of assays with Poractant Alfa, rabbit anti-SP-D (5 μg/ml) in TBST-C for 1 h at room temperature. Wells were washed three times with TBST-C prior to incubation with HRPO-conjugated donkey anti-goat IgG or donkey anti-rabbit IgG in TBST-C for 30 min at room temperature. Wells were washed with TBST-C, developed with TMB substrate (eBioscience, Hatfield, UK) and quenched with 1M H_2SO_4 prior to reading at 450 nm on a MRX TC Revelation plate reader (Dynex Technologies, West Sussex, UK).

Statistical analysis

All data are expressed as medians and IQRs unless otherwise stated. Statistical analysis was performed with GraphPad Prism V.5.01. Differences in the medians of continuous data were analysed by Mann–Whitney U test. In all cases, significance was achieved at p values <0.05.

RESULTS

Expression of SP-D in BALF from term and preterm ventilated infants

Full patient demographics are described in table 1. SP-D expression in term BALF on day 1 of life (11 730 ng/ml; 2788–20 340) was greater than in preterm BALF (3401 ng/ml; 2330–7582), but not significantly so (figure 1A). SP-D expression was significantly lower on day 1 of life in preterm infants who developed CLD (2460 ng/ml; 1301–3696) compared with those who did not (4334 ng/ml; 3265–9478; p=0.015) (figure 1B). Expression increased over the first 6 days of life in the majority of infants, particularly in the preterm cohort (figures 1C,D).

An assay to measure the lectin activity of SP-D

The ability of native purified SP-D to interact with zymosan and migrate to a pellet with these particles under centrifugation was tested. Following SDS-PAGE and western blot of pellet and supernatant fractions under reducing conditions a band of approximately 48 kDa, corresponding to monomeric subunits of SP-D, mobilised to the zymosan pellet in the presence Ca^{2+} (figure 2A and see online supplementary figure S1). The highest concentration of SP-D tested in preliminary experiments was 20 μg/ml (data not shown). The majority of SP-D remained in the supernatant fraction in the presence of EDTA and maltose, confirming the calcium and carbohydrate dependence of the interaction (figure 2A and see online supplementary figure S1).

A large proportion of BALF SP-D from term and preterm infants is incapable of binding to zymosan

Zymosan binding activity was tested in 80 BALF samples from 28 preterm infants and 16 BALF samples from five term infants. In addition to zymosan-bound SP-D, a band of identical molecular weight was observed in supernatant fractions from all BALF samples tested (figure 2B). A minor band approximately 2 kDa below the main band was frequently observed in pellet and supernatant fractions (figure 2B) while a band mobilising 4–5 kDa higher than the main SP-D monomer was often observed, but only in supernatant fractions (figure 2B). Sedimentation of SP-D did not occur independently of zymosan interactions which were calcium and carbohydrate dependent (figure 2C). Supernatant fractions containing non-bound SP-D did not exhibit substantial additional binding in a second round of zymosan binding, ruling out the possibility that ligand binding sites on the initial zymosan particles had been saturated (see online supplementary figure S2).

Therapeutic surfactant or other factors intrinsic to BALF do not inhibit SP-D zymosan binding activity

Pulmonary surfactant, previously reported to interact with SP-D,[24] had no effect on SP-D binding to mannan in a solid phase assay (see online supplementary figure S3). Furthermore, BALF samples in which all or most of the SP-D was found in the supernatant fraction were unable to inhibit exogenously spiked native SP-D binding to zymosan (figure 2D). These data suggest that an intrinsic inhibitor of SP-D binding within such BALF samples was not responsible for the lack of zymosan binding activity.

SP-D binding activity in term and preterm infants increases over the first week of life

Zymosan binding assays undertaken for all term and preterm BALF samples collected over the first 6 days of life were

