Differential Regulation of IL-8 by IL-1 β and TNF α in Hyaline Membrane Disease

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Mechanisms that regulate cytokine-mediated inflammation in the lungs of preterm infants, including factors which regulate production of the chemokine IL-8, remain poorly defined. Sequential bronchoalveolar lavage samples were obtained from preterm newborns with hyaline membrane disease over a 28-day period. Bronchoalveolar lavage cell cytokine relationships were evaluated and the differential regulation of IL-8 by IL-1 β and TNF α was studied in a short-term culture system. *In vivo*, IL-8 and IL-1 β protein levels correlated closely with each other and with macrophage counts. In cell culture, exogenous anti-IL-1 β antibody led to a 40% maximum inhibition (approximately) of IL-8 production by lipopolysaccharide stimulated lung inflammatory cells. Comparable amounts of exogenous anti-TNF α antibodies achieved a 15% maximum inhibition (approximately) of IL-8 production. Anti-IL-1 β and anti-TNF α antibodies in combination did not inhibit IL-8 production beyond that achieved by anti-IL-1 β antibody alone. These results, in preterm newborns, support the concept of lung inflammation mediated in part by a macrophage, IL-1 β , and IL-8 cell cytokine pathway. The results also suggest that factors other than IL-1 β and TNF α regulate IL-8 expression in the lungs of preterm infants.

KEY WORDS: Interleukin (IL)-1 β ; tumor necrosis factor- α ; IL-8; hyaline membrane disease; inflammation.

INTRODUCTION

Premature infants exposed to risk factors such as hyperoxia, barotrauma, and infection frequently develop hyaline membrane disease (HMD) and lung injury. These injuries, occurring in developmentally and structurally immature lungs, are thought to contribute to the pathogenesis of chronic lung disease (CLD) (1–4). A growing body of literature suggests that persistent lung inflammation may play an important role in the pathogenesis of HMD and the subsequent development of CLD (5–7). Proinflammatory cytokines such as tumor necrosis factor- α (TNF α), interleukin (IL)-1 β , IL-6, and IL-8 have been recovered from the bronchoalveolar lavage (BAL) fluid of premature infants with HMD and may be responsible for the recruitment of inflammatory cells into the pulmonary milieu (8–11). In preterm newborns, inflammation and the mechanisms which regulate it remain poorly defined.

The cytokines TNF α , IL-1 β , and IL-8 are believed to play major roles in the pathogenesis of various diffuse inflammatory diseases in mature hosts, such as adult respiratory distress syndrome (ARDS), cystic fibrosis (CF), and sepsis (12–17). Under these conditions, it is speculated that activation of the inflammatory cascade involves the expression of the peptide mediators TNF α and IL-1 β . These proinflammatory cytokines in turn induce and amplify the expression of chemokines such as IL-8 (12, 15). IL-8 is thought to be responsible for the recruitment of inflammatory cells, such as neutrophils, into the pulmonary environment, resulting in tissue injury (18, 19). Although *in vivo* data have demonstrated the presence of all three cytokines in the lungs of preterm newborns, it is unclear if a similar sequential inflammatory pathway is involved in the pathogenesis of HMD. Furthermore, regulation of IL-8 by TNF α and IL-1 β may be different in the neonate with HMD compared to the mature host with ARDS. These differences may be due to

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immature lungs, partially developed host immune responses and developmental regulation of certain inhibitory cytokines, such as IL-10, unique to neonates (8, 20–22).

A sequential paradigm involving TNF α , IL-1 β , and IL-8 is likely to be simplistic considering the multiple biologic activities demonstrated by these cytokines (18, 19, 23, 24). Other cytokines found in the lungs of newborns with HMD, such as IL-6, are also capable of regulating similar inflammatory mechanisms (10, 25). Nevertheless, since this pathway has been studied extensively and found to be intact in many inflammatory diseases such as ARDS and sepsis (12, 15), it is likely that it may also be associated with the pathogenesis of HMD. A better understanding of this system may provide insight into mechanisms of chronic lung inflammation in the newborn and improved treatment modalities.

