Bronchopulmonary dysplasia (BPD) occurs in approximately 80% of infants born between 22 and 24 weeks of gestation, and despite advances in ventilation to minimize barotrauma and supportive oxygen therapy to reduce oxidative stress, as well as supportive care, its incidence has not decreased. As more extremely premature infants survive, they increasingly are burdened by lifelong impaired lung health and an increased risk of lung disease, including chronic obstructive pulmonary disease. Retinopathy of prematurity (ROP) is a sight-threatening condition that affects 80% of premature neonates with a birthweight <1000 grams. ROP and BPD share many risk factors and the diseases often arise concurrently, suggesting a shared, or related, pathogenesis, which also is reflected in disease models. There is no effective treatment for either condition.

Inflammation, linked to the clinical pathology of alveolar simplification and enlargement, and microvascular damage, is common in BPD, suggesting a related pathogenesis, but specific mechanisms of BPD and ROP are obscure. A neonatal mouse hyperoxic model of coincident BPD and retinopathy was used to screen for candidate mediators, which revealed that granulocyte colony-stimulating factor (G-CSF) was up-regulated significantly in mouse lung lavage fluid and plasma at postnatal day 14 in response to hyperoxia. Preterm infants with more severe BPD had increased plasma G-CSF. G-CSF–deficient neonatal pups showed significantly reduced alveolar simplification, normalized alveolar and airway resistance, and normalized weight gain compared with wild-type pups after hyperoxic lung injury. This was associated with a marked reduction in the intensity, and activation state, of neutrophilic and monocytic inflammation and its attendant oxidative stress response, and protection of lung endothelial cells. G-CSF deficiency also provided partial protection against ROP. The findings in this study implicate G-CSF as a pathogenic mediator of BPD and ROP, and suggest the therapeutic utility of targeting G-CSF biology to treat these conditions. (Am J Pathol 2023, 1–15; https://doi.org/10.1016/j.ajpath.2023.07.006)
have long been implicated in BPD. In humans, BPD tracheal aspirates and lung washes have revealed increased proinflammatory cytokines, chemokines, soluble adhesion molecules, and growth factors including granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF, linked to neutrophil-mediated and monocyte-/macrophage-mediated inflammation.  

Inflammatory microbial activation, neutrophil recruitment, and mediators, including angiopeptidins and G-CSF, are implicated in ROP. Despite the link to inflammation, the use of anti-inflammatory steroids remains controversial because steroids have been linked to iatrogenic harm, notably optic nerve damage and neurocognitive impairment, and, thus, new, more effective, treatments are needed. Oxidative stress is linked inextricably to BPD in humans because ambient room air is a hyperoxic environment compared with lung oxygen tension in utero, and oxidative defense mechanisms develop late in gestation; this is compounded postpartum when supportive oxygen therapy is indicated. Accordingly, hyperoxic exposure of neonatal rodents, whose lung development lags humans at normal-term birth, is widely considered a rational and useful model of human BPD. In larger rodent models, inhibition of neutrophil influx improved alveolar development. Thus, blocking mediators of neutrophil-driven inflammation holds promise for the treatment of BPD.

G-CSF (CSF3) co-regulates the mobilization, survival, growth, and activation state, in particular, the capacity for oxidative injury, of neutrophils and mononuclear/macrophage lineages. It previously was shown that G-CSF directly causes severe multisystem pathology in experimental chronic obstructive pulmonary disease, asthma, and autoimmune/inflammation-PLCγ2-associated antibody deficiency immune dysregulation in adult mice. The current study tested whether G-CSF was up-regulated in human BPD neonates and whether its deficiency in mice could protect the lungs and eyes, which identified G-CSF biology as a potential therapeutic target for BPD and ROP.

