Endothelial Monocyte-Activating Polypeptide II Mediates Macrophage Migration in the Development of Hyperoxia-Induced Lung Disease of Prematurity

Daniel D. Lee^{1*}, Charitharth V. Lal^{2,3*}, Elizabeth A. Persad³, Chinn-Woan Lowe¹, Anna M. Schwarz³, Niranjan Awasthi⁴, Roderich E. Schwarz^{4,5}, and Margaret A. Schwarz¹

¹Departments of Pediatrics and ⁴Surgery, Indiana University, South Bend, Indiana; ²Department of Pediatrics, University of Alabama-Birmingham, Birmingham, Alabama; ³Department of Pediatrics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas; and ⁵IU Health Goshen Center for Cancer Care, Goshen, Indiana

Abstract

Myeloid cells are key factors in the progression of bronchopulmonary dysplasia (BPD) pathogenesis. Endothelial monocyte-activating polypeptide II (EMAP II) mediates myeloid cell trafficking. The origin and physiological mechanism by which EMAP II affects pathogenesis in BPD is unknown. The objective was to determine the functional consequences of elevated EMAP II levels in the pathogenesis of murine BPD and to investigate EMAP II neutralization as a therapeutic strategy. Three neonatal mouse models were used: (1) BPD (hyperoxia), (2) EMAP II delivery, and (3) BPD with neutralizing EMAP II antibody treatments. Chemokinetic function of EMAP II and its neutralization were assessed by migration *in vitro* and *in vivo*. We determined the location of EMAP II by immunohistochemistry, pulmonary proinflammatory and chemotactic gene expression by quantitative polymerase chain reaction and immunoblotting, lung outcome by pulmonary function

testing and histological analysis, and right ventricular hypertrophy by Fulton's Index. In BPD, EMAP II initially is a bronchial club-cell-specific protein-derived factor that later is expressed in galectin-3⁺ macrophages as BPD progresses. Continuous elevated expression corroborates with baboon and human BPD. Prolonged elevation of EMAP II levels recruits galectin-3⁺ macrophages, which is followed by an inflammatory state that resembles a severe BPD phenotype characterized by decreased pulmonary compliance, arrested alveolar development, and signs of pulmonary hypertension. *In vivo* pharmacological EMAP II inhibition suppressed proinflammatory genes *Tnfa*, *Il6*, and *Il1b* and chemotactic genes *Ccl2* and *Ccl9* and reversed the severe BPD phenotype. EMAP II is sufficient to induce macrophage recruitment, worsens BPD progression, and represents a targetable mechanism of BPD development.

Keywords: endothelial monocyte-activating polypeptide II; lung; inflammation; bronchopulmonary dysplasia

Lung disease of prematurity is among the disease states driven by inflammation. Placed on supportive care, prematurely born children with underdeveloped lungs

commonly progress toward the development of chronic lung disease, specifically bronchopulmonary dysplasia (BPD). Currently, premature birth is the

leading cause of death in children under the age of 5 years, affecting 1 in 10 births and representing ~15 million births per year worldwide (1–4). In its most severe form,

(Received in original form March 4, 2016; accepted in final form May 11, 2016)

*These authors contributed equally to this work.

This work was supported in part by National Institutes of Health grant 5R01HL114977 (M.A.S.), the Lilly Endowment, Inc., Physician Scientist Initiative (M.A.S.), the Children's Clinical Research Advisory Committee (M.A.S.), and the University of Texas Southwestern Simmons Comprehensive Cancer Center (M.A.S.).

Author Contributions: D.D.L. designed, contributed to, and performed *in vivo* experiments, performed immunoblotting, designed and performed imaging experiments, and analyzed and interpreted data; C.V.L. participated in mouse handling, performed *in vivo* experiments, sample collection and lung morphometry, and contributed to the acquisition of Fulton's index of pulmonary hypertension data; E.A.P. participated in the bronchopulmonary dysplasia model experiment, analyzed data, and performed immunoblotting; C.-W.L. and A.M.S. participated in microscopy experiments; N.A. prepared recombinant endothelial monocyte-activating polypeptide II; R.E.S. critically reviewed the manuscript; M.A.S. conceived the project, designed the mouse studies, interpreted data, and designed and performed *in vivo* and imaging experiments; D.D.L. drafted and wrote the manuscript; and M.A.S. wrote and critically reviewed the manuscript.

Correspondence and requests for reprints should be addressed to Margaret A. Schwarz, M.D., Department of Pediatrics, Indiana University School of Medicine—South Bend, 1234 Notre Dame Avenue, South Bend, IN 46617. E-mail: schwarma@iu.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 55, Iss 4, pp 602–612, Oct 2016

Copyright © 2016 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2016-0091OC on June 2, 2016

Internet address: www.atsjournals.org

Clinical Relevance

Inflammation plays a central role in the progression of a lung disease of prematurity, bronchopulmonary dysplasia (BPD), but it lacks a targeted therapy to minimize the pulmonary inflammatory state while protecting alveolar formation. In previous lung pathophysiologic studies, the antiangiogenic properties of endothelial monocyte-activating polypeptide II (EMAP II) have been the focus, whereas inflammatory properties have been implicated only in cancerous and neurologic inflammatory settings. In a BPD murine model, we identified an up-regulated chemokine, EMAP II and its source, detailed a physiological mechanism by which it worsens BPD pathogenesis, and demonstrated evidence that its neutralization prevents the development of a BPD phenotype. These findings represent a specific therapeutic target upstream of inflammatory cytokines, which BPD is currently lacking.

BPD can result in secondary cardiovascular sequelae such as pulmonary hypertension (PH) that persist into adulthood and abnormal ventilatory response (5–10). Despite advances in clinical ventilator management, the introduction of surfactant, and antenatal glucocorticoids, there is a marked lack of adjunctive therapies.

