A Role for Matrix Metalloproteinase 9 in IFN γ -Mediated Injury in Developing Lungs

Relevance to Bronchopulmonary Dysplasia

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We noted a marked increase in IFN γ mRNA in newborn (NB) murine lungs after exposure to hyperoxia. We sought to evaluate the role of IFN γ in lung injury in newborns. Using a unique triple-transgenic (TTG), IFN γ -overexpressing, lung-targeted, externally regulatable NB murine model, we describe a lung phenotype of impaired alveolarization, resembling human bronchopulmonary dysplasia (BPD). IFN γ -mediated abnormal lung architecture was associated with increased cell death and the upregulation of cell death pathway mediators caspases 3, 6, 8, and 9, and angiopoietin 2. Moreover, an increase was evident in cathepsins B, H, K, L, and S, and in matrix metalloproteinases (MMPs) 2, 9, 12, and 14. The IFN γ -mediated abnormal lung architecture was found to be MMP9-dependent, as indicated by the rescue of the IFN γ -induced pulmonary phenotype and survival during hyperoxia with a concomitant partial deficiency of MMP9. This result was concomitant with a decrease in caspases 3, 6, 8, and 9 and angiopoietin 2, but an increase in the expression of angiopoietin 1. In addition, NB IFN γ TTG mice exhibited significantly decreased survival during hyperoxia, compared with littermate controls. Furthermore, as evidence of clinical relevance, we show increased concentrations of the downstream targets of IFN γ chemokine (C-X-C motif) ligands (CXCL10 and CXCL11) in baboon and human lungs with BPD. IFN γ and its downstream targets may contribute significantly to the final common pathway of hyperoxia-induced injury in the developing lung and in human BPD.

Keywords: newborn; hyperoxia; BPD; IFN γ ; MMP9

Bronchopulmonary dysplasia (BPD) in the newborn (NB) is attributable to a combination of factors causing lung injury that interferes with normal lung development, resulting in impaired alveolarization. Both genetic and environmental factors contribute to the development of BPD (1, 2). Environmental factors include prolonged invasive ventilation, sepsis, and exposure to hyperoxia. Physiologically, embryonic or fetal lungs in the canalicular or saccular stage are in the process of active

CLINICAL RELEVANCE

This is the first report of a murine model of overexpression of IFN γ in the developing lung, which mimics human bronchopulmonary dysplasia. The role of matrix metalloproteinase 9 in the murine model and other downstream targets of IFN γ in hyperoxia-induced lung injury are also highlighted in baboon and human lungs with bronchopulmonary dysplasia.

development involving pneumocyte multiplication, angiogenesis, and vasculogenesis, with a concurrent alignment of capillary endothelium and alveolar epithelium with the developing airways (3). Investigations demonstrate various mechanisms of injury caused by mediators of inflammatory, apoptotic, and angiogenic pathways (4). The exact mechanisms of lung development and its impairment, including the interactions of components of various pathways, remain to be elucidated.

Within the family of cytokines, IFN γ is capable of stimulating the release of multiple cytokines. IFN γ plays a significant role in the regulation of diverse immune responses, including pathogen and antigen processing and presentation, cellular proliferation, the activation of microbicidal effectors, and leukocyte trafficking (5). The dysregulated production of IFN γ was implicated in a large number of diseases, including atherosclerosis (6), Crohn's disease (7), celiac disease (8), and rheumatoid arthritis (9). Although an impressive amount of knowledge explains the mechanisms of IFN γ -induced immunomodulation, the mechanisms of IFN γ -induced tissue injury, remodeling, and destruction have not been adequately defined.

Recent studies detected increased concentrations of IFN γ in lungs exposed to hyperoxia before any neutrophil infiltration was evident. IFN γ is known to be released from lung cells, after exposure to hyperoxia (10, 11). Several studies showed that IFN γ induces apoptosis in several cell types, including lung epithelial cells, and is known to mediate lung injury (12–17). IFN γ was shown to play a role in the pathogenesis of pulmonary emphysema, in which alveolar septae are destroyed, the production of IFN γ is enhanced, and increased concentrations of the IFN γ target gene IFN γ -inducible protein 10 (IP10)/chemokine (C-X-C motif) ligand 10 (CXCL10) are juxtaposed with the epithelium (18–22). Studies also demonstrated that the transgenic (TG) overexpression of IFN γ in the adult murine lung causes pulmonary emphysema (23).