Materials and Methods

Study Design

These studies were designed to identify pathogenic factors that were induced in neonatal mice in response to a high-oxygen insult, and once G-CSF was identified as the most significantly up-regulated factor, it was validated in a cohort of preterm infants by proteomic analysis of plasma. To elucidate the specific role of G-CSF in BPD, studies used C57BL/6 mice or mice deficient in G-CSF. Mice are well suited for modeling BPD because their lung structural development is delayed compared with that of humans, with the saccular stage occurring in the first 5 days after birth. Thus, mice were exposed to 75% oxygen for the first 14 days of life to mimic the period of respiratory support received by human preterm infants. This protocol leads to a phenotype reminiscent of BPD, and although it does not induce classic ROP, it leads to retinal thinning as an indicator of retinopathy. For every litter assigned to a high-oxygen group, a corresponding age-matched litter reared solely in room air was used as a control group. The lungs, blood, and eyes were evaluated at postnatal day 14 (PN14), and lungs were assessed again on PN40. Lung and retinal histopathology were performed on PN14, together with chromogenic immunohistochemistry, flow cytometry, gene expression studies of the lung tissue, and lung function tests. Lung histopathology also was performed on PN40 to assess long-term disease protection. All in vivo and ex vivo experiments were performed under controlled laboratory conditions and used samples collected across multiple experiments with appropriate biological and technical replicates. Details on statistics analyses and group sizes are included in all figure legends.

Study Approvals

Animal and human studies were approved by the Alfred Research Alliance Animal Ethics Committee (E-1746-2017M; Australia) and the Ludwig-Maximilian University of Munich Ethics Committee (EC #195-07; Germany), respectively.

Mice and Hyperoxia Exposure Model

Equal numbers of male or female G-CSF-/- mice on a C57BL/6 background or C57BL/6 wild-type controls were assigned randomly to normoxia (21% oxygen) or hyperoxia (75% oxygen) and were exposed within 12 hours of birth, defined as PN1. Mothers and litters were cycled in room air for 3 hours daily to reduce oxygen-induced toxicity to the dam. Pup losses occurred but were equal in the hyperoxia and room air groups, and there were no differences in losses between C57BL/6 or G-CSF-/- mice. These largely were attributed to mouse physiologic behavior on the day of birth or shortly thereafter, and not to the effects of hyperoxia itself. Mice were analyzed on PN14 and a subgroup was analyzed on PN40 (young adults). Age-matched C57BL/6 and G-CSF-/- mice housed under normoxic conditions were used as controls.

Bronchoalveolar Lavage and Plasma Cytokines in Mice

Cytokine concentrations in bronchoalveolar lavage (BAL) and plasma on PN14 were measured using a Bio-Plex Pro Mouse Cytokine 23-plex Assay (M60009RDPD; Bio-Rad Laboratories, South Granville, New South Wales, Australia) following the manufacturer’s specifications. Plates were analyzed using the Luminex MAGPIX system (Thermo Fisher Scientific, Waltham, MA).

Assessment of G-CSF Levels in Premature Infants

Serial whole-blood samples (minimum, 200 µL each) were obtained from infants during routine laboratory blood
drawings using ethylenediaminetetraacetic acid neonatal collection tubes. The sampling time point was in the first week of life, except for one child who was sampled on day 65 (median, day 4; range, 0 to 65 days of life). After pseudonymization, samples were processed for proteomic screening by centrifugation (1000 × g, 5 minutes) before supernatants were aliquoted and stored at −80°C. Samples were analyzed with the SOMAscan (SomaLogic, Boulder, CO) assay (n = 30 samples). This assay uses 1124 individual high-affinity molecules (SOMAmers - slow-off rate modified DNA aptamer - reagents) quantified on a custom Agilent hybridization array.28,29 one of which was G-CSF. Preterm infants were included in the study prospectively at the Perinatal Center in Munich after informed parental consent following published inclusion and exclusion criteria.30 Clinical variables were monitored comprehensively from birth to discharge (Table 1). Premature infants were classified as having no BPD (grade 0, n = 10), mild BPD (grade 1, n = 11), moderate BPD (grade 2, n = 4), and severe BPD (grade 3, n = 5) according to international consensus criteria published by Jobe and Bancalari.33 Preterm infants also were classified as having no ROP (0) or ROP (1) according to international guidelines.34 These data are part of a previously published data set.30

