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Interleukin-4 and 13 concentrations in infants at risk to develop Bronchopulmonary Dysplasia

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Abstract

Background: An exaggerated inflammatory response occurs in the first few days of life in infants who subsequently develop bronchopulmonary dysplasia (BPD). The increase of inflammatory cytokines in many disease processes is generally balanced by a rise in anti-inflammatory cytokines. Interleukin-4 (IL-4) and interleukin-13 (IL-13) have been shown to inhibit production of several inflammatory cytokines important in the development of BPD.

Methods: We sought to determine if a correlation exists between the presence or absence of IL-4 and IL-13 in tracheal aspirates (TA) during the first 3 weeks of life and the development of BPD in premature infants. Serial TAs were prospectively obtained from 36 very low birth weight infants and IL-4 and IL-13 concentrations were determined by ELISA.

Results: Infants who developed BPD (n = 19) were less mature (25.3 ± 0.02 wks vs. 27.8 ± 0.05 wks; $p < 0.001$), and had lower birth weights (739 ± 27 g vs.1052 ± 41 g; $p < 0.001$). IL-4 and IL-13 were detectable in only 27 of 132 and 9 of 132 samples assayed respectively. Furthermore, the levels detected for IL-4 and IL-13 were very low and did not correlate with the development of BPD.

Conclusions: TA concentrations of IL-4 and IL-13 do not increase significantly during acute lung injury in premature infants.

Background

An exaggerated inflammatory response occurs in the first few days of life in infants who subsequently develop bronchopulmonary dysplasia (BPD). This response includes an increase in airway protein, inflammatory cells and cytokines [1–7]. Increased concentrations of inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), macrophage inflammatory protein 1α (MIP-1α), tumor necrosis factor-α (TNFα) and interleukin-8 (IL-8) have been found in the tracheal aspirates (TA) of infants who developed BPD [3–8]. Additionally, the magnitude of the inflammatory response seems to correlate with the infant's ability to recover fully from acute lung injury that occurs with hyaline membrane disease.

Inflammatory responses may be modulated by several anti-inflammatory or otherwise immunomodulatory cytokines. The role of these cytokines in the pulmonary inflammatory response of the preterm newborn with hyaline membrane disease or its progression to BPD has had little study. Interleukin-10, which inhibits cytokine production from activated macrophages, is undetectable or

present only at very low concentrations in the TAs from preterm infants during the first 3 days of life [9]. These observations suggest an absence of immunomodulatory cytokines, particularly those that down regulate inflammation in the premature infant's lung, may play a role in the development of BPD.

Interleukin-4 (IL-4) and interleukin-13 (IL-13) are structurally related cytokines that share similar properties and receptor components [10]. The actions of these cytokines are pleiotropic, some may be beneficial in moderating lung inflammation, and others may be detrimental. For example, IL-4 and IL-13 have both been shown to inhibit production of several inflammatory cytokines that may be important in the development of BPD in vivo and in vitro [11–18]. However, increased IL-4 and IL-13 expression and production is associated with the development of bronchial hyperreactivity and asthma, which are significant long term complications of BPD [19,20].

Since information on the possible role of IL-4 and IL-13 in the pathogenesis of BPD is unknown, we designed a study to determine the role of these cytokines in the development of BPD. Specifically, we prospectively determined tracheal aspirate concentrations of IL-4 and IL-13 during the first 3 weeks of life in mechanically ventilated very low birth weight infants who did and did not develop BPD. Our goal was to determine if a correlation exists between the presence or absence of these cytokines in tracheal aspirates and the development of BPD in premature infants.

Methods

The study population consisted of very low birth weight infants admitted to the neonatal intensive care unit (NICU) at the Louisiana State University Health Sciences Center in Shreveport. Patients were admitted to the NICU between February 1997 and March 1998. Eligibility criteria included: a birth weight of less than 1500 grams, mechanical ventilation (MV) during the first day of life, and informed consent. Infants who were not expected to survive for 24 hours and those with major respiratory, cardiovascular or neurological anomalies were excluded. The study was approved by the Institutional Review Board for Human Research at LSUHSC.