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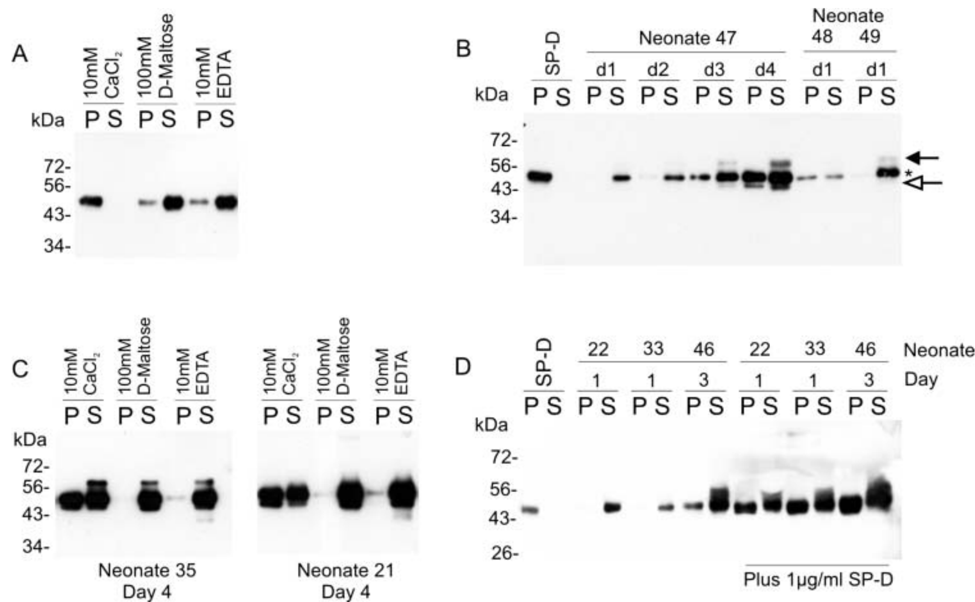

Figure 2 An assay for surfactant protein D (SP-D) lectin activity reveals significant amounts of SP-D in neonatal bronchoalveolar lavage fluid (BALF) which does not bind to zymosan. (A) Western blot of pellet (P) and supernatant (S) fractions from zymosan binding assays reveals binding of native SP-D to zymosan is inhibited by calcium chelation with ethylenediaminetetraacetic acid (EDTA) and by 100 mM D-maltose. A major band migrating between 43 and 56 kDa corresponds to the expected molecular weight for fully reduced SP-D monomeric subunits. (B) Native SP-D or BALF from preterm infants was added to a washed zymosan pellet in the presence of 10 mM $CaCl_2$. Bound (P) and non-bound (S) SP-D was visualised by western blot of respective fractions. In the native SP-D positive control, a major band migrating between 43 and 56 kDa (asterix) was observed only in the pellet fraction. An equivalent major band was observed in pellet and supernatant fractions from BALF. In some BALF samples all SP-D reactive material was present in the non-bound supernatant fraction (neonate 47, day 1 and 2; neonate 49, day 1). In others this band was present in bound pellet fractions and non-bound supernatant fractions. An additional band migrating 3–5 kDa above the major SP-D reduced monomer (closed arrow) was frequently observed, but exclusively in supernatant fractions (neonate 47, day 3 and 4; neonate 49, day 1). A further band migrating 1–2 kDa below the main SP-D band (open arrow) was also frequently observed but was present in supernatant and pellet fractions (neonate 47, day 3 and 4; neonate 49, day 1). Data are representative of all bands visible on duplicate blots from at least 21 independent experiments. (C) SP-D from neonatal BALF binds to zymosan in a D-maltose and EDTA inhibitable fashion. Blots represent two of three independent experiments. (D) Neonatal BALF containing SP-D which was predominantly unable to interact with zymosan (lanes 3–8) was spiked with native SP-D (1 μg/ml) for 30 min at 37°C, before adding it to a freshly washed zymosan pellet. The exogenous SP-D retained its capacity to interact with zymosan in the presence of BALF from neonates with endogenous SP-D which was unable to interact with zymosan (lanes 9–14). Data are representative of three independent experiments.

performed in duplicate to confirm reproducibility (representative western blots are presented in online supplementary figure S4A–D). Densitometry following assay of all available samples revealed the percentage of SP-D capable of binding to zymosan to be more than three times higher in term infants on day 1 of life (47.85%; 21.39–48.51) compared with preterm infants (13.35%; 2.03–28.13) (figure 3A). Close to 100% of positive control native SP-D mobilised to the pellet in these assays (data not shown). Binding activity on day 1 of life did not differ between infants who developed CLD and those who did not (figure 3B). A pattern of increased SP-D binding activity was evident in preterm BALF over the first 6 days of life, peaking on day 5 (median 50.76%, IQR 29.18–55.40) (figure 3C). A less obvious pattern punctuated by spikes in activity was noted in the term infants followed over the first 5 days of life (figure 3D).