Pulmonary inflammation contributes significantly to the multifactorial pathogenesis of BPD (11–15). As in other lung injuries such as asthma that are driven by inflammation, in BPD, bronchial epithelial cells and myeloid cells with macrophage lineage are key effectors that drive the secretion of both cytokines and chemokines such as IL-1 β and monocyte chemoattracting protein 1 (MCP-1), respectively.

Clinically, the current therapies administered to premature infants from birth include either surfactants to aid alveolar plasticity or glucocorticoids to limit inflammation and thereby prevent BPD progression. As expected, tracheal aspirates of infants exposed to hyperoxia had elevated inflammatory mediators primarily secreted by macrophages, notably IL-1 β and tumor necrosis factor- α

(14, 15). In infants with sepsis-induced inflammation, inhibitors against the two cytokines showed little improvement in survival rates; in mouse models treated with inhibitors against these cytokines, only some BPD features improved (16–20). This suggests that alternative, more broadly functioning or upstream targets are needed to prevent BPD.

Studies in BPD have identified candidate cytokines to be predictive of BPD onset. However, the source, function, and physiological mechanisms that drive the inflammatory state are poorly understood. In this study, we propose endothelial monocyte-activating polypeptide II (EMAP II) as a potential target in the prevention of BPD in infants undergoing supportive care with hyperoxia. EMAP II (*Aimp1*) encodes one component of the multi-aminoacyl transfer RNA synthetase complex, is ubiquitously expressed, and is conserved across species. EMAP II is defined by its secreted, cleaved extracellular moonlighting functions, with recent studies focusing on its antiangiogenic properties (21–25). EMAP II has also been shown indirectly to recruit macrophages in various injury models (26–28). EMAP II expression localizes between the epithelial/mesenchymal interface in the early stages of normal murine lung development, whereas later saccular and alveolar developmental stages find low levels of EMAP II expression confined to the perivascularity (29, 30).

Previously, we identified an association between elevated EMAP II levels and BPD in premature baboons and human infants (31). As a result, we hypothesized that EMAP II drives macrophage recruitment in BPD, which intensifies the inflammatory state. Using three mouse models, we identified sources of EMAP II throughout BPD progression and showed functional roles for EMAP II in the disease progression of severe BPD. We determined that its chemotactic role on macrophages not only leads to an inflammatory state exacerbating the development of BPD, but also represents a specific upstream, novel target for preventing BPD development.

Materials and Methods

Mice Studies

C57BL/6 mice were obtained from Jackson Laboratories. Studies complied with the

animal protocols approved by the Indiana University Institutional Animal Care and Use Committee. Newborn pups were selected randomly for treatment groups, and mice dams were exchanged every 24 hours to prevent oxygen toxicity. For details on normoxia and hyperoxia treatments, see Figure 1A. Regarding recombinant EMAP II injection studies, see Figure 2A. Antibodies neutralizing EMAP II were delivered according to Figure 4B. For further details, see online supplement.

Quantitative Polymerase Chain Reaction and Immunoblotting

RNA extraction and data collection and analysis were performed according to the methods used in a previous study (32). For further details on protein extraction and immunoblotting, see online supplement; Table E1 in the online supplement details antibodies and dilutions used in these studies.

Lung Microscopy and Morphometry Analysis

Lung tissue sections were prepared as described previously (33). Antigens on lung sections of 5 microns were retrieved and stained with antibodies according to Table E1. Mean linear intercepts (MLIs) and radial alveolar counts were calculated from hematoxylin and eosin-stained sections. Galectin (GAL)-3⁺ counts were performed in a blinded manner, decoded, and analyzed using Python 2.7 (see online supplement).

Lung Functional Studies

To avoid possible hormonal issues, only male mice were tested for pulmonary functions. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (6 mg/kg), followed by pancuronium (1 mg/kg) to induce paralysis. A metal cannulus was inserted through a small tracheal incision, followed by single-model and complex-model measurements of lung function using FlexiVent Software (SCIREQ Inc., Montreal, CA).

Transwell Migration Study

RAW264.7 cells (American Type Culture Collection, Manassas, VA) were cultured in phenol-red-free Dulbecco's modified Eagle medium media containing 10% fetal bovine serum, antimicrobial and antifungal supplement, 5 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid, and 5 mM L-glutamine until

Figure 1. Endothelial monocyte-activating polypeptide II (EMAP II) secreted by airway-conducting epithelial cells of bronchopulmonary dysplasia (BPD) mice recruits macrophages. (A) Experimental schematic of neonatal mouse oxygen exposure to induce BPD. (B) EMAP II protein expression and (C) quantification in whole-lung lysates of normoxia and hyperoxia mice (normalized to β -actin, pooled samples of at least $n = 3$ for Day 3, $n = 2$ for Day 30, $n = 3-4$ for Day 10, $n = 6-10$ for other days; at least two independent experiments). Main effect of oxygen, $**P = 0.0000322$, interaction of oxygen:age, $P = 0.788$. (D) Representative images of immunohistochemical co-staining for EMAP II expression (red) and Clara cell secretory protein (green). Purple indicates co-expression. Scale bar, 20 μm . (E) Representative images of immunohistochemical staining for EMAP II expression (red) and galectin-3 (green). Purple indicates co-expression. Scale bar, 100 μm . Note that compared with lungs exposed to normoxia, those exposed to hyperoxia and harvested on Day 15 were severely dysplastic so that both the bronchial epithelium and the alveoli could not be imaged in the same capture field, although the same magnification as that used in other images was used. (F) EMAP II concentration in tracheal aspirates by immunoblotting and quantification. Main effect of day, $P = 0.0187$; interaction of oxygen:day, $P = 0.711$; $n = 3$ per day. Data are presented as mean $\pm$ SEM. ACTB, protein name of β -actin.