Studies using adult IFN γ -deficient (IFN $\gamma^{-/-}$) mice reported that IFN γ plays a role in pulmonary inflammation. IFN $\gamma^{-/-}$ mice showed improved early survival compared with wild-type (WT) mice after exposure to hyperoxia (24). However, the role of IFN γ in injury and inflammation in the developing lung needs further elucidation.

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The remodeling of extracellular matrix plays a major role in the pathophysiology of BPD (25). A number of cytokines were implicated, along with a variety of pathways, in the destruction and maldevelopment of the lungs. Matrix metalloproteinases (MMPs) were found to be elevated in animal studies of IFN γ overexpression in lungs (23). MMP9 was shown to be a key proteinase, promoting the destruction of matrix and basement membranes (26). Experiments with MMP9 knockout (KO) NB mice indicated improved survival (27). MMP9 concentrations were found to be elevated in the tracheal aspirates of neonates with BPD (28, 29).

The importance of MMP9 as a possible “central agent” in cell damage, interacting with other inflammatory mediators, is poorly understood. IFN γ , by stimulating multiple pathways and multiple cytokines, creates a milieu of increased inflammatory response. We hypothesized that IFN γ mediates MMP9-dependent lung injury and impaired alveolarization in the NB lung.

We demonstrate the increased expression of IFN γ in the NB murine lung after exposure to hyperoxia. Using a unique triple-TG (TTG), IFN γ -overexpressing, lung-targeted, externally regulatable NB murine model, we describe a lung phenotype of impaired alveolarization, resembling human BPD. We also show that IFN γ -mediated abnormal lung architecture is associated with increased cell death, the upregulation of mediators of cell death, and angiogenic pathways, along with increased cathepsins and MMPs. These effects are MMP9-dependent, as shown by the rescue of the IFN γ -induced pulmonary phenotype, with a concomitant partial deficiency of MMP9. In addition, IFN γ TTG NB mice demonstrate significantly decreased survival in hyperoxia. Finally, as evidence of clinical relevance, we show increased concentrations of the downstream targets of IFN γ in baboon and human lungs with BPD.

MATERIALS AND METHODS

Transgenic and Genetically Modified Mice

We used C57BL/6J mice in our experimental studies. All animal work was approved by the Institutional Animal Care and Use Committee at Yale University School of Medicine. IFN γ -overexpressing TTG CC10-rtTA/tTs-IFN γ mice were generated in our laboratory. Details of the genetic constructs, the methods of microinjection and genotype evaluation, the inducibility, and the emphysematous and inflammatory phenotype of dual TG CC10-rtTA-IFN γ mice were previously described (16). We obtained the TTG IFN γ -overexpressing mice by breeding the dual TG and the tTs mice (the tTs mice were kindly donated by Jack Elias, MD). MMP-9^{-/-} mice, obtained as previously described (30), were bred with CC10-rtTA/tTs-IFN γ mice. This resulted in the generation of CC10-rtTA/tTs-IFN γ mice that were WT or heterozygous (HZ; +/-) for MMP9.

The administration of doxycycline (Dox) water, bronchoalveolar lavage (BAL), mRNA, Western blotting, histologic, densitometric, morphometric, and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) analyses, exposure to oxygen, and the human lung samples of BPD are described in more detail in SUPPLEMENTAL METHODS in the online supplement.

Baboon Model of BPD

Samples of frozen baboon lung tissue and bronchoalveolar lavage fluid (BALF) were provided by the Southwest Foundation for Biomedical Research (San Antonio, TX). All animal procedures were reviewed and approved by the animal care committees of the Southwest Foundation for Biomedical Research and the University of Texas Health Science Center (San Antonio, TX). In the “new” BPD model, baboons were delivered by hysterotomy at 125 days, intubated, treated with exogenous surfactant (Survanta; donated by Ross Laboratories, Columbus, OH), and maintained on pressure-limited, time-cycled infant ventilators for 6, 14, or 21 days (31, 32). Baboons that were delivered at 125 days, 140 days, or 146 days, and killed immediately,

served as age-matched gestational controls. A third control group consisted of baboons born via natural delivery at full-term gestation (~ 185 days) and killed 2–3 days later (full-term group). In the “old BPD” model, baboons were delivered at 140 days of gestation and were ventilated for a total of 10 days with 80–100% oxygen (33). BALF samples were obtained during necropsy, and processed as previously described (34). Whole lung tissue was homogenized in lysis buffer, as previously described (35). IP10 concentrations in BALF were measured using an ELISA kit (R&D Systems, Minneapolis, MN), following the manufacturer’s instructions.