Tracheal aspirates were collected when clinically indicated. Sterile normal saline (0.5 ml) was instilled into the endotracheal tube and 3–4 manual breaths were provided using a self inflating bag. Airway secretions were aspirated into sterile traps and any material remaining in the catheter was washed into the trap with an additional 0.5 cc saline. Serial aspirates were obtained at 0–24 hours, 48–72 hours, 4–5 days, 7–9 days, 12–14 days and on day 21 if the infant remained on mechanical ventilation. Routine bacterial and genital mycoplasma cultures were performed on tracheal aspirates collected in the first 24 hours. All TAs were frozen at -20°C until assayed for cytokines by enzyme linked immunoassay (ELISA).

Clinical management of the infants was directed by the attending neonatologists. The use and timing of steroids and indomethacin prophylaxis were not specified by the protocol. Demographic data collected in this study included; the need for surfactant therapy, duration of MV, use of corticosteroids, incidence of patent ductus arteriosus (PDA), use of indomethacin (prophylactic and therapeutic), presence and severity of intraventricular hemorrhage (IVH), and the presence of periventricular leukomalacia (PVL). Maternal data included mode of delivery, complications of pregnancy, the use of antenatal steroids and antibiotics.

Several definitions of BPD have been used through the years. Initially BPD was defined as oxygen dependency at 28 days of age [21,22]. More recently, the use of oxygen dependency at 36 weeks PCA has been proposed as a more suitable definition of BPD [23]. Both of these definitions however, predict long term respiratory abnormalities [24]. More recently a NIH consensus has proposed new definitions and diagnostic criteria that provide gradation of BPD into mild, moderate and severe forms [25]. Since even mild BPD may presage chronic respiratory problems we used the earlier broader definition of BPD (defined as oxygen dependency at 28 days postnatal age and a chest radiograph showing changes compatible with BPD) [24,21].

ELISA

The recombinant human cytokines IL-4 and IL-13, monoclonal and biotinylated monoclonal antibodies to IL-4 and IL-13 were purchased from Endogen (Woburn, MA). Streptavidin-horseradish peroxidase (Strep-HRP) conjugate was purchased from Pharmingen (San Diego, CA). Poly horseradish peroxidase-streptavidin (PolyHRP-Strep) and dilution buffer were obtained from Endogen. Horseradish peroxidase-conjugated rabbit anti-goat antibody, monoclonal and polyclonal goat anti-human secretory component antibodies, bovine serum albumin (BSA), and oxalic acid were purchased from Sigma Chemical Co (St. Louis, MO). Human secretory IgA was purchased from ICN Pharmaceuticals (Costa Mesa, CA). ELISA plates were obtained from Costar (Cambridge, MA). 2,2'-azino-di [3-ethyl-benzthiazoline-6-sulphonic acid] (ATBS) and 3, 3', 5, 5'-tetramethylbenzidine (TMB) peroxidase substrate kits were purchased from Bio-RAD (Hercules, CA).

Concentrations of coating and detecting antibodies were titrated to optimize assay conditions. The ELISAs were validated in TAs by adding 1000 pg of recombinant cytokine

to a pooled sample of TA with low or undetectable concentrations and comparing measured cytokine concentrations with what was added. Recovery was >80% of added cytokines. The R-squared values of the standard curves for these assays were generally greater than 0.95 with most assays having R-squared value of 0.99. Assays with standard curves with R-squared values less than 0.9 were repeated.

ELISA plates for IL-4 and IL-13 assays were coated with 100 μl of the appropriate monoclonal antibody in PBS (pH 7.4) overnight at 4°C. The coating antibodies were used at the following concentrations: IL-4, 3.4 μg/ml; IL-13, 3.8 μg/ml; and secretory component, 2.0 μg/ml. After coating, the ELISA plates were washed 3 times (5 minutes per wash) with 200 μl of phosphate buffered saline (PBS) containing 0.05% Tween 20 (wash buffer). All subsequent washes were performed similarly. The plates were then blocked using 200 μl PBS – Tween 20 containing 3% (w/v) BSA (blocking buffer) overnight at 4°C. After blocking, the plates were washed 3 times in wash buffer prior to use. All subsequent steps were performed in blocking buffer. Tracheal aspirates and recombinant cytokine standards were diluted in blocking buffer.