$\sim 70-80\%$ confluent. The media were exchanged for transmigration media containing phenol-red-free Dulbecco's modified Eagle medium and 1% fetal bovine serum for 2 hours before being scraped, incubated in CD16/32 to block nonspecific F'ab interactions on ice for ~ 15 minutes, washed, centrifuged at $400 \times g$ for 5 minutes at 4°C , and aspirated. Following this, 5×10^4 cells were resuspended in transmigration media and loaded into a single 5.0-micron pore Transwell insert. Inactivation of EMAP II protein was performed by boiling for 30 minutes at 100°C or by preincubating with EMAP II-neutralizing antibody at room temperature for 30 minutes at respective dosages. LPS (Serotype *Escherichia coli* 055:B4, Sigma, St. Louis, MO) was also preincubated with EMAP II-neutralizing antibody. The bottom inserts were filled with 500 microliters containing the listed treatments. Transmigration occurred for 4 hours at 37°C , fixed in 4% paraformaldehyde (wt/vol

in phosphate-buffered saline) overnight, and stained in crystal violet solution. Images were captured at $20\times$ magnification on DP70 using MicroSuite Biological Software (Melville, NY) ($n = 4-6$ for each treatment).

Results

EMAP II Levels in Lung Disease of Prematurity

To induce BPD formation, we exposed neonatal mice to an 85% O_2 saturation level (i.e., hyperoxia) and compared them with mice exposed to room air (normoxia) during lung alveologenesis (Figure 1A). EMAP II protein levels were quantified by immunoblotting (Figure 1B). Confirming previous studies, EMAP II expression was perivascular on normoxic Day 5 (Figure E1). Compared with levels in mice at normoxia, EMAP II levels were significantly elevated in the lungs of mice exposed to hyperoxia over time, peaking at

postnatal Day 15 (Figure 1C) (from hereon, mice exposed to hyperoxia and analyzed between 5 and 15 days are termed "early BPD mice," mice analyzed at later time points [i.e., between 15 and 20 days] are termed "BPD mice," and mice analyzed at 20 days and beyond are termed "late BPD"). However, analysis of EMAP II protein levels in tracheal aspirates of BPD mice revealed an early increase at Day 10 but a decline toward that of normoxia control mice by Day 15 (Figure 1F).

EMAP II expression differs in location during BPD formation. A significant increase over time in whole lung but a decreasing trend in tracheal aspirates suggested that EMAP II expression is localized and compartmentalized in response to hyperoxia. EMAP II has been shown to augment inflammatory cell counts (34). We proposed that the localization of EMAP II would be distributed in cells near the tracheal aspirate collection site and thus, histological analysis by co-staining

Figure 2. EMAP II protein mediates macrophage chemoattraction *in vivo*. (A–E) Mice treated with either EMAP II or vehicle (injection) from Days 3 to 15. (A) Schematic of EMAP II treatment in neonatal mice. (B) Representative immunohistochemical images of distal alveoli in lung sections of Day 15 mice showing macrophage (galectin-3, red) and (C) quantification by blinded analysis of galectin-3-positive cells per HPF ($n = 4$, **** $P = 0.00000235$). (D and E) Immunoblot probed for IL1 β in whole lung lysate of Day 15 mice (normalized to β -actin, ** $P = 0.01$, $n = 4$). Scale bar, 100 μ m. Results are representative of four (B and C) or two (D and E) independent experiments. Data are presented as mean $\pm$ SEM. HPF, high-powered field; PFT, pulmonary function tests; rEMAP II, recombinant EMAP II.

EMAP II with GAL-3, an activation and differentiation marker of macrophages, was performed. In contrast to normal perivascular localization of EMAP II expression, by Day 5, EMAP II expression was found in both proximal bronchiolar epithelial-rich regions, indicated by club-cell-specific protein expression, and in the perivascularity (Figure 1D). By Day 10, EMAP II expression was limited to GAL-3⁺ macrophages that were located in both the bronchiole and distal airways (Figure 1E); subsequently, by Day 15, EMAP II was localized only within macrophages of the distal airways. In agreement with the localization moving distally away from the bronchiolar airways, analysis of tracheal aspirates showed a significant decrease in EMAP II expression.

In Vivo Effect of EMAP II on Macrophages

Because a recruitment of macrophages over time was found in BPD mice (Figure 1E), we postulated that excess EMAP II in early

BPD directly recruited macrophages. We administered recombinant EMAP II to mice until the time point at which there was maximal EMAP II expression on Day 15 (Figure 2A). This dosage followed previous studies that determined EMAP II's alternate moonlighting antiangiogenic role (35); as observed previously, angiogenic genes were decreased without a compensatory effect on transcription (Figures E2A and E2B). We found a significant increase in the number of macrophages in the lungs of mice administered EMAP II as compared with control mice (Figures 2B and 2C). This suggested that there was macrophage chemoattraction by EMAP II.

In BPD, particular focus has been given to the proinflammatory cytokine, IL-1 β . Because it is secreted primarily by macrophages and because we saw a significant increase in macrophage recruitment, we evaluated IL-1 β expression in whole lung (Figure 2D). There was significantly elevated IL-1 β expression in lungs administered EMAP II (Figures 2D

and 2E), suggesting a contribution to macrophage pulmonary sequestration.