Lung Histology

At PN14 and PN40, lungs were inflation-fixed under constant pressure with 10% neutral-buffered formalin, and stored in 70% ethanol for 24 hours before processing. Paraffin-embedded lungs were cut (5 μm) at random intervals along the length of the lung and serial sections were mounted onto glass slides for histopathology and immunohistochemistry. Lung sections were stained with hematoxylin and eosin for evaluation of alveolar structure. Images were captured on an Olympus BX-51 brightfield microscope using 10× and 40× objectives (Olympus Australia, Notting Hill, Victoria, Australia). The mean linear intercept and septal wall thickness were calculated as previously described.5 To determine the mean linear intercept measurement, 10 images of each hematoxylin and eosin-stained lung cross-section were captured randomly at ×10 magnification and 10 equally distributed lines were drawn on each. The number of times each line crossed an airwall was counted and averaged for each lung. The mean linear intercept then was calculated by dividing the known length of the line that had been drawn across each lung cross-section (in micrometers) by the mean number of airwall intercepts. To define alveolar septal wall thickness, 10 randomly captured lung images at ×10 magnification were uploaded into ImageJ software version 1.37 (NIH, Bethesda, MD; http://imagej.nih.gov/ij), and for each image, 60 horizontal lines were drawn perpendicular to randomly chosen alveolar airwalls. The mean thickness of the airwall (in micrometers) was calculated for each lung sample.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Characteristics of Patients Used in This Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm cohort</td>
<td>Data*</td>
</tr>
<tr>
<td>n</td>
<td>30</td>
</tr>
<tr>
<td>GA, weeks</td>
<td>26.45 (23.2 to 29.4)</td>
</tr>
<tr>
<td>Birth weight, grams</td>
<td>795 (530 to 1510)</td>
</tr>
<tr>
<td>Sampling day</td>
<td>4 (0 to 65)</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>16/14</td>
</tr>
<tr>
<td>ANCS</td>
<td>21 (70)</td>
</tr>
<tr>
<td>Early onset infection</td>
<td>7 (23.3)</td>
</tr>
<tr>
<td>RDS</td>
<td>27 (90)</td>
</tr>
<tr>
<td>Mechanical ventilation, days</td>
<td>50 (5 to 109)</td>
</tr>
<tr>
<td>Oxygen supplementation, days</td>
<td>36 (0 to 186)</td>
</tr>
<tr>
<td>PDA</td>
<td>21 (70)</td>
</tr>
<tr>
<td>Postnatal steroids</td>
<td>13 (43.3)</td>
</tr>
<tr>
<td>ROP</td>
<td>8 (26.7)</td>
</tr>
<tr>
<td>IVH</td>
<td>5 (16.7)</td>
</tr>
<tr>
<td>ICU stay, days</td>
<td>71 (32 to 119)</td>
</tr>
<tr>
<td>BPD grade with coincident ROP</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>10 (33.3)</td>
</tr>
<tr>
<td>Mild</td>
<td>11 (36.7)</td>
</tr>
<tr>
<td>Moderate</td>
<td>4 (13.3)</td>
</tr>
<tr>
<td>Severe</td>
<td>5 (16.7)</td>
</tr>
<tr>
<td>BPD grade with coincident ROP</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1 of 10 (10)</td>
</tr>
<tr>
<td>Mild</td>
<td>1 of 11 (9.1)</td>
</tr>
<tr>
<td>Moderate</td>
<td>3 of 4 (75)</td>
</tr>
<tr>
<td>Severe</td>
<td>3 of 5 (60)</td>
</tr>
</tbody>
</table>