In this study we devised an *in vitro* cell culture system using BAL-derived inflammatory cells from newborns to determine the regulatory role of IL-1 β and TNF α on IL-8 protein expression. In addition, we measured the number of inflammatory cells and TNF α , IL-1 β , and IL-8 mRNA and protein levels from the same infants over the first 28 days of life to determine if there was any relationship between cell profiles and patterns of cytokine expression.

MATERIALS AND METHODS

Patient Selection

The subjects included in this study were preterm infants, 24–32 weeks of gestation and less than 24 hr of age, admitted to the Neonatal Intensive Care Unit of the LAC + USC Medical Center with a diagnosis of acute respiratory failure and roentgenographic findings consistent with HMD. The diagnosis of acute respiratory failure was defined by the presence of an arterial-to-alveolar partial pressure of oxygen ratio (A/a ratio) <0.4 for 2 hr, the requirement of a mean airway pressure >7 cm H₂O, or the need for a peak inspiratory pressure >30 cm H₂O at a ventilatory frequency of over 40 breaths/min to maintain normocarbia prior to subsequent administration of exogenous surfactant. All infants were intubated and ventilated with a time-cycled, pressure-limited, continuous-flow neonatal ventilator and/or a Sensor Medics 3100 high-frequency oscillatory ventilator. After initial stabilization, BAL was performed in each neonate and at 4-day intervals postintubation. This was continued until extubation or until conditions made BAL sampling contraindicated. The exact timing of the BAL on any particular day depended on the clinical

indications for patient care. All studies were performed with informed consent and approved by the Institutional Review Board of the LAC + USC Medical Center.

BAL Procedure

Three milliliters of normal saline was instilled in two 1.5-ml aliquots through a 5- or 8-French suction catheter introduced via a slide valve in the endotracheal tube connector until resistance was met. After the first aliquot, the infant was oxygenated and a second mini-BAL was performed. The amount of fluid recovered varied between 40 and 80% of the initial lavage instilled. The two aliquots were pooled in one sterile test tube and transported on ice to the laboratory within 30 min for processing.

BAL Sample Processing

Following transport to the laboratory, the samples were immediately centrifuged at 1000 rpm for 10 min. The cell-free supernatants were removed, aliquoted and stored at -80°C , and analyzed within 30 days. The cell pellets were washed in PBS and centrifuged again at 1000 rpm for 10 min. After the wash supernatants were discarded the cells were resuspended in 0.5 ml of RPMI + 10% fetal calf serum (FCS). Ten microliters of this cell suspension was diluted with 10 μl of trypan blue to determine the viable cell count. Cell cytopreps were prepared with 50,000 cells per slide in a Shandon 3 Cytospin at 500 rpm for 5 min. These were then air-dried and Wright-stained for cell differentials. The rest of the cells were frozen in 500 μl of Trizol reagent and kept at -80°C for reverse transcriptase-polymerase chain reaction (RT-PCR), which was performed within 30 days.

BAL Cell Differential Counts

Manual differential counts were determined for each BAL specimen. Following Wright staining, 300 cells per cytopsin were counted manually to determine the cell differential. Absolute cell counts were determined by multiplying the cell differential by the total cell count per milliliter of lavage fluid.