Effect of EMAP II on Lung Structure and BPD Pulmonary Outcomes

In addition to increased macrophage counts in mice administered EMAP II, a loss of lung structural integrity similar to that of BPD was observed. Because EMAP II has other reported functions, we sought to define the effects of sustained, elevated EMAP II on the lungs. Compared with that of control mice, the body weight of mice administered EMAP II was significantly lower, suggesting impaired overall growth (Figure E2B). Lungs of EMAP II-administered mice had severely dysplastic alveoli and increased elastin deposition (Figures 3A and 3F). There were larger distal airspaces, as evidenced quantitatively by both significantly decreased radial alveolar count and increased MLIs (Figures 3B and 3C). This suggested that excess amounts of EMAP II impaired the lung structure. However, structure does not

Figure 3. Lungs treated with EMAP II present BPD-like phenotype. The experimental design is the same as in Figure 2A. (A) Comparison of distal alveolar structure in inflation-fixed lungs (25 mm Hg) of mice killed on Day 15 by (B) mean linear intercept (**** $P = 0.00000719$), and (C) RAC by blinded observer analysis ($n = 8$, * $P = 0.03337$). (D) Biophysical parameters of lung function compliance, resistance, and elastance were assessed ($n = 3-6$; * $P = 0.011$, ** $P = 0.023$, * $P = 0.008$, respectively) and representative pulmonary flow loops presented. (E) Right ventricular hypertrophy quantified by Fulton's index ($n = 6$ mice per group, ** $P = 0.00520$) and (F) representative deposition of perivascular elastin (arrows) in distal lung tissue sections stained with Masson's trichrome. Scale bars, (A) 100 μ m and (F) 10 μ m. Results are representative of three (A–C and F) or two (D and E) independent experiments. Diamonds are data points past 75%. Data are presented as mean $\pm$ SEM. LV, left ventricular; RAC, radial alveolar count; RV, right ventricular.

always correlate with lung function or outcome measurements (36). Compared with control mice, those given EMAP II had significantly impaired pulmonary biophysical properties. The pressure

volume loop was shifted downward, suggesting an inability of the lungs to inflate maximally, in addition to other biophysical properties (Figures 3D and Figure E2C). To test whether impaired lung

biophysical properties were caused by surfactant expression, we measured surfactant protein-C (SP-C), a common indicator of type II alveolar epithelial cells that secrete surfactants. Compared with

Figure 4. Neutralizing EMAP II limits macrophage recruitment both *in vitro* and *in vivo*. (A) Quantification of Transwell-migrated macrophages in response to EMAP II vehicle (phosphate-buffered saline), nonspecific IgG, and EMAP II preincubated with various concentrations of anti-EMAP II ($n = 2-4$ replicates, $P = 0.0044$, one-way analysis of variance across treatments). (B) Schematic of neonatal hyperoxia exposure protocol used to induce BPD, inj. of anti-EMAP II or IgG. (C) Representative immunohistochemical images of distal alveoli in lung sections showing macrophages (galectin-3, red) and (D) galectin-3-positive cells per HPF, quantified by blinded analysis ($n = 4$ mice, $***P = 0.000457$). Results are representative of samples collected from four (D and E) and two (A) independent experiments. Data are presented as mean $\pm$ SEM. Inj., injection.

control mice, mice administered EMAP II had significantly elevated messenger RNA and protein levels of SP-C (Figures E1D and E1E). This suggested a compensatory mechanism in response to exogenous EMAP II; thus, the lung function change was independent of a lack of SP-C.

EMAP II-Treated Mice Presented with Signs of Pulmonary Hypertension

Macrophage counts were elevated after EMAP II injection, and previous studies link both the elevated counts and the subsequent inflammatory cytokine release to the pathogenesis of not only BPD but also its secondary sequel, PH (37, 38). Lungs of mice injected with EMAP II had impaired alveolarization and blood vessel formation

leading to decreased function, reflecting the antiangiogenic properties of EMAP II (Figure 3A); clinically, this implies cardiovascular sequelae, which are also prominent in the poor outcomes of patients with BPD (7, 10). We observed right ventricular hypertrophy in mice given EMAP II compared with control mice (Figure 3E). We also observed increased elastin deposition by Masson's Trichrome staining in distal vessels (Figure 3F), consistent with the right-heart hypertrophy found in PH.

We concluded that chronic, elevated EMAP II led to BPD-like disease, including the development of signs of secondary PH. Because SP-C levels were not decreased by EMAP II, but elevated EMAP II levels

and macrophage recruitment were found in BPD, an alternative mechanism of up-regulating EMAP II in early BPD must exist that modulates macrophage recruitment, negatively influencing lung and heart outcomes.

Neutralizing Excess EMAP II Limits Chemotactic Effects on Macrophages

We tested whether we could limit macrophage recruitment by neutralizing excess EMAP II. Using an EMAP II-neutralizing antibody (referred to as anti-EMAP II), we assessed macrophage transmigration *in vitro*. We found that, consistent with the *in vivo* findings, exogenous EMAP II significantly increased macrophage transmigration (Figures 4A and 4B).

However, anti-EMAP II incubated with excess EMAP II significantly neutralized this chemoattraction in a dose-dependent manner (Figures 4A and 4B). As a control, heat-inactivating EMAP II negated its function to increase transmigrated cells. Macrophage chemotaxis was specific to EMAP II and this was further confirmed by treating cells with LPS, an inflammatory agent that plays a role in macrophage migration and activation, and anti-EMAP II (Figures 2A and 2B).

To assess whether we could prevent hyperoxia-induced BPD formation, mice were randomly assigned and given the neutralizing anti-EMAP II antibody (Figure 4C). Delivery of antibody to lungs was confirmed (Figure E3A). Recruitment of macrophages in BPD mice was assessed by immunohistochemistry (Figure 4D). After treatment with anti-EMAP II, however, there was a significant decrease in the number of macrophages, as well as inhibition of a BPD-like phenotype (Figure 4E).