Statistical Analysis

For animal and human studies, values were expressed as means $\pm$ SEM, medians with 25th–75th centiles, or means $\pm$ SD. As appropriate, groups were compared using the Student two-tailed unpaired *t* test, the Mann-Whitney test, or the log-rank test, with GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA). In all analyses, *P* < 0.05 was considered statistically significant.

RESULTS

Experiments in NB IFN γ TTG (CC10-rtTA/tTs-IFN γ /MMP-9^{+/+}) Mice

Effects of hyperoxia on IFN γ expression in the NB WT lung. Because hyperoxia is a critical element in the pathogenesis of BPD, we first wanted to identify molecules that are regulated by hyperoxia in a developmentally appropriate murine model. We selected NB mice whose lungs were in the saccular stage of development (postnatal day [PN]1–PN4), somewhat akin to that of 23–28-week human preterm newborns, who are in the late canalicular/early saccular stage of lung development. We exposed NB mice to hyperoxia for 48 hours,

Figure 1. Effects of hyperoxia on IFN γ mRNA expression in the wild-type (WT) newborn (NB) murine lung. (A) WT NB mice were exposed to hyperoxia for 48 hours after birth. Each lane in the gel is representative of the condition described, with the corresponding β -actin for control purposes. (B) Effects of maternal exposure to doxycycline water (Dox) on BAL IFN γ concentrations in the NB lung. NB IFN γ triple-transgenic (TTG) mice receiving Dox water exhibited elevated BAL IFN γ concentrations, compared with control mice. IFN-g, IFN γ triple transgenic; Reg, regular water; BAL, bronchoalveolar lavage. Data are shown as box and whiskers plot (median, 25th–75th centiles, and range) of a minimum of four animals in each group. ****P* $\leq$ 0.001, Mann-Whitney test.

as described in MATERIALS AND METHODS, and detected increased lung IFN γ mRNA expression, as shown in Figure 1A.

Effects of maternal Dox exposure on BAL IFN γ concentrations in the NB lung. We initially used lung-targeted double TG NB IFN γ mice, and detected some degree of TG leakage in the system. To overcome this leak in the TG system and enhance the validity of our observations (given the fact that the lung is actively growing during this phase), we generated TTG mice, as described in MATERIALS AND METHODS. As shown in Figure 1B, impressive BAL IFN γ protein concentrations in the NB lung were evident, with minimal to no leakage in the system. The TTG NB mice exposed to Dox water exhibited a significant increase in BAL IFN γ concentrations (Figure 1B). The median BAL concentration of IFN γ in these mice was 63.9 pg/ml (25th–75th centiles, 16.8–185.9 pg/ml). The median BAL concentration of IFN γ in the three control groups of mice was ≤ 9.1 pg/ml.

Effects of increased IFN γ in the NB lung on TUNEL staining. We noted enlarged and simplified alveoli in the lungs of NB IFN γ TTG mice receiving Dox water. An increase in cell death and tissue injury may explain this phenotype. For a further understanding of the pathways involved in the alveolar simplification phenotype, we performed TUNEL staining on lung tissue slides, as shown in Figure 2A and quantified in Figure 2B. A significant elevation of TUNEL-positive cells occurred in NB IFN γ TTG mice, compared with control mice.

TUNEL-positive staining appeared to localize to epithelial and inflammatory cells.

Effects of increased IFN γ in the NB lung on caspases. Because of the increased number of TUNEL-positive cells, we evaluated the markers of cell death pathways by measuring the mRNA expression of caspases. The presence of increased IFN γ in the NB lung increased the expression of mRNA of caspases 3, 6, 8, and 9, as depicted in Figure 2C and quantified in Figures 2D–2G. In addition, we confirmed the increased caspase 3, 8, and 9 protein concentrations by Western blotting (see Figure E1 in the online supplement).