Sections for immunohistochemistry underwent heat-induced antigen-retrieval followed by immunostaining for markers of peroxidation (3-nitrotyrosine, 1:500; Abcam, Cambridge, UK), overnight at 4°C, which were captured on an Olympus BX-51 brightfield microscope using 10× and 40× objectives (Olympus Australia, Notting Hill, Victoria, Australia). The mean linear intercept and septal wall thickness were calculated as previously described.5 To determine the mean linear intercept measurement, 10 images of each hematoxylin and eosin-stained lung cross-section were captured randomly at ×10 magnification and 10 equally distributed lines were drawn on each. The number of times each line crossed an airwall was counted and averaged for each lung. The mean linear intercept then was calculated by dividing the known length of the line that had been drawn across each lung cross-section (in micrometers) by the mean number of airwall intercepts. To define alveolar septal wall thickness, 10 randomly captured lung images at ×10 magnification were uploaded into ImageJ software version 1.37 (NIH, Bethesda, MD; http://imagej.nih.gov/ij), and for each image, 60 horizontal lines were drawn perpendicular to randomly chosen alveolar airwalls. The mean thickness of the airwall (in micrometers) was calculated for each lung sample.

Diagnosis and severity of respiratory distress syndrome, scored on anterior–posterior chest radiographs according to Couchard et al.,11 Systemic infections (EOI), diagnosed according to Sherman et al.,12 based on one or more clinical and laboratory signs of infection. Bronchopulmonary dysplasia, defined according to Jobe and Bancalari12 was graded as mild (oxygen supplementation at 28 days postnatally), moderate (oxygen supplementation <30% and/or ventilator support at 36 weeks postmenstrual age), and severe (oxygen supplementation <30% and/or ventilator support at 36 weeks postmenstrual age).

*Data are given as the median and range or as the number and percentage of the total in group respective range.

1One child was sampled on day 65, but all other children were sampled within the first week of life.
2One male child had intrauterine growth restriction.
3ANCS, antenatal corticosteroids; BPD, bronchopulmonary dysplasia; GA, gestational age; ICU, intensive care unit; IVH, intraventricular hemorrhage; PDA, ______; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity (graded according to international criteria*).

*Indicates a role for G-CSF in BPD and Retinopathy.
determine the total lung area. The script then was run to calculate the percentage area of positive DAB tissue/cells in each image automatically. The selected thresholds were applied for all images to maintain consistency. Investigators were blinded to the experimental groups during morphometric analyses.

**Analysis of Pulmonary Function**

Mice were anesthetized by intraperitoneal injection of 125 mg/kg ketamine/10 mg/kg xylazine (Centravet, Taden, France), and, when deeply anesthetized, underwent a tracheostomy followed by insertion of a cannula (20G for neonates) between the first and second tracheal rings. Two sutures were tied, sealing the wall of the trachea around the cannula. To block spontaneous breathing, mice were administered pancuronium (1 mg/kg intraperitoneally) and then connected to an animal ventilator (Flexivent; SCIREQ, Montreal, Canada). Mice were ventilated mechanically (respiration rate, 300 breaths/minute; tidal volume, 10 mL/kg for neonates), and a positive end-expiratory pressure was set at 3 cm H$_2$O. Flexiware data acquisition software (v8.0.4) was used to perform respiratory system mechanics. $^{35}$ Initially, a deep inflation was performed to recruit closed lung areas and standardize lung volume history. This was followed by a snapshot-300 perturbation (1.26-second, 2.5-Hz, single-frequency forced oscillation measurement) to calculate respiratory system resistance, respiratory system compliance, and respiratory system elastance. An optimal ventilator waveform (quick-prime perturbation optimized for use with neonates; 2.8 seconds, extended range of input frequencies between 2 and 23 Hz; broadband low-frequency forced oscillation measurement) was performed to distinguish airway and tissue mechanics through measurements of central airway resistance (R$_n$, Newtonian resistance), tissue damping (G), and tissue elastance (H). Three independent measurements were taken for all perturbations for each mouse and the average was calculated.

**BAL and Lung Tissue Collection**

Mouse lungs were washed with ice-cold phosphate-buffered saline (5 x 200 µL) and the lavage was centrifuged immediately to isolate cells. The cell-free BAL fluid was frozen at -80°C for subsequent cytokine analysis. The cellular pellet was reconstituted in 1 mL FACS buffer (1 x phosphate-buffered saline, 2% fetal calf serum, 0.5 mmol/L EDTA), before counting via hemocytometer. Lungs were digested in Liberase (26 U/mL; Merck, Bayswater, Victoria, Australia) and DNase I (15 U/mL) for 30 minutes at 37°C, followed by rapid dissociation using the gentleMACS Dissociator (Miltenyi Biotec, San Jose, CA) as described. $^{36}$ The homogenized tissue was filtered through a 40-µm strainer and washed with FACS buffer. Cell preparations were counted using the Coulter counter (Beckman Coulter, Brea, CA). Cytospins were prepared for BAL and imaged with an Aperio Digital Pathology Slide Scanner (Leica, Mt Waverley, Victoria, Australia).