For the IL-4 ELISA, 50 μl TA (diluted 1:1 in blocking buffer) or recombinant cytokine standards (7.8–1000 pg/ml) were incubated in antibody coated ELISA plates for 1 hour at room temperature. Without washing or removing standards/samples (in accordance to manufacturers specifications), 50 μl biotinylated monoclonal anti-IL-4 antibody (500 ng/ml) was added and incubated for another 1 hour at room temperature. The plates were washed 4 times and then incubated with Strep-HRP (1:1000) for 30 minutes at room temperature. After washing 4 times, 100 ul TMB substrate was added to develop color. The reaction was stopped by adding 100 μl of 0.2 N sulphuric acid and the plates were read at 450 nm.

For the IL-13 ELISA, 50 μl TA (diluted 1:1 in blocking buffer) or recombinant cytokine standards (3.9–1000 pg/ml) were incubated in antibody coated ELISA plates for 1 hour at room temperature. Without washing or removing standards/samples, 50 μl biotinylated monoclonal anti-IL-13 antibody (500 ng/ml) was added and incubated for another 1 hour at room temperature. The plates were washed 4 times and then incubated with polyHRP-streptavidin conjugate (1:7000 in dilution buffer) for 30 minutes at room temperature. After washing 4 times, the plates were developed as described above.

The ELISAs for secretory component has been previously described [26]. TA samples were diluted 1:400 to 1:6400 (in blocking buffer) for the secretory component assay. Sensitivities of the assays were as follows: IL-4, 15 pg/ml; IL-13, 3.9 pg/ml; secretory component; 3 ng/ml.

Data Analysis

To adjust for variation in collection of tracheal aspirates, cytokine concentrations were normalized to secretory component [27]. Statistical analysis was performed using the SPSS for Windows version 6.0 (SPSS Inc., Chicago, IL). Chi square analysis was used to assess the statistical differences in categorical variables. The Student t-test was used to assess normally distributed variables. The duration of mechanical ventilation, oxygen use and hospitalization were not normally distributed therefore; the Wilcoxon Rank Sum test was used for analysis of these factors. The differences in TA cytokine concentrations were assessed using ANOVA. Differences in IL-4 concentrations between infants with and without BPD at each time point were also assessed post hoc using Wilcoxon Rank Sum test (data not normally distributed) without correction. A probability value of less than 0.05 was considered statistically significant. The data are presented as mean ± standard error of the mean (SEM).

Results

Thirty six (36) patients were enrolled in the study, 19 (53 %) developed BPD. Of the 19 patients with BPD, 3 infants met the criteria for severe BPD, the remainder were mild-moderate [25]. Infants who developed BPD were smaller, of lower gestational age, were more likely to have a tracheal aspirate culture positive for *Ureaplasma urealyticum* (Uu), and were more likely to have received treatment with indomethacin or postnatal dexamethasone (Table 1). *Ureaplasma urealyticum* was isolated from tracheal aspirate cultures in 8 (22%) study infants. Isolation of Uu from the TA was associated with an increased incidence of BPD (Table 1). There were three deaths among the study patients. One infant died at 14 days of age from pulmonary hemorrhage, Uu pneumonia and progressive respiratory failure. Two patients in the BPD group died, one of respiratory failure as a result of Uu infection, pulmonary hemorrhage and fungal sepsis at 43 days of age and another at 49 days of age died of sepsis and multi-organ failure.

IL-4 was detected in 17 of 89 (19.1%) samples from infants who subsequently developed BPD and 10 of 43 (23.3%) samples from infants who did not develop BPD. Raw concentrations of IL-4 ranged from undetectable (<16 pg/ml) to 587 pg/ml. Figure 1A shows the TA concentrations of IL-4 normalized to secretory component for all infants studied. When detected, TA concentrations of IL-4 were uniformly very low (< 6 pg/μg secretory component). TA IL-4 concentrations increased slightly over time in infants who developed BPD but these differences were not significant (Figure 1B). While TA IL-4 concentrations