Effects of increased IFN γ in the NB lung on angiogenic agents. An altered balance of the angiogenic factors angiopoietin 1 (Ang1) and angiopoietin 2 (Ang2) was implicated in hyperoxia-induced cell death and lung injury (41). We observed enhanced Ang2 mRNA expression, with no change in Ang1 mRNA expression, in the lungs of NB IFN γ TTG mice receiving Dox water (Figures 3A–3C). Our earlier studies implicated a role for increased Ang2 in human BPD (36, 37).

Effects of increased IFN γ in the NB lung on cathepsins. We also observed that cathepsins were induced by increased IFN γ in the NB lung (Figure 3D). This was confirmed by densitometry (Figures 3E–3I) and Western blotting (data not shown).

Effects of increased IFN γ in the NB lung on MMPs. We next evaluated MMPs 2, 9, 12, and 14 (Figure 4A). We found that IFN γ is a potent stimulator of MMPs in the NB lung. This

Figure 2. Effects of increased IFN γ in the NB lung. (A) terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)-stained lung histology of NB IFN γ TTG (designated as IFN γ +) or WT (designated as IFN γ -) on regular (designated as Dox -) or Dox (designated as Dox +) water is shown as dark blue nuclear staining. (B) Quantification of TUNEL-positive cells in lung histology. (C) mRNA expression of caspases 3, 6, 8, and 9 in lung tissue was ascertained on PN7 in NB IFN γ TTG (+) or WT (-) mice receiving regular (-) or Dox (+) water. Each lane in the gel is representative of the condition described, with the corresponding β -actin for control purposes. (D–G) Quantified mRNA expression of caspases 3, 6, 8, and 9 was ascertained on postnatal day 7 in NB IFN γ TTG (+) or WT (-) mice receiving regular (-) or Dox (+) water. Each bar represents the mean $\pm$ SEM for a minimum of four animals. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, t test.

Figure 3. Effects of increased IFN γ in the NB lung on angiogenic agents and cathepsins. mRNA expression and quantification of angiopoietins 1 and 2 (A–C) and cathepsins B, H, K, L, and S (D–I) in lung tissue were ascertained on PN7 in NB IFN γ TTG (+) or WT (–) mice receiving regular (–) or Dox (+) water. Each lane in the gel is representative of the condition described, with β -actin controls as in Figure 2. Each bar represents the mean $\pm$ SEM for a minimum of four animals. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, t test.

result was confirmed by densitometry (Figures 4B–4E) and Western blotting (Figure E1).

Taken together, our data suggest that the alveolar simplification phenotype in NB IFN γ TTG murine lungs may be attributable to increased cell death, perhaps secondary to elevated Ang2. In addition, enhanced concentrations of cathepsins and MMPs may contribute to tissue destruction. Because a role for MMP9 in the pathogenesis of BPD was recently envisioned (27–29), we undertook studies to understand the role of MMP9 in IFN γ -induced effects on the NB lung.

Experiments in IFN γ TTG/MMP9HZ (CC10-rtTA/tTs- IFN γ /MMP-9 $^{+/-}$) Mice

Effects of partial deficiency of MMP9 on BAL IFN γ concentrations in NB IFN γ TTG murine lungs. NB IFN γ TTG MMP9HZ mice manifested elevated BAL concentrations of

IFN γ (median, 45.5 pg/ml; 25th–75th centiles, 18.2–85.4 pg/ml), but these were similar ($P = 0.236$) to BAL IFN γ concentrations in NB IFN γ TTG mice (Figure 1B). NB MMP9 HZ mice exhibited median BAL IFN γ concentrations of 8.9 pg/ml, similar to control mice (Figure 1B). Hence, the effects subsequently noted were not attributable to a lack of IFN γ induction in the lungs. We also confirmed that lung proteolytic activity was reduced in NB IFN γ TTG MMP9HZ lungs according to zymography (see the 92-kD band in Figure E2).