**Flow Cytometry**

Flow cytometry was used to assess the structural cell compartment of the lung. The lungs were first digested at 37°C in Liberase, depleted of red blood cells by lysis, washed, filtered, incubated in 1:650 Fc block (in-house), and then stained using monoclonal antibodies, as detailed in Table 2 (all from BD Biosciences, San Jose, CA). Cells were analyzed using the LSR-Fortessa X-20 flow cytometer (BD Biosciences). Dead cells were excluded using Fluorogold Live/Dead, and CD45-expressing immune cells were gated and removed. The gating strategy used is detailed in Supplemental Figure S1. Because hyperoxia affected body weight gain of C57BL/6 mice to a greater extent than G-CSF-/-/ mice, which reflected smaller lungs, data are presented as proportions of CD45-negative cells, rather than absolute cell numbers.

BAL cells also were analyzed by flow cytometry, $^{22}$ with the gating strategy described in Supplemental Figure S2. Data were analyzed using FlowJo software (Windows v10, FlowJo, LLC, Ashland, OR) and cell proportions were calculated from the set gates. Expression of CD11b on myeloid cells was determined by normalizing the geometric mean fluorescence intensity on gated cell populations, by dividing the geometric mean fluorescence intensity of each individual sample by the mean geometric mean fluorescence intensity of the C57BL/6 normoxia group within each experiment, allowing data from multiple experiments to be pooled.

**Eye Histology and Retinal Thickness Quantitation**

Enucleated eyes from the same C57BL/6 and G-CSF-/- mice that were used for PN14 lung studies were fixed in 10% neutral-buffered formalin and submitted for alcohol processing. Paraform-embedded eye sections (3 µm) were cut 20 µm apart, and every 40th section was collected. Four sections were collected, stained with hematoxylin and eosin, and imaged using the Aperio Digital Pathology Slide Scanner (Leica). Quantitation of the central (approximately 100 µm from optic nerve on the left and right sides) and the midperipheral (middle area between the optic nerve and retina edge) region of the inner plexiform layer and the inner nuclear layer of the retina was performed as previously described. $^{37}$ Thickness was measured using the built-in line measurement tool within the Aperio ImageScope 12.3.3 Pathology Viewing Software. Three lines were drawn for each region and the average thickness was calculated per section. Investigators were blinded to the experimental groups.

**Quantitative RT-PCR**

Total RNA was extracted from frozen lung tissue using TRI-reagent (Sigma-Aldrich, Bayswater Victoria, Australia).
by established methods. \(^{38}\) RNA was converted to cDNA using the FIRESCRIPT Reverse Transcription cDNA synthesis kit (Solis Biodyne, Tartu, Estonia), as per the manufacturer’s instructions. Quantitative RT-PCR was performed using the following TaqMan prevalidated primers: NADPH oxidase 1 (Nqo1; Mm00549170_m1), NADPH quinone dehydrogenase 1 (Nqo1; Mm01253561_m1), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh; Mm99999915_g1), which was used as a housekeeping transcript. Gene expression studies assessing specific lung endothelial cells were performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Scoresby, Victoria, Australia). Primers for platelet endothelial cell adhesion molecule 1 (Pecam1) were from OriGene (Rockville, MD), with data normalized to hypoxanthine phosphoribosyl transferase (Hprt). \(^{40}\) Data were analyzed using the comparative 2\(^{-\Delta\DeltaCT}\) method.