Neutralizing Excess EMAP II Improved Lung Structure and Development of Altered Function

We considered that the inhibition of macrophages through neutralizing excess EMAP II in BPD would mitigate murine pulmonary damage. The body weight of hyperoxia mice treated with anti-EMAP II was comparable to that of control groups kept in room air (Figure E3B). After treatment (Figure 4C), there was an increase in the number of distal alveoli measured in a blinded manner, and a visible lack of bronchiolar vessel distension (Figure 5A). Associated with parameters of the qualitative findings, there was a significant decrease in MLI counts, which reflects a decrease in empty air space (Figure 5B). Radial alveolar counts in the lungs of mice treated with anti-EMAP II appeared to increase compared with control non-specific IgG (Figure 5C). By limiting macrophage recruitment, the hyperoxia mice treated with anti-EMAP II showed an improvement in pulmonary outcomes compared with mice treated with control IgG (Figure 5D and Figure E3E). It is possible that this improvement was not caused by limiting macrophage recruitment but perhaps by prevention of cellular apoptosis induced by either hyperoxia or EMAP II. We found increased apoptosis

caused by hyperoxia but an insignificant decrease after anti-EMAP II treatment (Figure E3C). An alternative mechanism might be an increase in surfactant production, but SP-C did not change significantly after anti-EMAP II treatment, suggesting that the treatment was independent of surfactant production (Figure E3D).

Anti-EMAP II Treatment Reduced Signs of PH

To test whether anti-EMAP II treatment could influence the development of PH, we assessed right ventricular hypertrophy. Significantly decreased right ventricular weight was seen in the hearts of hyperoxia mice treated with anti-EMAP II compared with that of mice treated with control IgG, which was comparable to that of mice in room air (Figure 5E). We observed that, consistent with right ventricular hypertrophy, there was elastin deposition in the distal alveolar vessels (Figure 5F).

Reducing Macrophage Numbers Resolved Inflammatory and Chemotactic Gene Expression

We proposed that by limiting macrophage recruitment through anti-EMAP II, we would reduce the levels of proinflammatory and chemotactic gene expression. Using immunoblotting, we detected IL-1 β levels in the lungs of hyperoxia mice (Figure 6A). Elevated IL-1 β levels were reduced significantly in the hyperoxia mice treated with anti-EMAP II (Figure 6B). In addition, the expression of proinflammatory genes *Tnfa*, *Il6*, and *Il1b* and chemotactic genes *Ccl2* and *Ccl9* was markedly decreased after anti-EMAP II treatment (Figure 6C).

Discussion

Premature birth, a major determinant of neonatal morbidity and mortality, is associated with long-term health consequences at an estimated expense of \$26 billion per year in the United States alone. BPD, a lung disease of prematurity, is a preterm complication without a specific targeted treatment. After a call for more directed studies on pulmonary inflammation in BPD, clinical studies determined that inflammatory markers are not only elevated in BPD but associated with prognosis (12, 15, 39). Some studies used untargeted antiinflammatory therapies

such as glucocorticoids, direct cytokines, or chemokines, which resulted in minimal improvement in some characteristics of BPD (19).

In contrast, our results provide an opportunity to consider targeting the pulmonary immune response by addressing macrophage infiltration as a therapeutic component of BPD. Our experiments show that EMAP II is a specific target that contributes directly to the pathogenesis of premature lung disease (e.g., BPD). This was manifested when elevated EMAP II was sustained in the lungs of BPD mice compared with those of control mice, corroborating the temporospatial-dependent role of EMAP II in BPD development in baboons and humans, specifically, in the bronchial epithelium rather than in the perivascularity, where it is normally expressed and declines over time (31). In addition to sustained levels, the direct effect of EMAP II on BPD development was evident when mice treated with EMAP II developed a BPD-like phenotype: arrested alveolar development, right ventricular hypertrophy consistent with PH, macrophage recruitment, and a heightened inflammatory state. Subsequently, anti-EMAP II-treated mice in hyperoxia presented with a significant reduction in the inflammatory state and of the BPD-like phenotype.

The bronchial epithelium has been identified recently as the initial source of an immune response in the context of various injuries (40, 41). Similarly, early, marked, elevated EMAP II expression in primary bronchial CCSP⁺ cells after hyperoxia supports EMAP II's role as an inflammatory modulator in BPD development. Improvement in both macrophage counts and inflammation after anti-EMAP II treatment attests to its chemotactic function in addition to its known antiangiogenic function (21–23, 25, 29, 31, 42). Neutralization of EMAP II limited chemotaxis of macrophages in cell culture and into the lung, ultimately limiting inflammation. Given the proximal CCSP⁺ cell expression of EMAP II followed by macrophages expressing EMAP II in BPD mice, there exists a possible positive reinforcing cycle. Epithelial cells such as the CCSP⁺ cells express EMAP II, which recruits macrophages; these cells, in turn, can produce more EMAP II, which further propagates and activates other immune cells. If this is the case, a novel

Figure 5. Rescued lung structure and function of BPD mice treated with anti-EMAP II. The experimental design is the same as in Figure 4C. (A) Comparison of distal alveolar structure in inflation-fixed lungs (25 mm Hg) of mice killed on Day 15 by (B) mean linear intercept and (C) radial alveolar count by blinded observer analysis ($n = 8$; **** $P = 0.0337$, $P = 0.08898$). (D) Biophysical parameters of lung function compliance, resistance, and elastance were assessed among hyperoxia groups ($n = 6-8$ mice; *** $P = 0.00642$, *** $P = 0.000209$, *** $P = 0.00183$) and representative pulmonary flow loops presented. (E) RV hypertrophy quantified by Fulton index (ratio of RV weight to LV plus septal weight, $n = 3$, ** $P = 0.00537$) and (F) representative deposition of perivascular elastin (arrows) in distal lung tissue sections stained by Masson's trichrome. Scale bars, (A) 100 μ m and (F) 10 μ m. Results are representative of four (A-F) or two (D-F) independent experiments. Diamonds are data points past 75%. Data are presented as mean $\pm$ SEM.

mechanism can be substantiated in clinical BPD development as a potential therapeutic target through the continuous presence of EMAP II.