RNA Extraction and RT-PCR Assay

Cytokine mRNA expression from BAL inflammatory cells was analyzed by RT-PCR. Total RNA was extracted from cells frozen in Trizol per the manufacturers' instructions (Life Technologies, Gaithersburg, MD). RNA samples were dissolved in DEPC-treated water and

quantified via spectrophotometry. cDNA was prepared via reverse transcription as follows. Equal amounts of RNA were dissolved in 15 μ l of water, heated at 70 to 80°C for 3 min, and immediately quenched on ice. Reverse transcriptase reaction was performed in a total volume of 30 μ l containing 1.67 μ M oligo(dT), 50 mM Tris-HCl (pH 8.3), 75 mM KCL, 3.0 mM MgCl₂, 0.01 M MDTT, a 0.2 mM concentration of each dNTP, 1 U/ μ l RNase inhibitor, and 10 U/ μ l MMLV reverse transcriptase. The reaction was then incubated for 1 hr at 37°C. TNF α , IL-1 β , and IL-8 primers were obtained from published sequences. PCR reactions were performed in a total volume of 25 μ l using 1 μ l of the cDNA product from each sample. The PCR reaction contained 20 mM Tris-HCl (pH 8.4), 50 mM KCL, a 250 μ M concentration of each dNTP, a 0.2 μ M concentration of each 5' and 3' oligonucleotide primer, and 1 U of Tag DNA polymerase. The optimized cycling conditions were 95°C for 1 min, 65°C for 2 min, and 72°C for 30 sec, for 72 cycles. Agarose gel electrophoresis was used to determine the size of the PCR product, which then underwent DNA sequence determination. The PCR results were not quantitative. The numbers of infants sampled for TNF α , IL-1 β , and IL-8 at the time of intubation and on days 4, 8, 12, 16, 20, 24, and 28 postintubation were 15, 15, 14, 14, 15, 12, 10, and 10, respectively.

Cytokine Protein Quantification

Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) were utilized to measure TNF α , IL-1 β , and IL-8. All kits work on a double-antibody sandwich principle with a peroxidase conjugate detection system and are sensitive to the picogram per milliliter concentration. Duplicate tests were run for each BAL supernatant analyzed. It is important to acknowledge that an acceptable method has not been well established to standardize solute determinations in BAL samples (26). For this reason, we report the cytokine concentrations as weight per volume of lavage fluid, as have other investigators (27). BAL ELISA measurements may vary depending on the percentage of lavage saline that is recovered in the BAL sample. RT-PCR determinations for cytokine mRNA should not be altered by the same processes which can affect standardization of solute concentration. The numbers of infants sampled for TNF α , IL-1 β , and IL-8 (for protein determination) at the time of intubation and on days 4, 8, 12, 16, 20, 24, and 28 postintubation were 15, 15, 14, 14, 15, 12, 10, and 10, respectively.

LPS Stimulation of TNF α , IL-1 β , and IL-8 Production

Eight infants were selected at day 12 postintubation and BAL was performed as described. The cells were processed as described up until the PBS wash. After the wash supernatants were discarded, the cells were resuspended in 500 μ l of complete medium (CM) consisting of RPMI plus 10% FCS and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Ten microliters of cell suspension was diluted with 10 μ l of trypan blue and viable cells were counted via hemocytometer. The cell suspensions were then adjusted to 1×10^6 cells/ml and 50,000 cells used for cytospin. Cell differentials were performed as described.

Two hundred fifty thousand cells in 250 μ l CM were cultured with 250 μ l CM plus 20 ng/ml lipopolysaccharide (LPS), producing a final concentration of 250,000 cells and 10 ng/ml LPS in a total volume of 500 μ l. Two hundred fifty thousand cells were also added to identical volumes of CM to serve as controls. These were incubated at 37°C with 5.6% CO₂ and 21% O₂ for 24 hr. The supernatants were then removed, frozen at -80°C, and analyzed within 30 days. Measurement of TNF α , IL-1 β , and IL-8 levels were performed via ELISA as described. The cell pellets were resuspended in 500 μ l RPMI and cytospins made with 50,000 cells. Cell differentials were performed as described. Six cultures were set up for IL-1 β , eight cultures were set up for TNF α , and eight cultures were set up for IL-8 cytokine determination as described.