Statistics

Human protein expression levels were log2 transformed and analyzed by t-test, assuming a log-normal distribution. The protein concentration was corrected by extracting the residuals from the linear regression model, which regressed the two confounders (early onset infection and oxygen) onto the log2 transformed protein expression. Mouse data were analyzed by nonparametric U-test and are presented as the median ± interquartile range. Differences induced by hyperoxia within the one genotype are indicated by asterisks only, and the effect of G-CSF deficiency on the hyperoxia response in C57BL/6 mice is indicated by a bar, with significance marked on top. Nonsignificant differences are unmarked. \(P\) values < 0.05 were considered statistically significant (GraphPad Prism v9.3.1, San Diego, CA).

Results

Increased G-CSF Levels in the Lung and Plasma of Neonatal Mice Exposed to Hyperoxia

C57BL/6 mice exposed from PN1 to 14% to 75% oxygen (hyperoxia) had a marked increase in G-CSF, KC/CXCL1, \(^{33}\) and macrophage inflammatory protein (MIP)-1\(\alpha\), and a lesser increase in IL-6, IL-1\(\alpha\), IL-17A, and MCP-1 in BAL fluid on PN14 compared with normoxic controls with no differences for granulocyte-macrophage CSF, MIP-1\(\beta\), and IL-1\(\beta\) (Figure 1A, Supplemental Figure S3). G-CSF, IL-6, \(^{32}\) MIP-1\(\beta\), and IL-10 also were increased in plasma (Figure 1A, Supplemental Figure S3).

Increased G-CSF Levels in Preterm Infants with Moderate and Severe BPD

To assess the role of G-CSF in human disease, early postnatal (median, 4 days after birth) plasma levels of G-CSF were measured in preterm neonates (patient characteristics are summarized in Table 1). Preterm infants diagnosed with moderate and severe BPD presented with significantly increased plasma G-CSF levels after birth when compared with mild BPD cases and infants without the disease (Figure 1B), while accounting for potential effects of early onset infection in the analysis.

G-CSF Deficiency Protects Against BPD-like Lung Pathology in Neonatal Mice

At PN14, the lungs of hyperoxia-exposed C57BL/6 mice showed marked alveolar simplification, as well as septal wall thickening and histologic evidence of cellular infiltrate (Figure 2, A–C). \(^{32}\) In contrast, the lungs of hyperoxia-exposed G-CSF-/- mice showed markedly reduced inflammatory cell infiltrates (Figure 2A), and only minor increases in alveolar septal wall thickness (Figure 2, A and B). Although G-CSF-/- mice also showed significantly increased airspace enlargement (mean linear intercept), this was reduced significantly compared with C57BL/6 mice (Figure 2, A and C). G-CSF-/- mice also showed improved body weight gain in hyperoxia conditions, suggesting they were less affected (Figure 2D).

G-CSF Deficiency Reduces Oxidative Stress in the Neonatal Mouse Lung After Hyperoxia

Protein peroxidation revealed by 3-nitrotyrosine immunostaining and oxidant defense gene balance are thought to have important roles in BPD pathogenesis. \(^{32}\) The lungs of hyperoxia-exposed G-CSF-/- mice showed less 3-nitrotyrosine immunostaining, indicating less-damaging oxidative tyrosine modification (Figure 2, E and F). Nox1 mRNA is known to be up-regulated by hyperoxia \(^{4}\) and contributes to superoxide and peroxide generation. Its

Table 2 Antibodies Used in This Study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>30–F11</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11c</td>
<td>N418</td>
<td>PE-Cy7</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>APC-e780</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Ly6G</td>
<td>1A8</td>
<td>BV510</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>c-fms/CD115</td>
<td>AF598</td>
<td>Biotin</td>
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<td>Siglec F</td>
<td>E50-2440</td>
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<td>390</td>
<td>Biotin</td>
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</tr>
<tr>
<td>CD45</td>
<td>30–F11</td>
<td>FITC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>EpCAM (CD326)</td>
<td>G08-8</td>
<td>APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Streptavidin N/A</td>
<td>BV711</td>
<td></td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>

ApC; EpCAM, epithelial cell adhesion molecule; FITC, fluorescein isothiocyanate; Ly6G, ________; PE-Cy7, ________; Siglec F, _______.
expression was increased significantly by hyperoxia in C57BL/6 lungs while remaining largely unchanged in G-CSF−/− lungs (Figure 2G). Gene expression of the antioxidant enzyme, Nqo1, was increased markedly in the lungs of hyperoxia-exposed C57BL/6 mice, whereas expression was induced only mildly in G-CSF−/− mice. Collectively, these results are consistent with a lower oxidative burden in the lungs of G-CSF−/− mice after hyperoxia exposure (Figure 2H).