Moving away from a simple dichotomy in macrophage activation reveals the many varying functional subsets not only

in BPD, but in other disease contexts. Two recent studies indicate that rather than being a simple dichotomy in macrophage activation, a threshold of varying functional subsets of unknown origin (e.g., blood-derived circulation, bone marrow egression) is at least sufficient for

BPD progression (19, 43). The first showed that elevated macrophage numbers in conjunction with proinflammatory gene expression resulted in BPD despite decreased counts of immune response cells (19). This suggests that a hyperactivated macrophage subset is crucial in

Figure 6. Neutralizing EMAP II limited macrophage recruitment and caused inflammation induced by high oxygen to subside. (A and B) Representative immunoblot probed for IL1 β in whole lung lysate of Day 15 mice and quantified ($n = 3$, normalized to β -actin, $*P = 0.0498$). (C) mRNA expression of inflammatory *Il1b*, *Il6*, and *Tnf* and chemokine genes *Ccl2* and *Ccl9* in lungs determined by quantitative polymerase chain reaction calculated on the basis of *Hprt*, *Eef2*, and *Rpl13a* expression ($n = 6-7$; $*P = 0.0195$ [*Il1b*], $**P = 0.0489$ [*Tnfa*], $**P = 0.00594$ [*Il6*], $**P = 0.00227$ [*Ccl2*], $P = 0.0889$ [*Ccl9*]). Samples are from three independent experiments (A–C). Data are presented as mean $\pm$ SEM. IB, immunoblot.

hyperoxia-induced inflammation. The second study defined an alternative macrophage-like CD11b⁺ monocyte origin that protected BPD mice independent of neutrophilia (43). Thus, the macrophage proinflammatory response is not only limited to lung disease of prematurity.

In agreement with the cited studies, our study shows significance in functional outcome

depending on the number of cells transduced by EMAP II. Before any meaningful function is assigned to subsets recruited by EMAP II, further studies are needed to determine the origin and functional consequence in normal lung development, including that of the CD11b mononuclear subset.

A previous study showed that elevated macrophage numbers in conjunction with

proinflammatory gene expression resulted in BPD, despite decreased counts of immune response cells (19). This suggests that hyperactivated macrophages are the major cell type in hyperoxia-induced inflammation. However, as noted, the macrophage proinflammatory response is not only limited to lung disease of prematurity. Using this study of BPD as a

working model, the complex interactions of macrophages and their environment can be implicated equally as contributing or driving factors in other chronic inflammatory diseases such as Crohn's disease or rheumatoid arthritis (44–46). Inhibition of excess macrophage numbers supports normal lung development, informing potential antiinflammatory therapies.

In BPD, hyperoxia-induced inflammation has also been linked to impaired lung biophysical properties, but with conflicting results; both increasing and decreasing compliance have been described (47–49). Some studies suggested that hyperoxia increased compliance as in the case of emphysematous lungs, whereas other studies concluded hyperoxia decreased compliance because the lungs are less pliable (47–50). We tested murine pulmonary outcomes at 6 weeks in accordance with previous studies (36, 47, 49).

Sustained EMAP II was associated with decreased compliance (Figure 3D). For this reason, other biophysical properties, such as resistance, also need to be taken into account. Because impaired biophysical properties are collective, insufficient oxygen exchange, inflammation, and subsequent right ventricular hypertrophy contribute to pulmonary dysfunction. However, after anti-EMAP II treatment, vessels were not thickened, which is an indication of PH. Suppression of EMAP II inflammatory properties alleviated these pulmonary biophysical abnormalities including decreased resistance, decreased tissue damping, and decreased airway space, which are associated with hyperoxia-induced BPD.

Conclusions

Our results highlight an EMAP II-mediated inflammatory mechanism as a significant component of the multifactorial

pathogenesis of BPD, a lung disease of prematurity. In contrast to other studies, the results of our experiments show not only robust protection from a BPD phenotype and signs of secondary PH, but also a reduction of macrophage recruitment and inflammatory status. Neutralization of EMAP II and the curbing of its ability to chemoattract macrophages are possible future therapeutic goals in the prevention of BPD and secondary PH in the context of necessary chronic oxygen supplementation. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank R. Singh for sample handling and H. Xu for sample acquisition. They thank Dr. Jerry Zimmerman for reviewing the manuscript. They thank the Notre Dame Genomics and Bioinformatics Core Facility through Genomics Services for Agilent Bioanalyzer 2100 analysis.

