

Role of elevated plasma soluble ICAM-1 and bronchial lavage fluid IL-8 levels as markers of chronic lung disease in premature infants

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Abstract

Background – Pulmonary neutrophilia characterises both the relatively transient inflammation associated with infant respiratory distress syndrome (IRDS) and the persistent inflammation of chronic lung disease. The possibility that persistently raised markers of inflammation indicate the development of chronic lung disease in low birth weight (<1730 g) pre-term (<31 weeks) infants was therefore investigated.

Methods – Soluble ICAM-1 (sICAM-1) levels in plasma, and interleukin (IL)-8 and myeloperoxidase (MPO) levels in bronchial lavage fluid (BLF) obtained from 17 infants on days 1, 5, and 14 following birth were measured and correlations with the number of neutrophils in BLF sought. Peripheral neutrophils were isolated on Polymorphoprep and chemotactic responsiveness to IL-8 was assessed using micro Boyden chambers.

Results – Sixteen infants developed IRDS and, of these, 10 infants subsequently developed chronic lung disease. Levels of IL-8 in BLF at 14 days of age correlated with the long term requirement for intermittent positive pressure ventilation (IPPV). Interleukin 8 levels in BLF correlated with neutrophil numbers and MPO concentration, suggesting both recruitment and activation in response to this cytokine. Antibody depletion studies showed that approximately 50% of total neutrophil chemotactic activity in BLF was due to IL-8. No difference in peripheral neutrophil chemotactic responsiveness at any age was observed for infants with IRDS or chronic lung disease. Plasma soluble intercellular adhesion molecule (sICAM-1) was higher at 14 days of age in infants who developed chronic lung disease than in those with resolving IRDS, and correlated with severity of disease, as indicated by duration of IPPV.

Conclusions – The results indicate that high levels of plasma sICAM-1 and IL-8 in BLF at day 14 correlate with the development of chronic lung disease and indicate the severity of disease.

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Keywords: chronic lung disease, interleukin 8, soluble intercellular adhesion molecule 1.

Chronic lung disease of the premature neonate is defined as oxygen dependency for 28 days, together with an abnormal chest radiograph.¹⁻³ This condition sometimes develops as a result of exposure to supplemental oxygen and positive pressure ventilation used to treat the respiratory failure of infant respiratory distress syndrome (IRDS) in premature neonates with surfactant deficiency.¹⁻³ Neutrophil accumulation in bronchoalveolar lavage fluid characterises the pulmonary inflammation associated with both diseases.^{4,5} However, the inflammatory processes in IRDS are self-limiting, and the raised number of neutrophils seen in bronchoalveolar lavage fluid at 2-4 days of age resolve completely within one week of birth.⁵ Conversely, neutrophil counts remain high for at least five weeks in chronic lung disease.⁵ Neutrophils are active participants in the inflammatory process and not innocent bystanders as evidenced by parallel changes in neutrophil elastase activity in bronchoalveolar lavage fluid.^{4,5} A functional role for neutrophils in lung damage associated with ventilation has also been demonstrated in an animal model where granulocyte depleted animals failed to develop lung disease after surfactant deficient respiratory distress.⁶

Recruitment of neutrophils from the circulation occurs in four main stages:^{7,8} (1) primary adhesion events or "rolling" of neutrophils on capillary endothelium mediated by neutrophil selectins; (2) chemoattractant-dependent activation of adhesion receptors (integrins) on neutrophils; (3) stable attachment mediated by neutrophil integrin binding to endothelial intercellular adhesion molecules (ICAM-1 and ICAM-2); and (4) diapedesis leading to localisation of neutrophils in the tissue matrix. Directed migration occurs under the influence of local concentration gradients of chemoattractants in tissues. Several neutrophil chemoattractants have been described, including the relatively short-lived lipid mediators leukotriene (LT)₄, platelet activating factor (PAF), and 5-hydroxy eicosatetraenoic acid (5-HETE), and mediators such as activated complement (C5a), formyl-peptides, and members of the chemokine family including IL-8. Some of these – for example, C5a, LT₄ and IL-8 – have been detected in sputum and/or lavage fluid of patients with a number of diseases characterised by a neutrophilic infiltration of the lungs such as cystic fibrosis,^{9,10} ARDS,^{11,12} and

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Table 1 Clinical parameters of patients in the study