Table 1: Clinical characteristics of Study Subjects

Characteristic	BPD n = 19	no BPD n = 17	p value
Birth weight (grams)	739 ± 27	1052 ± 42	<0.001
Gestation (weeks)	25.3 ± 0.2	27.8 ± 0.5	<0.001
Surfactant therapy	17 (89)	13 (76)	0.296
Ureaplasma isolated	7 (37)	1 (6)	0.026
Mycoplasma isolated	5 (26)	1 (6)	0.100
Chorioamnionitis	1/9 (11)	4/15 (27)	0.430
Antenatal Steroids	9 (53)	10 (53)	0.999
PDA	9 (47)	4 (24)	0.137
Indomethacin[a]	17 (89)	10 (59)	0.034
Days MV[b]	42 ± 13	7 ± 2	<0.001
Days oxygen[b]	64 ± 12	11 ± 2	<0.001
Days hospital[b]	104 ± 12	69 ± 6	0.009
MV > 28 days	9 (47)	0	<0.01
Pulmonary hemorrhage	6 (32)	0	0.011
Supplemental O_2 at 36 weeks PCA	3 (16)	0	0.061
Postnatal steroids	16 (84)	7 (41)	0.005
Age steroids started (days)	8 ± 2	9 ± 2	0.758
IVH	6 (32)	5 (29)	0.888
PVL	3 (16)	1 (6)	0.316

PDA-Patent ductus arteriosus; MV-mechanical ventilation, IVH-Intraventricular hemorrhage; PVL-Periventricular leukomalacia; PCA-Postconceptional age. [a]Indomethacin was used prophylactically in most infants. Indomethacin was used to close a clinically significant PDA in 2 control and 7 BPD infants. [b]Wilcox Rank Sum test was used because data was not normally distributed. Data are presented as Mean ± SEM or as numbers (percentages)

were slightly higher in infants who developed BPD than those infants who did not develop BPD, these differences were also not significant (ANOVA and post hoc Wilcoxon Rank Sum tests). Additionally, maximal TA concentrations of IL-4 were not different between groups (0.17 ± 0.07 pg/μg secretory component vs 0.85 ± 0.45 pg/μg secretory component, p = 0.183, control vs. BPD). Interleukin-13 was detected in only 9 of 132 TA samples (data not shown). There was no correlation between antenatal or postnatal steroids, gestational age, birth weight or isolation of Uu from tracheal secretions and concentrations of IL-4 and IL-13 (data not shown). Additionally, there were too few patients to analyze IL-4 concentrations in patients with severe BPD.

Discussion

Inflammation is the primary pathological process that precedes the development of BPD. Increased tracheal concentrations of inflammatory cytokines have been reported in infants who develop this disease [3–8]. Interleukin-4 and IL-13 have been shown to modulate lung inflammation in animal models and thus the presence or absence of these cytokines in the lung may be important determinants in the pathogenesis of BPD [16,28].

Interleukin-4 is a pleiotropic cytokine produced predominately by T-lymphocytes and alveolar macrophages [10,29]. Interleukin-4 stimulates production of some cytokines and growth factors while inhibiting others [11–17,30]. Although IL-4 may be inhibitory to some aspects of lung inflammation, the presence of IL-4 may be detrimental by inducing proliferation of, and collagen production by, subsets of lung fibroblasts [31,32]. Further, IL-4 is a central mediator in the development of asthma [20]. We detected very low concentrations of IL-4 in only one fifth of the tracheal aspirates. This suggests that IL-4 does not play a major role in the lung injury, or protection from lung injury, associated with BPD. The role of IL-4 later in the course of the disease was not assessed in this study.

Interleukin-13, which is similar in structure and function to IL-4, is produced by activated TH_2 lymphocytes and alveolar macrophages [10,33]. Interleukin-13 inhibits inflammatory cytokine production in macrophages and epithelial cells and upregulates the anti-inflammatory cytokine, interleukin-1 receptor antagonist [14]. Thus, IL-13 potentially moderates lung inflammation. In adult fibrotic lung diseases significantly increased concentrations of IL-13 can be detected in bronchoalveolar lavage (BAL) fluid suggesting an up-regulation of this cytokine in response to lung inflammation [33]. As with IL-4, it has been shown in animal models that IL-13 protects against inflammatory lung injury [16]. In contrast, IL-13 was rarely detected in tracheal aspirates of premature infants with acute lung injury, and when detected, was well below physiologic concentrations required for biologic activity.