Effects of partial deficiency of MMP9 on the histologic and structural effects of increased IFN γ in the NB lung. The histology of NB IFN γ TTG mice receiving Dox water showed a thinning of the alveolar walls and enlargement of alveolar spaces in mice with increased IFN γ lung concentrations, a phenotype suggestive of human BPD. A marked improvement was evident in alveolarization in NB IFN γ TTG MMP9HZ mice

Figure 4. Effects of increased IFN γ in the NB lung on matrix metalloproteinases (MMPs). (A) The mRNA expression of MMPs 2, 9, 12, and 14 in lung tissue was ascertained on PN7 in NB IFN γ TTG (+) or WT (-) mice receiving regular (-) or Dox (+) water. Each lane in the gel is representative of the condition described, with β -actin controls as in Figure 2. (B–E) Quantified mRNA expression of MMPs 2, 9, 12, and 14 was ascertained on PN7 in NB IFN γ TTG (+) or WT (-) mice receiving regular (-) or Dox (+) water. Each bar represents the mean $\pm$ SEM for a minimum of four animals. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, t test.

receiving dox water, compared with NB IFN γ TTG mice receiving Dox water (Figure 5A, panels 6 and 4, respectively). A greater than 50% increase was evident in chord length values upon TG activation, which markedly improved with a concomitant partial deficiency of MMP9 (Figure 5B). Rishikof and colleagues (38), using an elastase model of emphysema in mice, speculated that damaged areas of the lung represent a reactivation of myofibroblast proliferation, and increased with α -smooth muscle actin (α -SMA) content is normally associated with postnatal alveolar septation. Hence, we stained lung tissues with α -SMA for the relative proportion of myofibroblast-type cells, and found that the area of distribution of α -SMA staining was increased in NB IFN γ TTG mice receiving Dox water (Figure 5C). The increased proportion could attributable to a proliferation of existing myofibroblasts, or to a transition/transformation of other cells into myofibroblasts. We noted a partial normalization in the distribution of α -SMA staining in NB IFN γ TTG MMP9HZ mice receiving Dox water (Figure 5C). We further quantified this normalization, using the SMA distortion index (as described in MATERIALS AND METHODS, and as depicted in Figure 5D). In addition, we noted decreased alveolar budding after α -SMA staining in NB IFN γ TTG mice receiving Dox water, compared with control mice. This finding

indicated significant recovery in NB IFN γ TTG MMP9HZ mice (Figure E3).

Effects of partial deficiency of MMP9 in the presence of increased IFN γ in the NB lung on the expression of angiogenic agents and caspases. Along with the recovery of alveolar architecture, we noted increased Ang1 with decreased Ang2 and caspase 3, 6, 8, and 9 mRNA expression in NB IFN γ TTG MMP9HZ murine lungs (Figure 5E). The densitometry for Ang1 and Ang2 mRNA expression is shown in Figures E4A and E4B.

Taken together, our data suggest that the alveolar simplification phenotype in NB IFN γ TTG murine lungs may be rescued by a partial deficiency of MMP9. In addition, this normalization of alveolarization was accompanied by a reversal of angiogenic factors and cell death pathway markers. Hence, our data suggest that the impaired alveolarization attributable to increased IFN γ in the developing NB lung is dependent, at least in part, on MMP9.

Effects of partial deficiency of MMP9 in the presence of increased IFN γ in the NB lung on survival in hyperoxia. To mimic the clinical scenario in human preterm neonates, we evaluated the impact of increased IFN γ in the developing lung, in the presence of hyperoxia. We exposed NB IFN γ TTG to hyperoxia, as described in MATERIALS AND METHODS. Signifi-

Figure 5. Effects of partial deficiency of MMP9 on the histologic and structural effects of increased IFN γ in the NB lung. (A) Hematoxylin and eosin staining of lung histology of NB IFN γ TTG (+) and WT (-) mice receiving regular and Dox water, and of MMP9 WT (+/+) and MMP9 heterozygous (MMP9 HZ) (+/-) mice receiving Dox (+) water, is shown. (B) Alveolar size, as measured by chord length, demonstrates rescue of altered alveolar architecture in NB IFN γ TTG murine lungs via partial deficiency of MMP9. (C) Alpha-smooth muscle actin (α -SMA) staining of lung histology of NB IFN γ TTG (+), WT (-), MMP9 WT (+/+), and MMP9 HZ (+/-) mice receiving Dox (+) water is shown. (D) Quantification of α -SMA staining (by distortion index measurement) and some normalization of stained area in the NB IFN γ TTG mice lungs by partial deficiency of MMP9. (E) mRNA expression of angiopoietins 1 and 2, caspases 3, 6, 8, and 9 of NB IFN γ TTG (+), WT (-), MMP9 WT (+/+), and MMP9 HZ (+/-) mice receiving Dox (+) water. Each lane in the gel is representative of the condition described, with the corresponding β -actin for control purposes. Each bar represents the mean $\pm$ SEM for a minimum of four animals. Each group represents a minimum of four animals. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, t test.

cantly increased mortality was evident in the NB IFN γ TTG mice ($P = 0.003$; Figure 6). We also noted the significantly increased survival of NB IFN γ TTG MMP9 mice, compared with NB IFN γ TTG mice ($P < 0.003$, Figure 6). This result supports our contention that increased IFN γ in the developing lung exposed to hyperoxia is detrimental to survival, and the decreased survival can be rescued by a partial deficiency of MMP9.