Patient no.	Birth weight (g)	Gestational age (weeks)	Antenatal steroid	IRDS	Exosurf	IPPV (days)	Days in O ₂	CLD	Postnatal steroid
1	1100	30	No	Yes	Yes	2	4	No	None
2	1040	28	3 h	Yes	Yes	5	9	No	None
3	1300	28	3 h	Yes	Yes	4	7	No	None
4	1260	28	3 h	Yes	Yes	4	71	Yes	None
5	1120	28	8 h	Yes	Yes	15	56	Yes	None
6	940	26	No	Yes	Yes	28	82	Yes	4 wks
7	1320	28	No	Yes	Yes	16	105	Yes	8 wks
8	970	28	No	Yes	Yes	8	112	Yes	None
9	1620	30	3 days	No	No	0	0	No	None
10	1340	30	6 wks	Yes	No	0	5	No	None
11	1200	30	6 wks	Yes	No	1	168	Yes	None
12	1730	31	1 h	Yes	No	1	4	No	None
13	1270	29	3 h	Yes	Yes	9	150	Yes	8 wks 16 wks
14	1660	30	6 h 18 h	Yes	Yes	4	5	No	None
15	780	26	None	Yes	Yes	28	41	Yes	None
16	750	26	None	Yes	Yes	30	36	Yes	None
17	820	24	None	Yes	Yes	64	64	Yes	2 wks

IRDS = infant respiratory distress syndrome; CLD = chronic lung disease; Exosurf = synthetic surfactant.

bacterial pneumonia.^{12,13} Furthermore, increased levels of LTB₄, 5-HETE, PAF,^{14,15} C5a,¹⁵ and IL-8^{15,16} have been found in the tracheal aspirates of infants with chronic lung disease. However, chemotaxis of peripheral neutrophils¹⁷⁻¹⁹ and expression of L-selectin and the integrin MAC-1, a counter-receptor for ICAM-1, was decreased in term and preterm neonates compared with adults.¹⁹⁻²¹

Neutrophil migration across endothelial and epithelial cell barriers is dependent on the adhesion receptor ICAM-1, a molecule extensively upregulated in inflammatory disorders.²² Shedding of cell associated ICAM-1 can be induced by inflammatory cytokines²³ and detection of a soluble form of ICAM-1 in the circulation²⁴ has been proposed to be a useful marker of inflammation.²⁵ Thus, raised levels of sICAM-1 have been detected in serum samples from patients with a number of inflammatory disorders²⁶ including bronchial asthma.²⁷ Increased levels of leucocyte-derived L-selectin and endothelial-derived E-selectin in the circulation have similarly been described in systemic inflammation, although organ-specific inflammation did not correlate with increased concentrations of these adhesion molecules in the circulation.²⁶

We therefore investigated bronchial lavage fluid (BLF) levels of IL-8 and serum levels of sICAM-1 as potential markers of the progression of the acute inflammation of IRDS to the chronic phase associated with chronic lung disease. We also compared the IL-8 induced chemotactic responsiveness of peripheral neutrophils from patients with IRDS and those with chronic lung disease.

Methods

PATIENTS

Seventeen infants (26–31 weeks gestation, birth weight 940–1730 g) were consecutively recruited during a five month study which was approved by the local ethical committee. During the study period one infant was not recruited due to the presence of congenital abnormalities. Informed written consent was obtained from both parents at the time of

birth, or as soon as possible thereafter. Without parental consent on day 1, five infants were entered into the study on day 5 and three on day 14. Details of the patients and maternal steroid or infant surfactant therapy are shown in table 1.

STUDY DESIGN

Venous blood (1–2 ml) was obtained on days 1, 5, and 14 and collected into tubes containing EDTA. Bronchial lavage was performed on day 1, 2–4 hours after birth, prior to surfactant administration (Exosurf, an artificial surfactant, 2 × 5 ml/kg), and on days 5 and 14 on those infants who were intubated for intermittent positive pressure ventilation (IPPV) and for whom there were no contraindications. Bronchial lavage was carried out while the infants were supine, with the head turned to the left, and two aliquots (1 ml/kg) of normal saline were introduced via a 5 gauge catheter. Lavage samples were aspirated immediately after each aliquot. In most cases the ventilator circuit was not broken.