Combining these data with those from the ELISA, the amount of SP-D capable of binding to zymosan was seen to increase substantially over the first 5 days of life, particularly in preterm infants (figure 4C,D). The concentration of SP-D with binding activity was almost 10 times lower in preterm infants (373 ng/ml; 83–1537) than term infants (3668 ng/ml; 1030–7210) on day 1 of life (p=0.014) (figure 4A). Substantial (but not significant; p=0.12) differences in SP-D binding zymosan were observed between those who developed CLD (237 ng/ml; 27–399) and those who did not (1105 ng/ml; 143.5–1990) (figure 4B).

A large proportion of SP-D from preterm BALF also fails to bind to maltose-agarose

Maltose-agarose, a well described affinity matrix for SP-D, did not support the binding of a significant proportion of SP-D in BALF and in some cases supported the binding of less SP-D than zymosan binding assays carried out in parallel (see online supplementary figure S5). As observed in zymosan binding assays, an additional band mobilising 4–5 kDa higher than the main SP-D monomer was also observed in non-bound supernatant fractions (see online supplementary figure S5). A minor band migrating 1–2 kDa below the major band was also observed in pellet and supernatant fractions from both assays (see online supplementary figure S5).

SP-D which fails to bind zymosan exhibits characteristics of sub-dodecameric oligomeric form

SP-D in supernatant and pellet fractions from zymosan binding assays was examined by SDS-PAGE under non-reducing conditions to assess differences in oligomeric form (figure 5A). The majority of zymosan-bound SP-D was characterised by highly oligomerised forms too large to enter the running gel (figure 5B). By contrast, SP-D which did not bind to zymosan migrated with the predicted mass of trimers and also included apparent dimeric and monomeric material, indicating the presence of lower oligomeric forms (figure 5B). Monomeric material was also occasionally observed in the bound, pellet fraction.

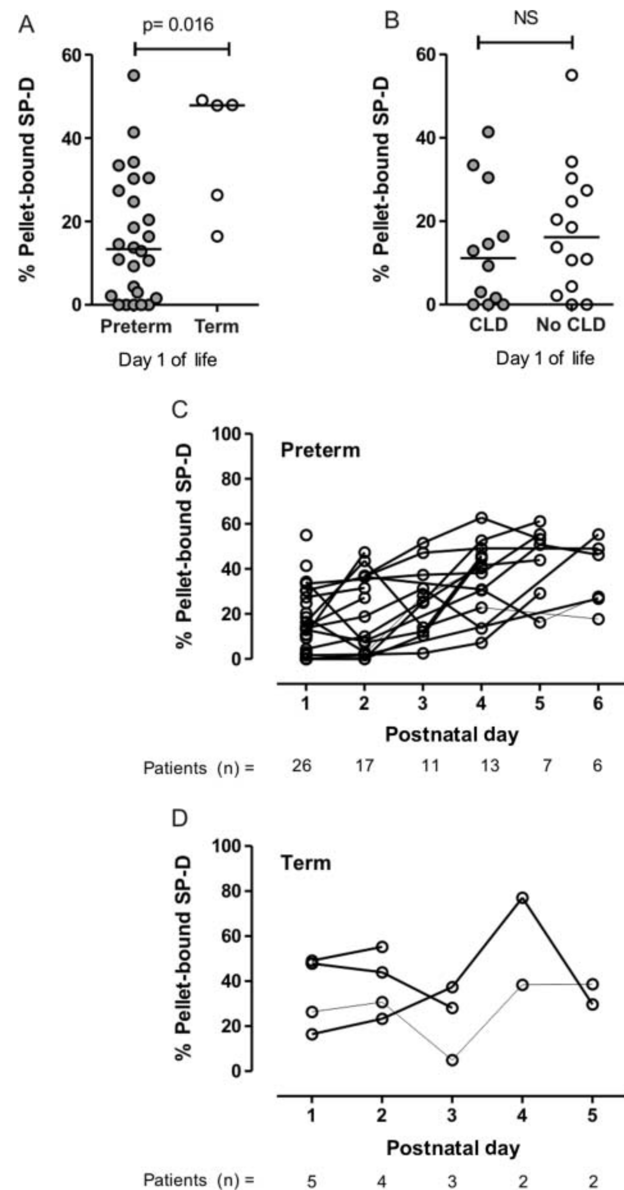

Figure 3 Analysis of surfactant protein D (SP-D) binding activity in all neonatal bronchoalveolar lavage fluid (BALF). Two independent zymosan binding assays were performed on each BALF sample from 28 preterm infants and five term infants. Native SP-D was used as a positive control in all assays. SP-D in bound pellet and non-bound supernatant fractions was visualised following sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and western blot. Individual western blots for each experiment were subjected to densitometry to quantify the relative proportions of bound and non-bound SP-D. Data presented refer to the percentage of total SP-D density associated with the pellet-bound fraction. (A and B) The percentage of zymosan bound SP-D was significantly higher in BALF from term infants on the first day of life (n=5) compared with preterm infants (n=26, p=0.016) but did not differ between infants who developed chronic lung disease (n=12) and those who did not (n=14). Bars refer to medians. (C and D) The percentage of pellet-bound SP-D for the indicated number of BALF samples was plotted against postnatal day for preterm and term infants.