Antibody Blocking of Cytokines in Cultured Cells

To determine the effective concentrations of blocking antibody to add to cell cultures, 10 cell culture conditions were set up using inflammatory lung cells from infants on day 12 postintubation. Serial concentrations of human recombinant anti-IL-1 β antibody and anti-TNF α antibody (R&D Systems) were added to individual cultures to determine the concentrations of antibody needed to achieve 20, 50, 80, and 100% inhibition of measurable IL-1 β and TNF α protein levels via ELISA (data not shown). Anti-IL-1 β antibody concentrations of 0.02, 0.2, 2, and 20 μ g/ml and anti-TNF α antibody concentrations of 0.06, 0.1, 1, and 10 μ g/ml inhibited the respective amounts of IL-1 β and TNF α protein in these cultures (Table I). These concentrations of anti-IL-1 β and anti-TNF α antibodies were then used to determine their impact on IL-8 production by LPS stimulated lung inflammatory cells. Culture conditions and cytokine protein measurements were as described in the previous section.

Table 1. Inhibition of Measurable IL-1 β and TNF α Protein by Human Anti-IL-1 β and Anti-TNF α Monoclonal Antibodies

Concentration added to cell culture (μ g/ml)		% Inhibition	
Anti-IL-1 β antibody	Anti-TNF α antibody	Measurable IL-1 β protein	Measurable TNF α protein
0.02	0	20	0
0.2	0	50	0
2.0	0	80	0
20.0	0	100	0
0	0.06	0	20
0	0.1	0	50
0	1.0	0	80
0	10	0	100
2.0	1.0	80	80
20.0	10.0	100	100

Statistical Analysis

For protein cytokine levels and cell counts, changes from time point to time point within each cytokine group and cell type were analyzed using the paired *t* test. Correlation of mean cytokine trends and mean cell count trends was analyzed with the Pearson correlation coefficient. Comparisons between cytokine levels in cell culture, with and without LPS stimulation, were analyzed using the Wilcoxon signed rank test. Comparisons between perinatal conditions were analyzed using the Mann-Whitney *U* test. Finally, comparisons of percentages of IL-8 inhibition by anti-IL-1 β versus anti-TNF α antibodies were analyzed using Student's *t* test.

RESULTS

Patient Data Sampling

Sequential BAL samples from 15 premature infants were obtained for study as described. The mean birth weight of the patients was 1019 ± 271 g and the mean gestational age was 29 ± 2 weeks. All the study patients had diffuse alveolar disease associated with HMD and fulfilled the entry criteria. The first samples were taken close to the time of intubation, ranging from 1.5 to 12 hr following birth, with most obtained within 6 hr of birth. All patients tolerated the BAL sampling well, without any adverse reactions reported. There were no changes in oxygen saturation, ventilatory requirements, or vital signs temporally associated with the BAL sampling procedure.

Inflammatory Cell Profile from Sequential BAL Samples

Sequential BAL samples showed a predominance of alveolar macrophages and neutrophils. Flow cytometry on five representative samples near the time of intubation

Fig. 1. Total numbers of neutrophils and macrophages found in BAL fluid from infants with HMD sampled at 4-day intervals. At the time of intubation there was a large number of neutrophils in the BAL fluid, which declined to low levels by day 8 but peaked again on days 16 and 28. The number of macrophages was low at the time of intubation but gradually peaked by day 12 and gradually declined to a low value by day 28. Data expressed as mean $\pm$ SD.

and on day 12 demonstrated a predominance of macrophages and neutrophils and a total absence of lymphocytes using CD3, CD4, and CD8 positive cells as markers (data not shown).

The mean number of alveolar macrophages (AM) was approximately 1×10^5 AM/ml in the initial BAL samples. The number trended toward approximately 8.5×10^5 AM/ml by day 12, then declined to 2×10^5 AM/ml and continued to decrease gradually to 1×10^5 AM/ml by day 28. The mean neutrophil (PMN) cell profile had a less definitive trend, with approximately 8×10^5 PMN/ml in the initial BAL samples, which declined to approximately 3×10^5 PMN/ml by day 8 and increased again to approximately 7×10^5 PMN/ml by day 16 (Fig. 1). Due to the wide variation in the number of AM and PMN among individuals, there were no true statistical alterations in the AM and PMN number at any time.