The total cell count of BLF was performed on fresh lavage fluid using Kimura stain in a haemocytometer. Differential cell counts were carried out on cytopsin slide preparations fixed in methanol and stained with Haema-Gurr differential stain (BDH Ltd, Poole, Dorset, UK). The remaining BLF was centrifuged at 200 g for 10 minutes and the supernatant was frozen in 100 µl aliquots at –80°C.

Chronic lung disease was diagnosed if the infant received IPPV, had persisting oxygen requirement on day 28, and had an abnormal chest radiograph.¹

BLF dilution factor

The dilution of the epithelial lining fluid by lavage medium was calculated as the ratio of urea concentration in the BLF to urea concentration in the plasma,²⁸ which was measured using an in-house urease-based assay. Concentrations of IL-8 and MPO in BLF and neutrophil numbers were adjusted using this ratio.

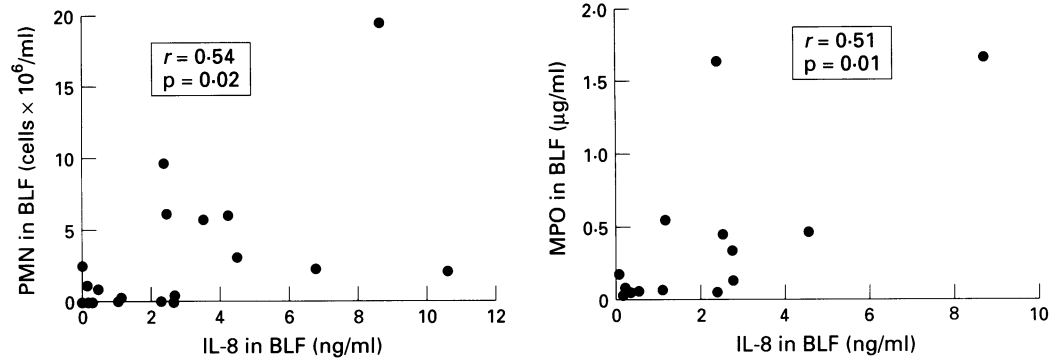


Figure 2 Correlation of IL-8 concentration in bronchial lavage fluid (BLF) with neutrophil number (PMN) and MPO activity.

BLF could be obtained for analysis. No significant difference was detected in the samples obtained on day 1 between infants who subsequently developed chronic lung disease (median 305 pg/ml, range 0–2340 pg/ml) and those in whom RDS resolved (median 2717 pg/ml, range 1066–2738 pg/ml). All of the infants sampled on day 5 were also sampled on day 1, and IL-8 concentrations were significantly ($p < 0.05$) higher than on day 1. In addition, there was a significant ($p < 0.05$) correlation of IL-8 concentration with postnatal age in infants who developed chronic lung disease. It was also evident that at 14 days of age the IL-8 concentration in BLF (median 5519 pg/ml, range 41–10 615 pg/ml) was significantly correlated ($p < 0.05$) with the duration of IPPV (day of extubation indicated in brackets).

The concentrations of free IL-8 measured in BLF samples correlated significantly with both the number of neutrophils and the MPO concentration in BLF (fig 2).

The neutrophil chemotactic activity of five BLF samples from patients with chronic lung disease was assayed using adult neutrophils and was compared with the response to an optimal concentration of IL-8 (fig 3). The neutrophil chemotactic activity of BLF (26.8–52.7 neutrophils/hpf) was greater than that observed with 62.5 ng/ml IL-8 (16 neutrophils/hpf) (fig 4). The highest IL-8 concentration measured in the BLF used was 1.6 ng/ml, indicating that neutrophil chemoattractants other than IL-8 were present in BLF. This was tested by treating the samples of BLF with a neutralising IL-8

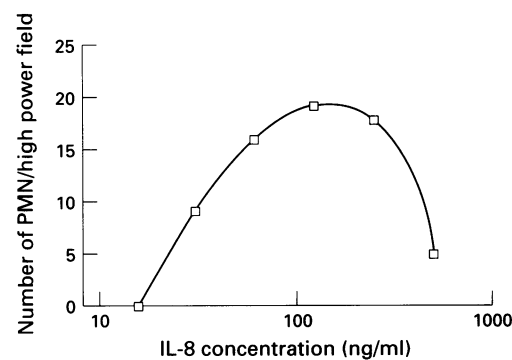


Figure 3 IL-8 dose-response curve. Neutrophils (PMN) from a single donor were used to demonstrate the migratory response of neutrophils to hr IL-8 in Boyden chambers.

antibody. Antibody treatment almost completely blocked (85%) the chemotactic response to IL-8. The specificity of the antibody was demonstrated using fMLP as chemoattractant, for which no inhibition of neutrophil chemotactic activity was observed following antibody treatment. The neutrophil chemotactic activity of BLF was inhibited by 52% (SE 5.8%) by the treatment with IL-8 antibody.