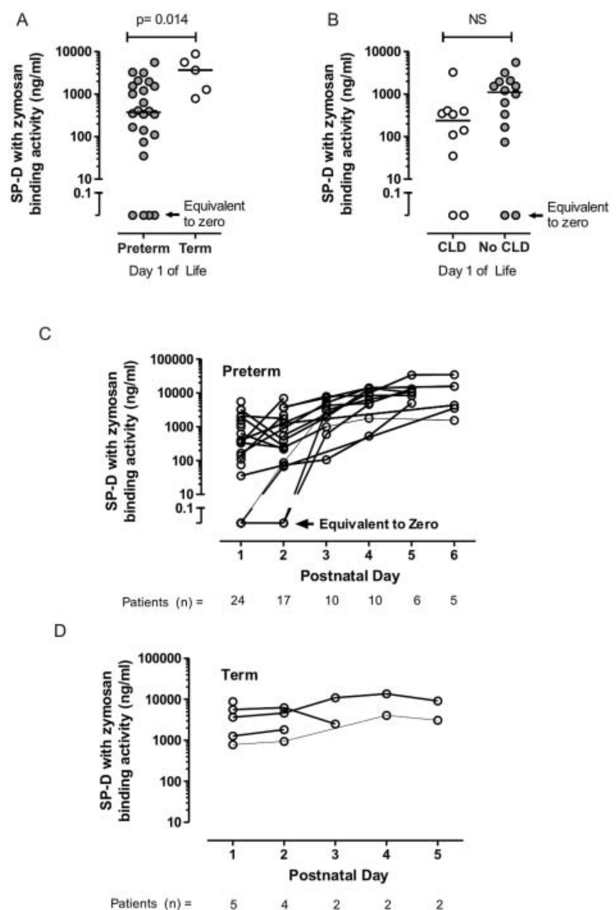

Figure 4 Total surfactant protein D (SP-D) with zymosan binding activity is higher in term than preterm neonatal bronchoalveolar lavage fluid (BALF). Data from ELISA presented in figure 1 and the zymosan binding assay described in figure 3 were used to calculate the amount of SP-D in BALF with zymosan binding activity. (A) Term neonatal BALF (n=5) contained significantly more SP-D with the capacity to bind zymosan on day 1 of life compared with preterm neonatal BALF (n=24, p=0.014). (B) The concentration of SP-D with zymosan binding activity in BALF from infants who subsequently developed chronic lung disease (n=10) was not significantly different from those who did not (n=14, p=0.12). (C) Preterm neonates exhibited a pattern of increasing concentrations of SP-D capable of binding to zymosan over the first 6 days of life. (D) The amount of SP-D capable of binding to zymosan in BALF from term neonates remained relatively stable across the first 5 days of life.

SP-D which fails to bind zymosan is also unable to bind mannan

SP-D in supernatant fractions from zymosan binding assays was tested in plate-based mannan binding assays. BALF with approximately 50% of SP-D capable of interacting with zymosan was selected for these assays (zymosan binding assays run in parallel are shown in online supplementary figure S6B). Following zymosan binding, supernatant fractions were adjusted to achieve 1 μg/ml SP-D (ELISA equivalent) and this was used as source material for binding assays. Also included was non-assayed BALF adjusted to 2 μg/ml total SP-D (giving the equivalent of 1 μg/ml of material capable of binding to zymosan and 1 μg/ml of non-binding material). Native SP-D was used as a positive control. In all cases, post-zymosan supernatant fractions exhibited negligible binding to mannan (see online supplementary figure S6A). By contrast, SP-D from equivalent non-assayed BALF exhibited EDTA and maltose inhibitable binding to mannan comparable to native SP-D.