Expression of Proinflammatory Cytokine mRNAs in BAL Cells by RT-PCR Analysis

The mRNA for TNF α and IL-8 was detectable in 80 to 90% of the BAL samples including the initial sampling points. IL-1 β mRNA was detectable in the initial BAL fluid from nearly half of the newborns and detectable in 80 to 90% of all sampling points thereafter. Consistent with the absence of lymphocytes by flow cytometry, there was no IL-2 mRNA detectable in any of the samples. Detection of IL-2 mRNA by the RT-PCR method employed in this study was confirmed in phytohemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells from one of the investigators (data not shown).

Analysis of Cytokine Concentration by ELISA

TNF α , IL-1 β , and IL-8 protein was detectable in the BAL fluid from the preterm infants. The supernatants from the same BAL samples used to determine mRNA were used for cytokine protein measurement. There were detectable quantities of TNF α and IL-8 near the time of intubation in all the infants. IL-1 β was detectable in only 56% of infants near the time of intubation. All three cytokines were, however, detectable by day 4 postintubation (second sampling time point). IL-1 β levels gradually peaked at day 16 and decreased to low levels by day 28. IL-8 levels peaked at day 12 and decreased to low levels by day 28. TNF α did not peak until day 20 and decreased to low levels by day 28 (Fig. 2). IL-1 β levels correlated most strongly with IL-8 levels ($r = 0.85$). TNF α levels did not correlate with either IL-1 β or IL-8 levels (Fig. 3). Macrophage count correlated most strongly with IL-8 levels ($r = 0.79$) (Fig. 5) but not with IL-1 β and TNF α levels. The number of neutrophils did not correlate with any cytokine levels (Table II).

Based upon the presence or absence of IL-1 β at the time of intubation, the infants could be divided into two groups. Clinically, the population of infants who expressed IL-1 β near the time of intubation had a higher incidence of maternal intrauterine infection, based upon pathological examination of the placentas (data not shown), than the population who did not express IL-1 β near the time of intubation. In the former group 90% of the placentas examined had evidence of infection, whereas in the latter group, only 42% of the placentas examined had any evidence of infection ($P = 0.02$). There was no statistical difference in maternal profile or whether steroids or antibiotics were given prior to delivery between the two groups. IL-1 β levels in the former group peaked on day 16, in contrast to the IL-1 β levels of the latter group, which peaked on day 12. There was a very strong correlation between IL-1 β levels from those infants with no detectable IL-1 β protein near the time of intubation and IL-8 levels in the same infants ($r = 0.95$) (Fig. 4).

Due to the wide variations in cytokine levels measured from the *in vivo* BAL source, the correlations observed were between mean cytokine levels and cell numbers. These trends, however, were not statistically significant.

In Vitro Cytokine Expression (by ELISA) Following LPS Stimulation

Lung inflammatory cells were obtained via BAL on day 12 postintubation for cell culture, as this sampling time point yielded the highest number of macrophages.

Fig. 2. Cytokine protein levels in sequential BAL samples from preterm newborns with HMD sampled over 28 days. (a) IL-8 protein levels from BAL fluid were detectable near the time of intubation in all infants, peaked on day 12, and gradually declined to low levels by day 28. (b) TNF α protein levels were also detectable near the time of intubation in all infants, peaked on day 20, and declined to low levels by day 28. There were two distinct subpopulations of infants with regard to IL-1 β expression. In 56% of all infants IL-1 β protein was undetectable near the time of intubation. The remaining 44% of infants had detectable levels of IL-1 β protein near the time of intubation. All infants had detectable levels of IL-1 β protein by day 4. (c) This figure shows the IL-1 β protein levels sampled over 28 days from infants from both subpopulations (those expressing and not expressing IL-1 β near the time of intubation). IL-1 β protein levels peaked on day 16 and declined to negligible levels by day 28. Data expressed as mean $\pm$ SD.