IL-8 IN PLASMA

The peripheral plasma concentration of IL-8 of all the infants in the study remained below 120 pg/ml. There was no trend with postnatal age and no difference between infants with IRDS or chronic lung disease.

SOLUBLE ICAM-1 IN PLASMA

Soluble ICAM-1 concentration in plasma increased in all patients between days 1 and 5. In the group with chronic lung disease there was a highly significant ($p < 0.001$) trend of increasing concentration with postnatal age (fig 5). Additionally, at 14 days of age, levels of sICAM-1 in the plasma of patients with chronic lung disease was significantly ($p = 0.02$) higher than in the group with IRDS. For all patients,

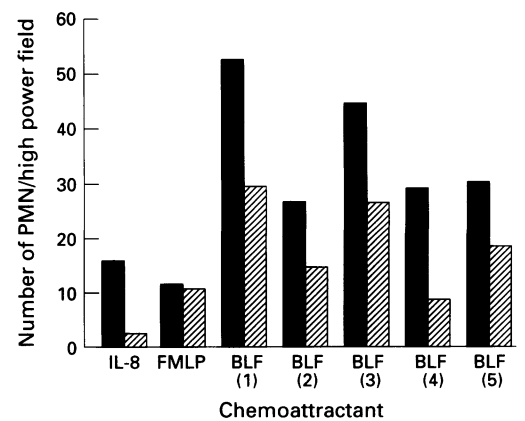


Figure 4 Effect of a neutralising IL-8 antibody on the total neutrophil (PMN) chemotactic activity of bronchial lavage fluid (BLF), assayed using neutrophils from a single donor. Bars indicate response to IL-8 (62.5 ng/ml), fMLP ($10^{-7}M$), and BLF samples ($n = 5$) before (solid) and after (hatched) treatment of chemoattractants with an IL-8 neutralising antibody.

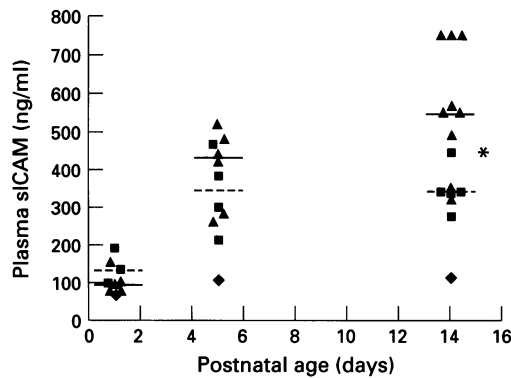


Figure 5 Plasma sICAM concentration as a function of postnatal age in premature babies with IRDS (■), chronic lung disease (▲), or neither disease (◆). The dotted and solid lines indicate the median value for the IRDS and the chronic lung disease groups, respectively. * $p=0.02$ difference between the patient groups.

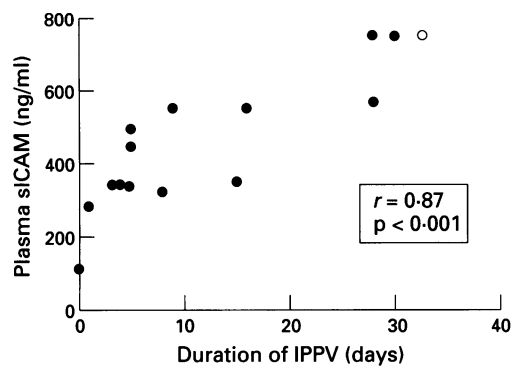


Figure 6 Correlation of plasma sICAM concentration with duration of IPPV. The open symbol indicates the patient who died.

at all ages, sICAM-1 concentrations in plasma correlated highly significantly ($p=0.005$) with the number of neutrophils in BLF. Also, plasma sICAM-1 concentrations at 14 days of age correlated with the severity of disease as reflected by the duration of IPPV (fig 6). In the IRDS group the raised sICAM levels at five days of age did not significantly increase further. The infant who did not require ventilation maintained the lowest level of sICAM in plasma throughout the study.