DISCUSSION

We have presented the first systematic evaluation of SP-D functionality in any clinical cohort. Our data reveal substantial and

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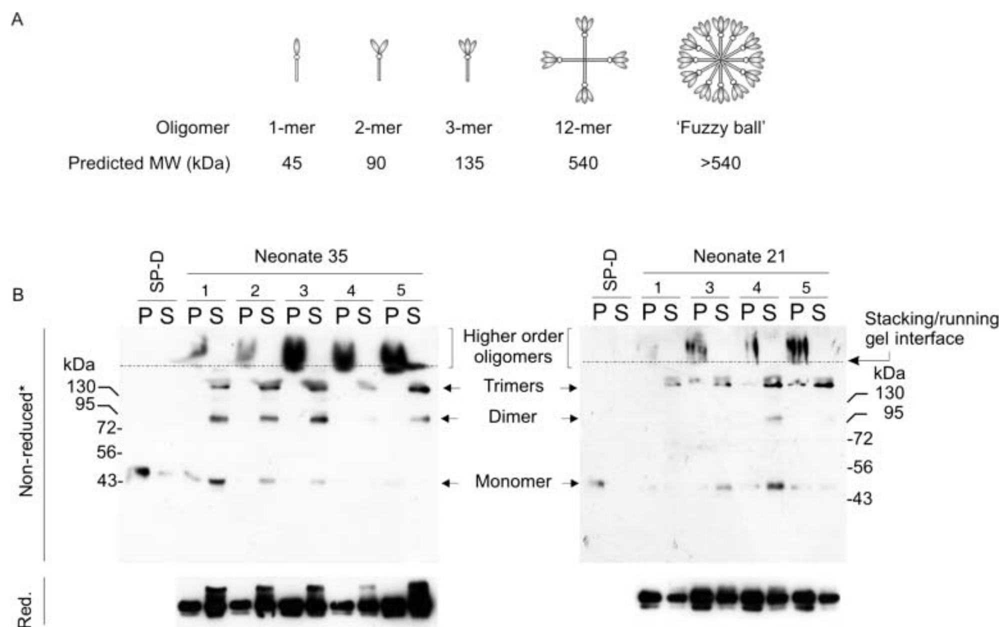

Figure 5 Surfactant protein D (SP-D) which fails to bind zymosan is of a lower oligomeric state than bound SP-D. (A) Schematic representation of oligomeric variants of SP-D and their approximate predicted molecular weights (MWs). These MWs can differ from those observed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) due to differences in post-translational modification, buffer system and molecular weight standards used. (B) A series of bronchoalveolar lavage fluids from two different neonates were assessed in a zymosan binding assay and pellet-bound and supernatant non-bound fractions were subsequently separated by SDS-PAGE under non-reducing (top panels) or reducing (bottom panels) conditions, followed by western blot for SP-D. *The native SP-D positive control was only run under reducing conditions and is presented in the upper panels. Dashed lines on non-reduced gels indicate the approximate position of the interface between the 4% stacking gel and 10% running gel. Under non-reducing conditions SP-D eluted from the zymosan pellet appears predominantly either as large oligomers which fail to enter the running gel (neonate 35) or as a combination of these large oligomers and forms migrating with a MW indicative of trimers (neonate 21). This denatured banding pattern indicates the presence of dodecameric and larger forms. By contrast all material in the non-bound supernatant fractions consisted of bands consistent with trimeric, dimeric and monomeric forms of SP-D, indicative of lower oligomeric forms. Occasionally, monomeric forms were also visible in the zymosan-bound pellet fraction (neonate 21, days 4 and 5). Data for non-reducing conditions are representative of three independent experiments.

significant reduction of SP-D expression in preterm infants who develop CLD and also identifies a significant restriction of SP-D lectin activity in preterm infants on day 1 of life related to the oligomeric state of that molecule.

BALF was collected from preterm and term infants undergoing mechanical ventilation as previously reported,[21] and according to published guidelines.[25] Although the cohort under investigation was relatively small, the reduced SP-D expression in preterm infants who develop CLD and the increased SP-D expression over the first 6 days of life broadly agree with data from the only previous study in a similar population.[20] Although SP-D expression varied considerably in the limited number of term infants available to us, a pronounced increase in expression over the first 5 days was not observed. Increased BALF SP-A and SP-B expression over the first week of life has also been reported in a preterm population, [20] suggestive of increased secretory protein expression as the lung matures. In addition to being more gestationally mature, only term neonates exposed to ≤28% O_2 during respiratory support were included here and so were subject to milder ventilation than the preterm cohort. Lung maturation over the first week of life combined with exposure to mechanical ventilation may explain some of the increased SP-D expression in the preterm population, however the limited number of term infants available demands a cautious interpretation until a larger cohort enables a more detailed analysis. SP-D expression in lung tissue increases in late gestation and is glucocorticoid responsive in humans [26] and rodents.[27] Here, SP-D expression in preterm infants on day 1 of life did not correlate with gestational age and did not differ depending on mode of delivery or in those born to mothers who received antenatal steroids (>24 h) (data not shown). Furthermore, although interactions between SP-D and inflammatory cells have been reported,[5 28] statistically significant differences in total cell, neutrophil or macrophage numbers were not observed between infants who developed CLD and those who did not on day 1 (data not shown), suggesting that separation of cells and supernatant during BALF processing does not explain differences in SP-D expression between groups, a possibility experimentally ruled out in other inflammatory lung diseases.[29]