These inflammatory cells (mononuclear cells and neutrophils) expressed detectable amounts of TNF α , IL-1 β , and IL-8 protein in complete media. The unstimulated *in vitro* levels of these cytokines were comparable to the corresponding *in vivo* levels except for IL-8, which was 10 times higher. The explanted cells were responsive to

Fig. 3. The relationship between IL-8 and IL-1 β protein expression in the lungs of preterm infants with HMD. (a) IL-8 protein levels sampled at 4-day intervals over 28 days correlated with IL-1 β protein levels ($r = 0.85$) more closely than with (b) TNF α protein levels ($r = 0.26$) in the BAL fluid of these preterm infants. (c) In the subpopulation of infants with undetectable levels of IL-1 β protein near the time of intubation, subsequent IL-8 and IL-1 β protein levels displayed the closest correlation ($r = 0.95$) compared to (d) the subpopulation of infants with detectable levels of IL-1 β protein near the time of intubation ($r = 0.4$). Data expressed as mean $\pm$ SD.

LPS stimulation *in vitro*, producing higher levels of TNF α , IL-1 β , and IL-8 than corresponding *in vivo* levels (Fig. 5). At the end of the culture period (24 hr) the number of viable cells as determined by trypan blue staining decreased by approximately 30%. The different (various) culture conditions had no specific impact on cell viability.

In Vitro Cytokine Protein Expression (by ELISA) Following Antibody Blockade

Anti-TNF α and anti-IL-1 β antibodies both significantly inhibited the expression of IL-8 following LPS stimulation (Fig. 6). There was a dose-dependent inhibition of IL-8 by anti-IL-1 β antibody, while inhibition of IL-8 by anti-TNF α antibody was steady over the range of

antibody concentrations. The addition of anti-IL-1 β antibody led to greater inhibition of IL-8 (27–46% inhibition of IL-8) than anti-TNF α antibody (7–18% inhibition of IL-8). The combination of anti-IL-1 β and TNF α antibodies did not achieve any higher level of IL-8 inhibition than anti-IL-1 β antibody alone. A significant decrease in IL-8 production was observed when 20% of the measurable IL-1 β was inhibited, whereas inhibition of 100% of the measurable TNF α was required to observe a significant reduction in IL-8 production.

DISCUSSION

In the current study, we investigated the expression of the cytokines IL-1 β , TNF α , and IL-8 in the lungs of premature neonates with HMD. Furthermore, we used an

Table II. Correlation Coefficient Analysis of BAL Cytokine Levels and Cell Counts over Time^a

	TNF α	IL-1 β	IL-8	AM	PMN
TNF α	—	0.164427	0.261211	0.024399	0.068162
IL-1 β	0.164427	—	0.856279*	0.500375	0.164691
IL-8	0.261211	0.856279*	—	0.790376 [#]	0.012062

^aThe closest correlations were found between IL-8 and IL-1 β ^(*) and IL-8 and macrophage cell counts^(#). AM, mean macrophage count; PMN, mean neutrophil count.

Fig. 4. IL-8 protein levels correlated closely with the number of macrophages in the lungs of preterm newborns with HMD sampled over 28 days. Sequential analysis of BAL supernatant IL-8 protein levels yielded a close correlation with the number of macrophages found in the BAL fluid ($r = 0.79$) compared with the number of neutrophils found in the BAL fluid ($r = 0.012$). Data expressed as mean $\pm$ SD.

in vitro cell culture system to determine the regulation of IL-8 by $\text{TNF}\alpha$ and IL-1 β in cells recovered by BAL from the lungs of the same newborns. Both the *in vivo* and the *in vitro* results are consistent with the following conclusions: (a) IL-1 β , $\text{TNF}\alpha$, and IL-8 are expressed in the lungs of preterm newborns during the first 28 days of