References

- Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L, *et al*. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet* 2012;379:2162–2172.
- Liu L, Oza S, Hogan D, Perin J, Rudan I, Lawn JE, Cousens S, Mathers C, Black RE. Global, regional, and national causes of child mortality in 2000–13, with projections to inform post-2015 priorities: an updated systematic analysis. *Lancet* 2015;385:430–440.
- World Health Organization. Preterm birth. Factsheet No. 363 [updated 2015 Nov; accessed 2015 Nov 20]. Available from: <http://www.who.int/mediacentre/factsheets/fs363/en/>
- Stoll BJ, Hansen NI, Bell EF, Walsh MC, Carlo WA, Shankaran S, Laptook AR, Sánchez PJ, Van Meurs KP, Wyckoff M, *et al*; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993–2012. *JAMA* 2015;314:1039–1051.
- Bates ML, Farrell ET, Eldridge MW. Abnormal ventilatory responses in adults born prematurely. *N Engl J Med* 2014;370:584–585.
- del Cerro MJ, Sabaté Rotés A, Cartón A, Deiros L, Bret M, Cordeiro M, Verdú C, Barrios MI, Albajara L, Gutierrez-Larraya F. Pulmonary hypertension in bronchopulmonary dysplasia: clinical findings, cardiovascular anomalies and outcomes. *Pediatr Pulmonol* 2014;49:49–59.
- Khemani E, McElhinney DB, Rhein L, Andrade O, Lacro RV, Thomas KC, Mullen MP. Pulmonary artery hypertension in formerly premature infants with bronchopulmonary dysplasia: clinical features and outcomes in the surfactant era. *Pediatrics* 2007;120:1260–1269.
- Mourani PM, Sontag MK, Younoszai A, Miller JI, Kinsella JP, Baker CD, Poindexter BB, Ingram DA, Abman SH. Early pulmonary vascular disease in preterm infants at risk for bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2015;191:87–95.
- Austin ED, Kawut SM, Gladwin MT, Abman SH. Pulmonary hypertension: NHLBI Workshop on the Primary Prevention of Chronic Lung Diseases. *Ann Am Thorac Soc* 2014;11:S178–S185.
- Bhat R, Salas AA, Foster C, Carlo WA, Ambalavanan N. Prospective analysis of pulmonary hypertension in extremely low birth weight infants. *Pediatrics* 2012;129:e682–e689.
- Jobe AH, Ikegami M. Lung development and function in preterm infants in the surfactant treatment era. *Annu Rev Physiol* 2000;62:825–846.
- McEvoy CT, Jain L, Schmidt B, Abman S, Bancalari E, Aschner JL. Bronchopulmonary dysplasia: NHLBI Workshop on the Primary Prevention of Chronic Lung Diseases. *Ann Am Thorac Soc* 2014;11:S146–S153.
- Desmarquest P, Chadelat K, Corroyer S, Cazals V, Clement A. Effect of hyperoxia on human macrophage cytokine response. *Respir Med* 1998;92:951–960.
- Eriksson L, Haglund B, Odland V, Altman M, Kieler H. Prenatal inflammatory risk factors for development of bronchopulmonary dysplasia. *Pediatr Pulmonol* 2014;49:665–672.
- Ambalavanan N, Carlo WA, D'Angio CT, McDonald SA, Das A, Schendel D, Thorsen P, Higgins RD; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. *Pediatrics* 2009;123:1132–1141.
- Abraham E, Anzueto A, Gutierrez G, Tessler S, San Pedro G, Wunderink R, Dal Nogare A, Nasraway S, Berman S, Cooney R, *et al*; NORASEPT II Study Group. Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. *Lancet* 1998;351:929–933.
- Opal SM, Fisher CJ Jr, Dhainaut JF, Vincent JL, Brase R, Lowry SF, Sadoff JC, Slotman GJ, Levy H, Balk RA, *et al*. Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. *Crit Care Med* 1997;25:1115–1124.
- Oncel MY, Yurtutan S, Alyamac Dizdar E, Gokce IK, Gonul II, Topal T, Canpolat FE, Dilmun U. Beneficial effect of etanercept on hyperoxic lung injury model in neonatal rats. *J Invest Surg* 2016;29:1–5.
- Nold MF, Mangan NE, Rudloff I, Cho SX, Shariatian N, Samarasinghe TD, Skuza EM, Pedersen J, Veldman A, Berger PJ, *et al*. Interleukin-1 receptor antagonist prevents murine bronchopulmonary dysplasia induced by perinatal inflammation and hyperoxia. *Proc Natl Acad Sci USA* 2013;110:14384–14389.
- Johnson BH, Yi M, Masood A, Belcastro R, Li J, Shek S, Kantores C, Jankov RP, Tanswell AK. A critical role for the IL-1 receptor in lung injury induced in neonatal rats by 60% O₂. *Pediatr Res* 2009;66:260–265.