In addition to this quantitative deficiency, a significant proportion of term and preterm BALF SP-D failed to bind the *Saccharomyces cerevisiae* derived particle, zymosan. Zymosan saturation or a competing factor within the BALF did not appear to explain the functional defect and the SDS-PAGE migration pattern did not suggest proteolytic degradation, previously reported to alter SP-D activity.[10] However, the frequent presence of an apparent isoform of SP-D migrating 4–5 kDa above the main monomeric subunit was reminiscent of a differentially glycosylated 50 kDa form of SP-D described by Mason *et al*[30] and thought to limit SP-D oligomerisation to trimers. Furthermore, SP-D which failed to bind zymosan migrated on SDS-PAGE with a pattern previously reported for low oligomeric forms of SP-D,[7] consisting predominantly of subdodecameric species. Conversely, zymosan-bound SP-D migrated with a pattern described for dodecameric and highly oligomerised variants of SP-D.[7] Restricted oligomerisation of SP-D

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limits its ability to interact with a range of pathogens[7 8] and purified carbohydrate ligands,[7 9] however this is the first time this phenomenon has been systematically addressed in a relevant clinical population. The restricted binding activity of SP-D from term and preterm BALF towards maltose-agarose and mannan is also likely to represent lower oligomeric forms as previously reported.[7 9]

In addition to its lectin activity, SP-D exhibits additional activity in regulating inflammatory processes, most clearly evident in the emphysematous, inflammatory lung disease observed in the SP-D knockout mouse.[6 31] The mechanism underlying this phenotype is not fully understood but is thought to involve interactions between SP-D and inflammatory cells either to regulate their activity[5 32] or to promote clearance of apoptotic bodies during inflammatory resolution.[28 33] Genetic reconstitution of knockout mice with a version of SP-D lacking residues required for full oligomerisation failed to rescue the emphysematous phenotype,[34] implying a critical role for full oligomerisation in pulmonary homeostasis. However, recombinant trimeric fragments of SP-D with a minimal collagenous tail region also exhibit significant immunomodulatory activity in vivo[28 35]

We identified substantial variation in the status of SP-D in preterm infants at risk of CLD. Existing therapeutic surfactant used in this population lacks SP-D. Replacement therapy has shown promise in the treatment of endotoxic shock and ventilator-induced inflammation in preterm lambs,[18 19] and pulmonary overexpression of SP-D protects mice from acute hyperoxic lung injury.[17] In this context a quantitative and functional deficiency of SP-D in the preterm population may limit their ability to appropriately regulate pulmonary inflammation, a key factor in the development of CLD. The importance of oligomeric size in mediating the anti-inflammatory effects of SP-D remains unclear but should be further investigated before considering the best approach to SP-D therapy in this population.

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Contributors We confirm that all named authors made a significant contribution to each of the following areas: conception and design, or analysis and interpretation of data; drafting the article or revising it critically for important intellectual content; final approval of the version to be published. We also confirm that no person who fulfils these criteria has been omitted as an author. Eamon McGreal conceived and designed the experimental aspects of the study, undertook experimental work, analysed the data and drafted the manuscript. He acts as guarantor. Philip Davies analysed and interpreted clinical aspects of the study, revised the article for intellectual content and gave final approval of the version to be published. Howard Clark collaborated in the conception and design of experimental aspects of the study, revised the article for intellectual content and gave final approval of the version to be published. Sailesh Kotecha conceived and designed clinical aspects of the study, was involved in analysis and interpretation of the data, revised the article for intellectual content and gave final approval of the version to be published.

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Competing interests None.