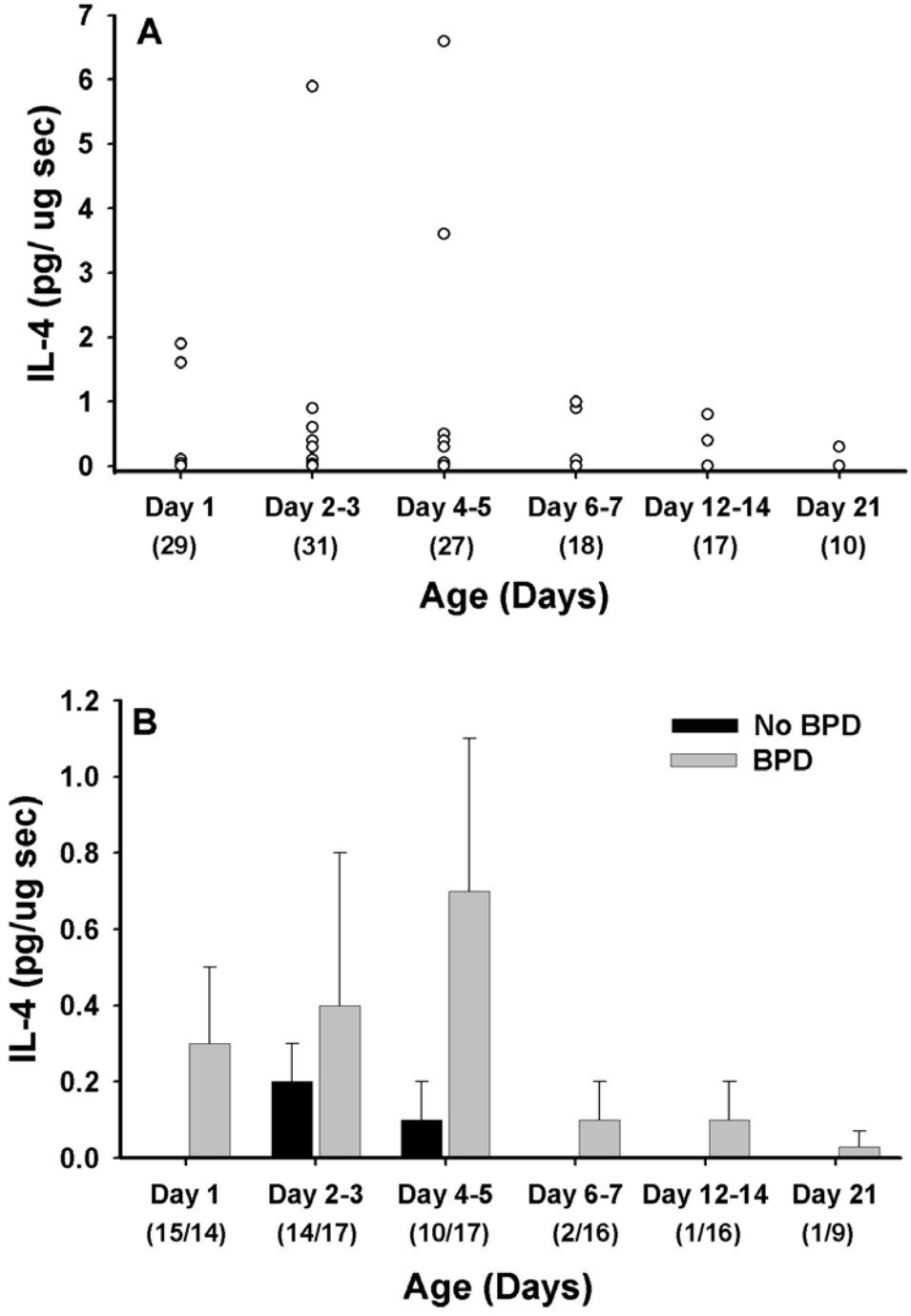

Figure 1
A. Tracheal aspirate concentrations of IL-4 normalized to secretory component in mechanically ventilated very low birth weight infants during the first 21 days of life. Number in parenthesis represents the number of TA samples assayed at each time point. B. Tracheal aspirate concentrations of IL-4 normalized to secretory component in infants who developed BPD (grey bars) and those who did not (black bars) during the first 21 days of life. Concentrations are expressed as pg of cytokine per μg secretory component ± SEM. Number in parenthesis represents the number of TA samples assayed at each time point.

These observations suggest that very premature neonates may be unable to produce physiologic concentrations of this cytokine in the lung. This suggests that proinflammatory processes in the lungs of the premature infant may be relatively unchecked compared to the adult. Thus, as with IL-4, IL-13 does not appear to have a role in modulating pulmonary inflammatory responses in preterm infants at risk of developing BPD.