PERIPHERAL NEUTROPHIL FUNCTION EX VIVO

Neonatal peripheral neutrophil chemotactic responsiveness to IL-8 was determined in preliminary experiments to be maximal at an IL-8 concentration of 0.5 µg/ml (6 nM). At this concentration of IL-8 there was no significant

difference in the magnitude of the response by neutrophils from infants with IRDS or chronic lung disease (table 2), neither was a difference observed in ED₅₀ for neutrophils from each group of patients.

Discussion

Interleukin 8 has been proposed as the major neutrophil chemoattractant in human lung³⁰ and has been associated with a number of inflammatory conditions in adults.¹⁰⁻¹² Recent studies have indicated relatively high concentrations of IL-8 in the pulmonary lavage fluids of premature infants who develop chronic lung disease, ranging from 41.7 ng/ml on day 1 (22 hours after birth),¹⁴ to 11.9 ng/ml at 37 days of age.³¹ Others have reported up to approximately 500 pg/µg secretory component at 15 days of age.¹⁵ These studies did not, however, correlate IL-8 levels in lavage fluids with numbers of neutrophils nor with indices of neutrophil activation such as MPO or elastase activity in lavage fluid. Elastase and MPO activity were previously shown to be increased in chronic lung disease compared with IRDS, but no correlation with IL-8 concentrations was made.³²

We measured lower levels of IL-8 in BLF than previously reported,^{16,31} which probably reflects the specificity of our ELISA reagents for free IL-8, and also the fact that many factors present in BLF (such as immunoglobulins, α₂-macroglobulin, secretory component, heparan sulphate and hyaluronic acid) reduce detectable IL-8 (unpublished data). Assay of uncomplexed IL-8 is relevant since this is an active form of the soluble cytokine, while complexation with IgG autoantibodies, for example, renders the ligand unable to bind to its receptor on neutrophils.²⁹ However, not all complexing molecules inhibit IL-8 activity – for example, heparan sulphate enhances the neutrophil response.³³ Additionally, we found that although secretory IgA reduced detectable IL-8 by 85%, no IgA-induced inhibition of the neutrophil response to IL-8 could be demonstrated (results not shown). We have previously detected IL-8 autoantibodies of both the IgG and IgA class in bronchoalveolar lavage fluid from adults,³⁴ and it is conceivable that immunoglobulins regulate levels of free IL-8 and thus IL-8 bioactivity in the immature lung.

Levels of free IL-8 correlated overall with both neutrophil numbers and MPO concentration in BLF. While not excluding a role for other neutrophil activators in BLF, a role for IL-8 in both recruitment and activation of neutrophils in the inflammation associated with hyperoxia and barotrauma is suggested. This is supported by the observations that IL-8 production is increased by hyperoxia,³⁵ reactive oxygen metabolites,³⁶ and trauma.¹¹ Considering the data obtained at the earliest time point, which was up to four hours after birth, we found no correlation of IL-8 levels with numbers of neutrophils or MPO concentration, nor significant differences between infants who developed IRDS and those who developed chronic lung disease. Others have suggested

Table 2 Peripheral neutrophil IL-8 induced chemotactic responsiveness

Patient group	Postnatal age (days)	n	Neutrophils/hpf	ED ₅₀ (µg/ml)
IRDS	1	3	46.1 (14.6–88.2)	0.11 (0.09–0.20)
	5	5	11.4 (4.6–42.4)	0.18 (0.02–0.50)
	14	4	20.9 (9.1–49.7)	0.06 (0.04–0.20)
CLD	1	5	37 (15.3–73.8)	0.04 (0.03–0.18)
	5	6	10.4 (8.2–19.2)	0.1 (0.02–0.55)
	14	6	13.2 (0–70.6)	0.9 (0.11–0.9)

IRDS=infant respiratory distress syndrome; CLD=chronic lung disease. Values are expressed as median (range).

that measurement of elevated IL-8 concentrations 22 hours after birth is a good predictor of chronic lung disease in premature infants.¹⁵ However, at the four hour time point used in our study, the lack of correlation of IL-8 with other markers of inflammation indicated, firstly, the lack of an inflammatory response at this time point⁵ and, secondly, that IL-8 in BLF may be derived by aspiration of amniotic fluid and therefore of maternal origin.^{37,38}