Association of downstream targets of IFN γ in murine and baboon models of BPD. The increased expression of the downstream target of IFN γ , namely IP-10, was evident in our NB IFN γ TTG mice lungs (Figures E5A and E5B). Although the stages of lung development in our murine model of BPD are

equivalent to those in the human neonate predisposed to BPD, our model contains certain drawbacks. The murine lung is not surfactant-deficient and was not undergoing ventilation, and these two factors play an important role in the pathogenesis of BPD (1). The premature baboon model (31) is well-recognized as an excellent model of human BPD. Increased mRNA expression and protein concentrations of IP10 were evident in baboon lungs and airways with BPD, respectively, compared with gestational age-matched controls (Figures 7A and 7B).

Association of downstream targets of IFN γ in the human lung with BPD. We attempted to detect IFN γ by ELISA from tracheal aspirates (TAs) obtained in the first postnatal week from premature human neonates at risk for BPD. TA samples

Figure 6. Effects of increased IFN γ and concomitant partial deficiency of MMP9 in the NB lung on survival during hyperoxia. Significantly increased mortality occurred in NB IFN γ TTG mice exposed to 100% O₂ over PN1–7, compared with WT littermate controls, while receiving Dox water. In contrast, NB IFN γ TTG MMP9 HZ mice demonstrated significantly improved survival over NB IFN γ TTG mice exposed to 100% O₂ over PN1–7. All animals were exposed to Dox water. Each group represents a minimum of four animals. * $P \leq 0.003$, log-rank test.

were analyzed from 52 ventilated preterm infants (mean $\pm$ SD, gestational age of 25.7 ± 1.7 weeks; birth weight, 739 ± 155 g). Fourteen infants manifested no BPD, and 38 infants either developed BPD or died before 36 weeks of postmenstrual age. The clinical characteristics of the two groups are summarized in Table E2. IFN γ was detected in TA samples from only three infants (at concentrations of 4.7, 12.3, and 14.3 pg/ml) who developed BPD or died. IFN γ was undetectable in the other infants, including all 14 who did not develop BPD.

Although some investigators were able to demonstrate the immunohistochemical expression of IFN γ in human tissue (39), we did not see evidence of that in our human NB lung tissue samples, presumably because of low-level expression or lack of retention of protein within cells. Other systems (e.g., atherosclerosis) in which IFN γ was shown to play a role in humans are known to involve concentrations of IFN γ not detectable by immunostaining (George Tellides, personal communication). Hence, to ascertain the clinical relevance of our findings further, we used immunohistochemistry to detect another downstream target of IFN γ , namely, IP-9/CXCL11/I-TAC, in the human neonatal lung. As depicted by representative microphotographs in Figure 7C, increased brown staining of the epithelial and inflammatory cells occurred in the BPD lung, compared with control lungs.

DISCUSSION

Lung injury caused by an imbalance in the release of proinflammatory and anti-inflammatory cytokines, occurring as a result of volutrauma or barotrauma, sepsis, pulmonary edema, or hyperoxia, damages the immature lung of the NB. This is followed either by healing (resolution of injury) or by repair of the lung (BPD) (2). The pathology of the “new” BPD involves dysregulated angiogenesis and impaired alveolarization (1). Limited information is available regarding the mechanisms that cause structural remodeling in BPD. Although the infiltration of lung tissue by inflammatory cells was demonstrated in hyperoxia-induced lung injury in adult mice, the key finding in early stages before neutrophil infiltration was an increased expression of IFN γ in lungs (10, 11).

Hence, we postulated that increased IFN γ could constitute a critical early response molecule in the pathogenesis of BPD. Using developmentally appropriate animal models, we showed that IFN γ -induced impaired alveolarization is MMP9-dependent.