21. Schwarz MA, Zhang F, Gebb S, Starnes V, Warburton D. Endothelial monocyte activating polypeptide II inhibits lung neovascularization and airway epithelial morphogenesis. *Mech Dev* 2000;95:123–132.
22. Schwarz MA, Kandel J, Brett J, Li J, Hayward J, Schwarz RE, Chappey O, Wautier JL, Chabot J, Lo Gerfo P, et al. Endothelial-monocyte activating polypeptide II, a novel antitumor cytokine that suppresses primary and metastatic tumor growth and induces apoptosis in growing endothelial cells. *J Exp Med* 1999;190:341–354.
23. Reznikov AG, Chaykovskaya LV, Polyakova LI, Kornelyuk AI. Antitumor effect of endothelial monocyte-activating polypeptide-II on human prostate adenocarcinoma in mouse xenograft model. *Exp Oncol* 2007;29:267–271.
24. Tsai BM, Wang M, Clauss M, Sun P, Meldrum DR. Endothelial monocyte-activating polypeptide II causes NOS-dependent pulmonary artery vasodilation: a novel effect for a proinflammatory cytokine. *Am J Physiol Regul Integr Comp Physiol* 2004;287:R767–R771.
25. Clauss M, Voswinkel R, Rajashekhar G, Sigua NL, Fehrenbach H, Rush NI, Schweitzer KS, Yildirim AO, Kamocki K, Fisher AJ, et al. Lung endothelial monocyte-activating protein 2 is a mediator of cigarette smoke-induced emphysema in mice. *J Clin Invest* 2011;121:2470–2479.
26. van Horssen R, Rens JA, Brunstein F, Guns V, van Gils M, Hagen TL, Eggermont AM. Intratumoural expression of TNF-R1 and EMAP-II in relation to response of patients treated with TNF-based isolated limb perfusion. *Int J Cancer* 2006;119:1481–1490.
27. Schikorski D, Cuvillier-Hot V, Boidin-Wichlacz C, Slomianny C, Salzet M, Tasiemski A. Deciphering the immune function and regulation by a TLR of the cytokine EMAPII in the lesioned central nervous system using a leech model. *J Immunol* 2009;183:7119–7128.
28. Clarijs R, Schalkwijk L, Ruiter DJ, de Waal RM. EMAP-II expression is associated with macrophage accumulation in primary uveal melanoma. *Invest Ophthalmol Vis Sci* 2003;44:1801–1806.
29. Schwarz M, Lee M, Zhang F, Zhao J, Jin Y, Smith S, Bhuva J, Stern D, Warburton D, Starnes V. EMAP II: a modulator of neovascularization in the developing lung. *Am J Physiol* 1999;276:L365–L375.
30. Zhang F, Schwarz MA. Temporo-spatial distribution of endothelial-monocyte activating polypeptide II, an anti-angiogenic protein, in the mouse embryo. *Dev Dyn* 2000;218:490–498.
31. Quintos-Alagheband ML, White CW, Schwarz MA. Potential role for antiangiogenic proteins in the evolution of bronchopulmonary dysplasia. *Antioxid Redox Signal* 2004;6:137–145.
32. Lee DD, Schwarz MA. Adapted approach to profile genes while reconciling *Vegf-a* mRNA expression in the developing and injured lung. *Am J Physiol Lung Cell Mol Physiol* 2015;308:L1202–L1211.
33. Bennett KM, Afanador MD, Lal CV, Xu H, Persad E, Legan SK, Chenaux G, Dellinger M, Savani RC, Dravis C, et al. Ephrin-B2 reverse signaling increases $\alpha 5\beta 1$ integrin-mediated fibronectin deposition and reduces distal lung compliance. *Am J Respir Cell Mol Biol* 2013;49:680–687.
34. Nührenberg TG, Langwieser N, Schwarz JB, Hou Y, Frank P, Sorge F, Matschurat S, Seidl S, Kastrati A, Schömig A, et al. EMAP-II downregulation contributes to the beneficial effects of rapamycin after vascular injury. *Cardiovasc Res* 2008;77:580–589.
35. Schwarz RE, Schwarz MA. *In vivo* therapy of local tumor progression by targeting vascular endothelium with EMAP-II. *J Surg Res* 2004;120:64–72.
36. Manali ED, Moschos C, Triantafyllidou C, Kotanidou A, Psallidas I, Karabela SP, Roussos C, Papiris S, Armaganidis A, Stathopoulos GT, et al. Static and dynamic mechanics of the murine lung after intratracheal bleomycin. *BMC Pulm Med* 2011;11:33.
37. Jankov RP, Luo X, Belcastro R, Copland I, Frndova H, Lye SJ, Hoidal JR, Post M, Tanswell AK. Gadolinium chloride inhibits pulmonary macrophage influx and prevents O₂-induced pulmonary hypertension in the neonatal rat. *Pediatr Res* 2001;50:172–183.
38. Masood A, Yi M, Lau M, Belcastro R, Li J, Kantores C, Pace-Asciak CR, Jankov RP, Tanswell AK. Cyclooxygenase-2 inhibition partially protects against 60% O₂-mediated lung injury in neonatal rats. *Pediatr Pulmonol* 2014;49:991–1002.
39. Walsh MC, Szefer S, Davis J, Allen M, Van Marter L, Abman S, Blackmon L, Jobe A. Summary proceedings from the bronchopulmonary dysplasia group. *Pediatrics* 2006;117:S52–S56.
40. Evans CM, Williams OW, Tuvim MJ, Nigam R, Mixides GP, Blackburn MR, DeMayo FJ, Burns AR, Smith C, Reynolds SD, et al. Mucin is produced by Clara cells in the proximal airways of antigen-challenged mice. *Am J Respir Cell Mol Biol* 2004;31:382–394.
41. Gibbs J, Ince L, Matthews L, Mei J, Bell T, Yang N, Saer B, Begley N, Poolman T, Pariollaud M, et al. An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. *Nat Med* 2014;20:919–926.
42. Yuan C, Yan L, Solanki P, Vatner SF, Vatner DE, Schwarz MA. Blockade of EMAP II protects cardiac function after chronic myocardial infarction by inducing angiogenesis. *J Mol Cell Cardiol* 2015;79:224–231.
43. Eldredge LC, Treuting PM, Manicone AM, Ziegler SF, Parks WC, McGuire JK. CD11b⁺ mononuclear cells mitigate hyperoxia-induced lung injury in neonatal mice. *Am J Respir Cell Mol Biol* 2016;54:273–283.
44. Thiesen S, Janciauskiene S, Uronen-Hansson H, Agace W, Högerkorp CM, Spee P, Håkansson K, Grip O. CD14^{hi}HLA-DR^{dim} macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. *J Leukoc Biol* 2014;95:531–541.
45. Murthy A, Li Y, Peng I, Reichelt M, Katakam AK, Noubade R, Roose-Girma M, DeVoss J, Diehl L, Graham RR, et al. A Crohn's disease variant in *Atg16l1* enhances its degradation by caspase 3. *Nature* 2014;506:456–462.
46. Vande Walle L, Van Opdenbosch N, Jacques P, Fossoul A, Verheugen E, Vogel P, Beyaert R, Elewaut D, Kanneganti TD, van Loo G, et al. Negative regulation of the NLRP3 inflammasome by A20 protects against arthritis. *Nature* 2014;512:69–73.
47. Yee M, Chess PR, McGrath-Morrow SA, Wang Z, Gelein R, Zhou R, Dean DA, Notter RH, O'Reilly MA. Neonatal oxygen adversely affects lung function in adult mice without altering surfactant composition or activity. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L641–L649.
48. Yee M, White RJ, Awad HA, Bates WA, McGrath-Morrow SA, O'Reilly MA. Neonatal hyperoxia causes pulmonary vascular disease and shortens life span in aging mice. *Am J Pathol* 2011;178:2601–2610.
49. Ramani M, Bradley WE, Dell'Italia LJ, Ambalavanan N. Early exposure to hyperoxia or hypoxia adversely impacts cardiopulmonary development. *Am J Respir Cell Mol Biol* 2015;52:594–602.
50. Woyda K, Koebrich S, Reiss I, Rudloff S, Pullamsetti SS, Rühlmann A, Weissmann N, Ghofrani HA, Günther A, Seeger W, et al. Inhibition of phosphodiesterase 4 enhances lung alveolarisation in neonatal mice exposed to hyperoxia. *Eur Respir J* 2009;33:861–870.