The mechanisms responsible for the inability of premature infants to increase concentrations of IL-4 or IL-13 in response to a significant lung inflammation are unclear. It could be that the cells that produce these cytokines are not present in the airways and lungs of the premature newborn. Alveolar macrophages, one of the cells types that produce IL-4 are not usually present in the lung at birth in the absence of congential pneumonia but populate the lungs shortly after birth. An influx mononuclear cells and macrophages are part of the inflammatory response that presages BPD [1]. Thus, it seems likely that the right cell types are present in the lung after birth. This suggests that the ability to produce IL-4 or IL-13 in the lung may be developmentally regulated. Therefore, a functionally immature lung may lack the ability to produce these down regulators of inflammation. This may in part explain the exaggerated inflammatory response frequently observed in infants who develop BPD. Consistent with this hypothesis is the report that cord blood mononuclear cells do not produce significant IL-4 in response to endotoxin [34]. Further, we were not able to demonstrate IL-13 production by lipopolysaccharide (LPS) stimulated cord blood cells (unpublished observations).

The role of airway colonization with Uu in the development of chronic lung disease is unclear. Most, but not all studies, suggest that isolation of Uu from tracheal aspirates is associated with an increased incidence of chronic lung disease in very low birth weight infants [35–37]. The frequency of Uu isolation in our infants and the increased incidence of BPD is in agreement with other reports [35–37].

Postnatal steroids were used frequently in this study. At the time when these infants were cared for in our NICU, it was our practice to treat infants who were ventilator dependent for more than 7 days with systemic dexamethasone. Corticosteroid administration (either antenatal or postnatal) could potentially inhibit IL-4 production [38]. We did not observe an effect of antenatal or postnatal steroids on IL-4 concentrations. The lack of effect of postnatal steroids on IL-4 may be due to the time (on average 8 postnatal days) when these were started. At that time IL-4 concentrations were declining.

All studies of this nature suffer from limitations inherent in their design. Tracheal aspirate cytokine concentrations may not reflect what is happening at the alveolus or interstitium of the lung. In addition, values of IL-4 or IL-13 normalized to secretory component do not directly estimate the concentration of these compounds in the epithelial lining fluid (ELF) of the lung. In a few samples the raw concentration of IL-4 was greater than 100 pg/ml. This is within the range of biological activity of IL-4. IL-4 concentrations as low as 10 pg/ml have been reported to cause partial inhibition of LPS induced IL-8 production in peripheral blood monocytes *in vitro*[13]. Thus, it is possible that biologically significant concentrations of these cytokines are present in some infants with developing BPD.

This study examined a relatively small number of tracheal aspirates in a small number of infants. This sample size which was sufficient to detect large differences in cytokine production between BPD and control infants is not sufficiently powered to detect smaller differences [26]. Thus it is possible that smaller magnitude differences in IL-4 (and IL-13) do exist between control and BPD infants and would have been detected with a larger sample. The clinical importance of these small magnitude differences is uncertain. Additionally, most patients had mild-moderate BPD and there were few patients who developed severe BPD. These infants (severe BPD) could have different patterns in IL-4 or IL-13 production that are not obvious in our study.

In clinical practice infants are extubated as soon as feasible to limit potential damage from continuing ventilation. Therefore, infants who have less severe lung disease or recover from their acute lung injury rapidly are not sampled. Thus, there are disproportionately fewer samples and fewer time points in infants who recover or do not develop chronic lung disease. This may introduce bias into our results limiting the conclusions that may be drawn. However, we believe that our results are consistent with other similar clinical studies and in animal models of lung injury providing further insight into the mechanisms of chronic lung injury in the newborn.

Conclusions

In summary we have shown that TA concentrations of IL-4 and IL-13 do not increase significantly during acute lung injury in premature infants. Further studies are required to determine the role (if any) of these and other anti-inflammatory mediators in the pathogenesis of chronic lung disease.

Competing Interests

None declared.

Authors' Contributions

RJB was responsible for the design of the study, laboratory and statistical analysis, primary preparation of the manuscript. JL supervised collection of TA samples, helped with obtaining informed consent and editing of manuscript.

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