Ethics approval South East Wales Research Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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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 ≤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 50µl 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: 10µl 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 10µl of TBS-Ca and 10µl of either freshly thawed BALF or, as a positive control, 10µl of native SP-D diluted to 2µg/ml in 154mM NaCl with 1.5mg/ml protease free BSA (Sigma, Dorset, UK) to give a final

concentration of 10mM $CaCl_2$ in all binding assays. Where Ca^{2+} 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 $CaCl_2$ 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 $CaCl_2$ 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 $CaCl_2$ 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 $CaCl_2$ (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 $CaCl_2$ or, in the case of assays with Poractant Alfa, 50μl rabbit anti-SP-D (5μg/ml) in TBS-Tween/10mM $CaCl_2$. Wells were washed three times with TBS-Tween/10mM $CaCl_2$ prior to incubation with 50μl HRPO conjugated donkey anti-goat IgG or donkey anti-rabbit IgG in TBS-Tween/10mM $CaCl_2$ for 30 minutes at room temperature. Wells were washed three times and developed with TMB substrate (eBioscience, Hatfield, UK). Development was quenched with 1M H_2SO_4 and the plate was read at 450nm on a MRX TC Revelation plate reader (Dynex Technologies, West Sussex, UK).

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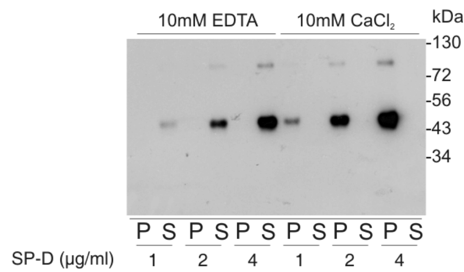
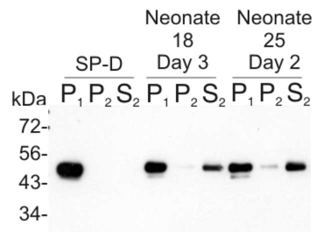
Supplementary Figure 1) An assay for SP-D lectin activity: Western blot of pellet (P) and supernatant (S) fractions from zymosan binding assays reveals binding of increasing concentrations of native SP-D to zymosan is inhibited by calcium chelation with EDTA. A major band migrating between 43 and 56kDa corresponds to the expected molecular weight for fully reduced SP-D monomeric subunits. Minor bands mobilising at approximately 95kDa correspond to partially unreduced SP-D dimers.

Supplementary Figure 1



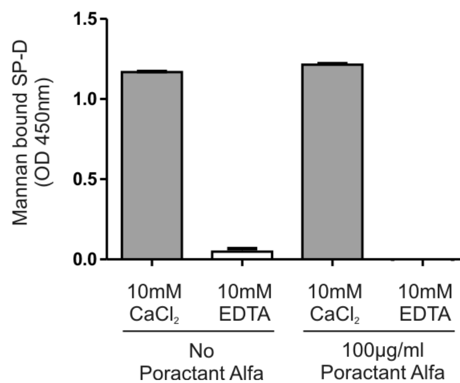
Supplementary Figure 2) Zymosan saturation does not explain the restricted SP-D lectin activity: Following one round of zymosan binding, supernatants from experiments carried out with native SP-D (lanes 1-3) or BALF from two neonates (lanes 4-6 & 7-9) were subjected to a second binding step. Following Western blot under reducing conditions, SP-D found in the supernatant of BALF from the first round showed negligible binding to zymosan in a second round (P_2) and was mainly found in the supernatant from the second round (S_2). All native SP-D bound the zymosan pellet in the first round (P_1). Blots are representative of three independent experiments.

Supplementary Figure 2



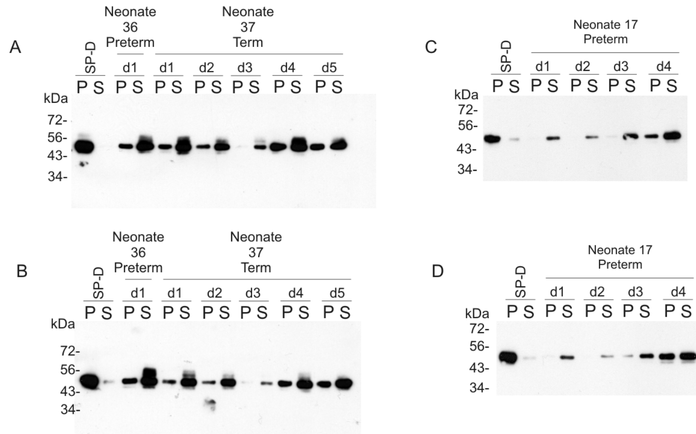
Supplementary Figure 3) Exogenous surfactant does not inhibit native SP-D lectin activity: Mannan (50μg/ml) was coated onto a 96-well plate and used as a substrate to measure the impact of the exogenous surfactant, Poractant Alfa, on SP-D lectin activity. Native SP-D bound equally well to mannan in the presence or absence surfactant, however binding was almost completely abrogated by 10mM EDTA. Data represents the mean of triplicate values from one of three independent experiments.