Fig. 5. Cytokine production by BAL derived lung inflammatory cells in culture. (a) Explanted cells in complete medium continued to produce $\text{TNF}\alpha$, IL-1 β , and IL-8. (b) When stimulated with lipopolysaccharide (LPS) these cells produced higher values of IL-1 β , $\text{TNF}\alpha$, and IL-8 protein compared to the same cells in unstimulated culture. Note: Some points with similar values are overlaid. Error bars $\pm$ SD.

life, and IL-1 β levels correlate with the magnitude of IL-8 protein expression; and (b) consistent with these *in vivo* findings, IL-1 β appears to be a predominant regulator of IL-8 in explanted cells treated with LPS compared to $\text{TNF}\alpha$.

A number of studies have examined the expression of various inflammatory cytokines in the lungs of adult and neonatal subjects with acute and/or chronic lung disease. In contrast, information regarding inflammatory pathways and their regulation in human preterm newborns remains limited. IL-8 is a potent chemokine whose expression has been implicated in a variety of acute and chronic inflammatory conditions. In cell culture studies, regulation of IL-8 was shown to be influenced by both IL-1 β and $\text{TNF}\alpha$ (18, 19–30). Herein, we demonstrate that expression of all three cytokines, IL-1 β , $\text{TNF}\alpha$, and IL-8, occurs early in the lungs of preterm newborns at risk for development of chronic lung disease. More specifically, based on whether or not IL-1 β protein could be detected in the initial BAL sample taken within 6 to 12 hr postpartum, it was evident that the preterm neonates examined in this study could be divided into two groups. The first group exhibited detectable levels of IL-1 β protein near the time of intubation and included a predominant number of neonates with maternal chorioamnionitis. In contrast, IL-1 β protein was undetectable in the initial BAL of the second group, which had a significantly lower incidence of maternal chorioamnionitis. Thus, early expression of IL-1 β may provide a potential marker for detection of maternal chorioamnionitis in preterm neonates.

Elevated IL-8 levels in BAL from neonates with HMD are consistent with the findings of other investigators. Previous studies have found a correlation between the development of CLD and increased levels of IL-8 in the BAL of infants with HMD (10, 11). However, neither *in vivo* nor *in vitro* data exist regarding the relationship between the expression of IL-8 and other proinflammatory cytokines such as IL-1 β and $\text{TNF}\alpha$ in premature neonates. In the current study, protein levels of IL-8 closely correlated with IL-1 β protein levels and macrophage cell counts in serial samples obtained from preterm newborns over a 28-day course. In contrast, no apparent correlation was observed between the levels of $\text{TNF}\alpha$ and IL-8 and the macrophage cell counts in the same patients. These results indicate that in the lungs of preterm newborns, IL-1 β and $\text{TNF}\alpha$ may differ in their abilities to affect the regulation of downstream target genes such as IL-8.

Measurement of protein levels in BAL are often subject to variability and are hence difficult to quantify. A significant variable relates to the volume of the

Fig. 6. In primary cell culture, the addition of anti-IL-1 β and anti-TNF α monoclonal antibodies demonstrated differential regulation of IL-8 expression by LPS-stimulated, BAL-derived lung inflammatory cells. (a) Anti-TNF α antibodies significantly inhibited IL-8 protein production (compared to baseline) at only higher levels of antibody

recovered fluid used in the BAL. This variability applies to multiple samples taken from the same patient as well as samples obtained from different patients. A number of attempts have been made to normalize BAL samples based on various denominators, including secretory IgA and urea (31–33). Despite these attempts, an appropriate denominator has not as yet been widely agreed upon. Thus we have chosen to report the BAL cytokine concentrations as weight per volume of fluid recovered, similar to other investigators (26, 27). In contrast, *in vitro* studies are more easily amenable to standardization and quantification. For this reason, we developed an *in vitro* cell culture system to study the regulation of IL-8 by TNF α and IL-1 β in lung inflammatory cells from the same infants as used in our *in vivo* BAL measurements.