Interleukin 8 is not, however, the only neutrophil chemoattractant in bronchial lavage fluid and the magnitude of the IL-8 dependent chemotactic response to BLF, compared with hr IL-8 alone, indicates either active IL-8 complexes and/or a synergistic interaction between IL-8 and other neutrophil chemoattractants or activators in BLF. Chemotactic activity that was not inhibited by an IL-8 neutralising antibody may reflect PAF, LTB₄, or 5-HETE activity, previously shown to be present in tracheal aspirates of patients with chronic lung disease,¹⁴ or other neutrophil chemoattractants such as C5a or ENA-78, a chemokine produced by bronchial epithelial cells.³⁹ Although LTB₄ was proposed by Groneck *et al*⁴⁰ to be an important neutrophil chemoattractant in the early stages of chronic lung disease, they observed that late treatment of premature infants at day 16 with dexamethasone resulted in a decrease in neutrophil accumulation in pulmonary fluid which did not correlate with a decrease in LTB₄ levels. The decreased neutrophil chemotactic activity of pulmonary effluent⁴⁰ may, however, be related to the significant decrease in IL-8 levels observed following dexamethasone treatment.³¹ The cellular source of IL-8 in BLF is unknown, but may be derived from bronchial epithelial cells,⁴¹ alveolar macrophages,⁴² or pulmonary fibroblasts.⁴³ Interleukin 8 synthesis by these cells is upregulated by the proinflammatory cytokines IL-1 β and TNF- α and inhibited by dexamethasone. The cytokines IL-1 β ^{40,44} and TNF- α ⁴⁵ were present in bronchial aspirates of preterm infants at low concentrations at birth which increased at day 4 in parallel with other inflammatory processes. Higher concentrations occurred in infants requiring oxygen for a long time,⁴⁵ and levels of IL-1 β and TNF- α were reduced by dexamethasone therapy.^{40,45} These observations support our finding that IL-8 in BLF appears to be a marker of disease progression. In addition to the importance of established cytokine networks in the control of pulmonary IL-8 production,⁴⁶ increasing neutrophil elastase levels⁴⁵ may play a part in amplification of IL-8 production by bronchial epithelial cells⁴⁷ as inflammation progresses to the chronic phase.

ICAM-1 expression is upregulated by inflammatory cytokines²² and is abundant in bronchial endothelium and epithelium.^{48,49} In an animal model, hyperoxia induced increased expression of ICAM-1 on alveolar epithelium.^{50,51} Additionally, raised levels of sICAM in tracheal aspirates of infants with chronic lung disease were suggested to be a consequence of lung injury.⁵² Levels of sICAM-1 in serum are diagnostic for various inflammatory and

immune disorders,^{25,26} and our results indicate that plasma sICAM-1 levels may also be diagnostic for the development of chronic lung disease. Higher levels of plasma sICAM-1 in infants with chronic lung disease than in those with IRDS were significantly correlated with the severity of disease, as determined by the subsequent IPPV requirement. A significant increase in plasma sICAM-1 levels was observed on day 5 in infants with IRDS and chronic lung disease. Those with IRDS, in whom inflammation resolved, showed no further increase in this parameter. Only in those with chronic lung disease was a significant increase in plasma sICAM observed at day 14. Thus, sICAM-1 in plasma may reflect endothelial damage at sites of inflammation, particularly in infants with chronic lung disease where persistent neutrophil infiltration of the lungs appears to be mediated by sustained elevated levels of IL-8. The lack of difference in neutrophil chemotactic response to IL-8 indicated that this neutrophil function was not responsible for differences in the course of inflammation in the patient groups. Our experiments did not, however, take into account the possible differential expression of adhesion molecules or other functions in neutrophils from the two groups. Thus, we propose that increased levels of IL-8 in BLF and sICAM-1 in plasma mark the progress of IRDS to chronic lung disease. Factors which may be important in the regulation of inflammation in IRDS include alpha-1 protease inhibitor⁴⁵ and IL-1 β receptor antagonists.⁴⁴ In view of the importance of IL-8 in the development of chronic lung disease, we propose that IL-8 autoantibodies in BLF may be important regulators of the activity of this cytokine and this is the subject of our current research.

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