Supplementary Figure 3



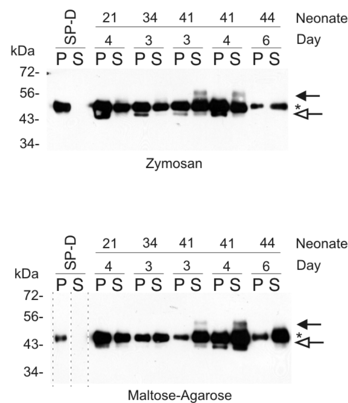
Supplementary Figure 4) Analysis of SP-D binding activity in neonatal BALF: Term and preterm BALFs were assessed in duplicate independent zymosan binding assays. Following SDS-PAGE and Western blot for SP-D, native SP-D positive control was predominantly found in the bound pellet (P) fraction. Occasionally a minor proportion was observed in the non-bound supernatant fraction (S). In the case of BALFs, a large proportion of SP-D was consistently found in the non-bound supernatant fraction. These findings were reproducible across duplicate independent experiments undertaken for all BALFs and representative examples are shown here (A&B, C&D).

Supplementary Figure 4



Supplementary Figure 5) A high proportion of neonatal BALF SP-D also fails to bind maltose-agarose: Neonatal BALF or native SP-D was tested in parallel for binding to zymosan (upper panel) or maltose-agarose (lower panel). SDS-PAGE followed by Western blot of bound pellet fractions (P) and non-bound supernatant (S) fractions revealed that a substantial proportion of SP-D in neonatal BALF failed to bind to maltose-agarose. In some cases (neonate 41, day 3 & 4) less material appeared capable of binding to maltose-agarose than zymosan. Bands migrating 3-5kDa above (closed arrows) and 1-2 kDa below (open arrows) the main reduced monomeric subunit of SP-D (asterix), were visible in the maltose-agarose binding assay as well as the zymosan binding assays. The higher molecular weight band was found exclusively in the non-bound supernatant fraction in both zymosan and maltose-agarose binding assays. Data are representative of 4 independent experiments assessing 15 BALF samples. Dotted lines in the maltose-agarose blot indicate where lanes have been moved within the gel image to facilitate comparison with the zymosan blot above due to a discrepancy in the original loading of SDS-PAGE lanes.

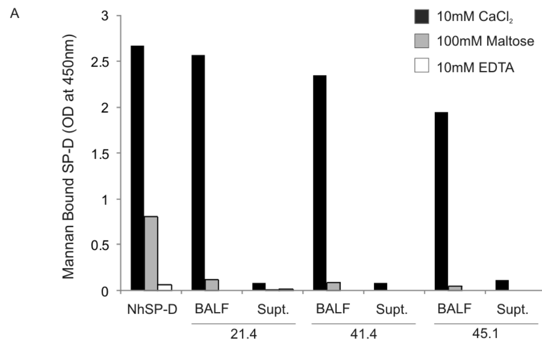
Supplementary Figure 5



Supplementary Figure 6) SP-D which fails to bind zymosan does not exhibit substantial additional binding activity towards mannan: Neonatal BALF containing an approximately equal proportion of bound and non-bound SP-D in zymosan binding assays (panel B) was selected for input into a solid phase mannan binding assay in 96 well plates. Input material for the binding assays were: 1) Native SP-D (NhSP-D) (1µg/ml), 2) BALF (adjusted to achieve 1µg/ml ELISA equivalent each of zymosan-binding and non-binding SP-D) or 3) supernatant (Supt.) aspirated following a zymosan binding assay (adjusted to achieve 1µg/ml ELISA equivalent of SP-D). All input materials were diluted either in TBS-Tween plus 10mM $CaCl_2$ (black bars), 10mM $CaCl_2$ plus 100mM D-maltose (grey bars) or 10mM EDTA (open bars). After a 90 minute binding step, bound SP-D was detected by sequential incubation with goat anti-SP-D and donkey anti-goat-HRPO, followed by the addition of TMB substrate. Presented data are expressed as mannan bound SP-D minus background binding to wells coated with PBS alone.

Supplementary Figure 6

A



B