Specifically, we confirmed that IFN γ increases in response to hyperoxia exposure in the developing NB lung. Next, using a unique, lung-targeted, externally regulatable TTG murine model of IFN γ overexpression, we show impaired alveolarization. The IFN γ -induced NB murine lung phenotype mirrors human BPD, as exemplified by alveolar simplification. Our study showed that IFN γ causes structural remodeling along with the activation of enzymes involved in cell death, angiogenesis, and protein degradation. In addition, significant rescue of the pulmonary phenotype occurs secondary to a partial deficiency of MMP9, and is associated with a reversal of changes evident in cell death pathways and angiogenic markers. The clinical relevance of our work is indicated by the increased mortality of NB mice exposed to hyperoxia, in the presence of increased IFN γ , and with the increased mortality being rescued by a partial deficiency of MMP9. Furthermore, the clinical significance of our findings was supported by data regarding the enhanced expression of downstream targets of IFN γ in murine and baboon models and in the human neonate with BPD.

A significant increase of IFN γ in BALF within 48–72 hours of exposure to hyperoxia was reported in adult mice (11, 24). Hyperoxia-induced lung injury is believed to result from the direct toxic effects of reactive oxygen species and the indirect effects of inflammatory cell activation, with a resultant synthesis of cytokines. A previous study in adult mice (24) showed that the depletion of IFN γ resulted in attenuated neutrophil accumulation in the lung airspace and in an inhibition of hyperoxia-induced increases in pulmonary alveolar permeability in the early phase of hyperoxic injury. Whereas earlier reports in adult mice also noted that increased caspases, cathepsins, and MMPs (23, 40) followed increased IFN γ in the lung, this finding had not been established in the developing NB lung. This is important, because the developmental regulation of molecules and their targets is well-described. In fact, significant differences occur in the response to the same stimulus and molecule by the mature versus the developing lung, as shown elsewhere (41–44). Hence, independent testing in developmentally appropriate *in vivo* modeling systems is important, as opposed to interpolating the results of *in vitro* experiments or data derived from adults.

We emphasize that we used a neonatal murine model of hyperoxia-induced alveolar development to mimic human BPD. Differences secondary to additional volutrauma/barotrauma may be attributable to invasive ventilation in preterm human lungs. However, the baboon model attempts to mimic closely what is practiced in most neonatal units, and our data support a role for IFN γ -induced effects.

Angiogenic factors play a significant role in remodeling and tissue repair (36). We found a significant elevation of Ang2 in lung tissue, secondary to IFN γ induction. We previously reported on the important role of Ang2 in hyperoxia-induced cell death and lung injury (36) and human BPD (36, 37). IFN γ -induced Ang2 may affect caspase-dependent cell death pathways, and contribute to alveolar simplification. It may also contribute to the increased mortality in NB IFN γ TTG mice exposed to hyperoxia.

Although we could not find any reports of a role for IFN γ in lung tissue of animal models or human BPD, elevated serum concentrations of IFN γ were associated in extremely preterm infants with BPD or death (45). We are not aware of any studies of IFN γ concentrations in the TAs of premature infants, to help us predict the kinetics of IFN γ production and release. IFN γ produced in the lung in preterm neonates may dissipate quickly (before our collection of the TA sample), and hence we could only detect it in a few samples (albeit all with BPD or death). However, after IFN γ is released, it triggers a cascade of specific

Figure 7. Expression of downstream targets of IFN γ in baboons and humans with BPD. (A) Inducible protein (IP) 10 chemokine (C-X-C motif) ligand 10 (CXCL10) real-time RT-PCR used RNA isolated from 125-day gestational control (GC), 140-day GC, natural delivery (ND), 125-day bronchopulmonary dysplasia (BPD), and 140-day BPD baboon lungs. (B) IP10 protein concentration was measured using ELISA in BAL fluid from 125-day GC, 140-day GC, 146-day GC, ND, 125-day/6-day PRN O₂, 125-day/14-day pro re nata (PRN) O₂, 125-day/21-day PRN O₂ delivered and ventilated with O₂ as needed for 6 days, 14 days, and 21 days, respectively, and 140-day BPD group baboon lungs. Each bar represents the mean $\pm$ SEM for a minimum of six animals. * P < 0.05, ** P $\leq$ 0.01, *** P $\leq$ 0.001, t test. (C) Immunohistochemical IP9 (I-TAC/CXCL11) staining of human lungs in NBs full-term (Term), NBs with respiratory distress syndrome (RDS), NBs with prolonged RDS (RDS prolonged), and bronchopulmonary dysplasia (BPD) is shown. Each microphotograph is representative of two lung samples from each of the four groups. Increased brown staining occurred in epithelial (green arrows) and inflammatory (red arrows) cells of BPD lungs, compared with control lungs.