Each culture comprised of a total of 250,000 BAL-derived cells. Flow cytometry and the absence of IL-2 mRNA were used to verify that the cultured cells consisted predominantly of neutrophils and macrophages. The number of inflammatory cells recovered from the neonates was insufficient to allow separation into specific cell types. Thus the expressed cytokines were derived from a combination of cell types. Nevertheless, this shortfall may prove to be a strength. Unlike studies on clonally derived, relatively homogeneous cell lines, the current *in vitro* system allows the investigator to examine cytokine dynamics in a population of cells more accurately resembling the composition of cells found in the native pulmonary environment in which pathogenesis of HMD presumably occurs. These cultured inflammatory cells were responsive in LPS *in vitro*, producing high levels of TNF α , IL-1 β , and IL-8 upon stimulation (Fig. 5). In this *in vitro* system, both anti-TNF α and anti-IL-1 β antibodies significantly inhibited the expression of IL-8 following LPS stimulation (Fig. 6). Inhibition of IL-8 by anti-IL-1 β antibodies was found to be dose dependent and twice as effective compared to that accomplished by anti-TNF α antibodies. Simultaneous addition of both anti-IL-1 β and anti-TNF α antibodies to the cells in culture did not result in a corre-

concentration ($P = 0.02$)(*). (b) Anti-IL-1 β antibodies, in contrast, led to a dose-dependent inhibition of IL-8 protein production (compared to baseline). This inhibition of measurable IL-8 was significant at all concentrations of anti-IL-1 β antibodies ($P < 0.05$ at all concentrations)(^W). Blocking 80 and 100% of measurable IL-1 β (with the addition of 2 and 20 μ g/ml anti-IL-1 β antibody) led to significantly higher inhibitions of IL-8 expression compared to blocking 80 and 100% of measurable TNF α (with the addition of 1 and 10 μ g/ml anti-TNF α antibody) ($P = 0.04$ and $P = 0.02$ respectively). (c) Anti-IL-1 β and anti-TNF α antibodies added to the cell culture in combination did not inhibit IL-8 production more than the equivalent amount of anti-IL-1 β antibody added to the culture alone. Data expressed as mean $\pm$ SD.

spondingly higher inhibition of IL-8 compared to that achieved by anti-IL-1 β antibody alone. We did not assess the possibility that TNF α regulation of IL-8 may be via regulation of IL-1 β (23, 24), however, since complete abrogation of both cytokines did not lead to a greater decline in IL-8 expression than blockade of IL-1 β alone, it is unlikely that this potential pathway has a more predominant role than the role of IL-1 β in the regulation of IL-8. Therefore, consistent with the *in vivo* results, while both TNF α and IL-1 β are capable of regulating IL-8 gene expression by preterm lung inflammatory cells in culture, IL-1 β exerts a more predominant influence.

Congruent with these results, other investigators have also demonstrated differential regulation of IL-8 by IL-1 β and TNF α in cell types isolated from mature hosts, such as pulmonary stromal cells and astrocytes (34–36). In contrast, studies using isolated human bronchial epithelial cells have reported equivalent expression of IL-8 protein and mRNA in response to TNF α and IL-1 β (37). These variable results highlight the complexity of IL-8 gene regulation. Differential regulation of IL-8 by IL-1 β and TNF α may vary according to specific cell types. In addition, since total inhibition of both IL-1 β and TNF α did not completely abrogate the production of IL-8 *in vitro*, the impact of the local environment, such as the presence of LPS, may also play a role in the expression of IL-8. With further studies, the regulation of IL-8 during the pathogenesis of HMD may be further understood and potential therapeutic methods developed, aimed at interrupting the expression of this potent chemokine.

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