events, including the production of downstream markers such as IP9 and IP10. Therefore, we focused on detecting those markers in human and baboon lungs. Elevated MMP9 concentrations were detected in BALF collected from preterm infants who subsequently developed BPD (46), in alveolar Type II epithelial cells in adult rats after 85% hyperoxia exposure (47), and in the lungs of NB rat pups after hyperoxic exposure (48, 49). Increased MMP concentrations were evident when IFN γ was overexpressed in our animals. MMP9 caused damage by degrading the extracellular matrix (ECM). In the lung, the ECM is composed of Type I collagen, and Type III collagen is also present in the alveolar walls. Both are substrates for MMP9. The destruction of the ECM may result in impaired alveolar and vascular development (50, 51). In NB IFN γ TTG MMP9HZ mice, we observed a reversal of the damage noted in the NB IFN γ TTG mice, despite their similarly elevated IFN γ lung concentrations. Our data are in accordance with studies of NB MMP9 null mutant mice exposed to hyperoxia (27), but not with the IL-1 β -induced murine model of BPD, where a deficiency of MMP9 worsened alveolar architecture (52). Variations in the types, timing, and amounts of cytokines under study could account for the different results. Importantly, the baboon model (53) and human BPD (28, 29) studies consistently showed increased lung MMP9 concentrations. We

also detected the elevated expression of other MMPs (i.e., MMPs 2, 12, and 14 in our NB IFN γ TTG murine lungs) (Figure 4A). The contributions of these other MMPs to the “BPD” phenotype call for further experimentation, which was beyond the scope of the present study.

In utero sepsis (chorioamnionitis) also contributes to the pathogenesis of BPD (1). In the endotoxin-exposed lamb model of chorioamnionitis, increased IP10 was evident in the bronchiolar epithelium of preterm lambs, and was speculated to contribute to lung injury (54). Our data of increased IP10 and IP9 in murine, baboon, and human BPD lungs suggest that IFN γ may contribute to lung injury and repair responses.

The present study enhances our understanding of the mechanism of IFN γ -induced tissue remodeling and destruction. We demonstrate that IFN γ , a potent inducer of multiple caspases, proteases, and cathepsins, causes injury in the developing lung, and that these responses are partly mediated by an MMP9-dependent mechanism. By demonstrating an amelioration of lung injury after reducing the activity of MMP9 in NB IFN γ TTG MMP9HZ mice, we show that MMP9-induced ECM damage is a critical event in IFN γ -induced lung pathology. In a cause-and-effect fashion, states of inflammation, enhanced protease activity, cellular DNA injury, cell death, and tissue destruction define a novel pathway of IFN γ -induced tissue

remodeling that may be operative in diverse biologic settings. In neonatal lung disease, these observations provide the first pathogenetic construct that can unify the seemingly disparate inflammatory, protease/antiprotease, and cell death theories of disease pathogenesis.

BPD of the premature neonate and emphysema of the adult lung are characterized by increased airspace size and respiratory insufficiency. BPD and emphysema exhibit striking similarities in their pathophysiology, including the effects of oxidative stress, sustained inflammation, enhanced apoptosis, protease-antiprotease imbalance, elastic fiber deterioration, and altered microvascularization (55). Hence, our findings may also be relevant to pulmonary emphysema and chronic obstructive lung disease (COPD). In fact, emerging data indicate that BPD may be a precursor to COPD as infants become older (1).

In conclusion, our study highlights an MMP9-dependent lung injury pathway, and demonstrates that MMP9 plays a key role in IFN γ -induced alveolar remodeling and chronic lung disease. These findings validate IFN γ , its downstream targets (including MMP9), and cell injury response regulators as potential targets for therapies directed at the treatment of neonatal lung disease, COPD, and other IFN γ -mediated diseases as diverse as atherosclerosis, rheumatoid arthritis, and Crohn's disease. Additional investigations of the potential therapeutic utility of anti-IFN γ -based and anti-MMP9-based therapies for lung disease are warranted.

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