G-CSF Deficiency Reduces Alveolar Cell Proliferation and Endothelial Damage from Hyperoxia

Alveolar epithelial cell (AEC) proliferation reflects the extent of microvascular damage from hyperoxia. Immunostaining on PN14 revealed a marked decrease in Ki-67+ cells in hyperoxia-exposed G-CSF−/− mice (Figure 3, A and B), suggesting that there is less damage to their lungs. AEC and endothelial cell compartments then were assessed by flow cytometry of whole-lung tissue digests (Supplemental Figure S1, Figure 3C). After hyperoxia, proportions of epithelial cell adhesion molecule (EpCAM)highCD31neg AECs were increased in C57BL/6 mice but remained unchanged in G-CSF−/− mice (Figure 3D), whereas EpCAMintCD31neg AECs were expanded similarly (Figure 3E). Hyperoxia reduced the proportions of lung EpCAMhighCD31+ endothelial cells to a lesser degree in G-CSF−/− mice (Figure 3F). To analyze the endothelial compartment further, gene expression studies were performed to examine genes known to be expressed specifically in different endothelial cell subsets but not by other cells in the lung. Strikingly, 14 days of hyperoxia significantly reduced expression of Pecam1 (all endothelial cells), VWF (arterial and venous endothelial
Figure 2  Neonatal mice deficient in granulocyte colony-stimulating factor (G-CSF) are protected from oxygen-induced alveolar structural damage. C57BL/6 and G-CSF−/− mice were reared in normoxia or hyperoxia conditions and their lungs were analyzed on postnatal day 14. A: Representative photomicrographs of lung cross-sections of the indicated mice stained with hematoxylin and eosin (blue arrowheads represent thickened alveolar walls and black arrowheads depict immune cells). B–D: Measurements of alveolar septal wall thickness (B), mean linear intercept on the groups in panel A (C), and body weight in grams (D). E: Top: Representative photomicrographs taken with a Olympus BX-51 bright field microscope of lungs stained for 3-nitrotyrosine (pink arrowheads) and counterstained with hematoxylin. Bottom: Selected regions of interest are magnified. F: Quantitation of the percentage of 3-nitrotyrosine staining per lung area on the groups in panel E. G and H: Gene expression of reactive oxygen species–producing enzyme Nox1 (G) and anti-oxidant Nqo1 in lung tissue measured by quantitative RT-PCR (H). Data are shown as median ± interquartile range. *n = 4 mice per group from two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus the same genotype mice in normoxia conditions. Scale bars = 50 μm. Original magnification, ×40.
cells), and Gpihpb1 (general capillary endothelial cells), but not Ca4 (alveolar aerocytes) in C57BL/6 mice, but expression was unaffected by hyperoxia in G-CSF−/− mice (Figure 3, G–J). Collectively, these data indicate that G-CSF deficiency partially protects alveolar and endothelial compartments to reduce lung damage in response to hyperoxia exposure.

**G-CSF Deficiency Reduces Inflammation in the Lungs of Neonatal Mice**

BAL cytospins on PN14 showed that small quiescent alveolar macrophages (AMs) were the main cell type in C57BL/6 and G-CSF−/− mice in normoxic conditions (Figure 4A). In hyperoxia-exposed C57BL/6 mice, BAL cytopsins showed red blood cells, consistent with microvascular fragility; neutrophils; and activated, enlarged, and vacuolated AMs (Figure 4A). In contrast, the BAL of hyperoxia-exposed G-CSF−/− mice showed no red blood cells, fewer infiltrating neutrophils, and AMs were of a more quiescent appearance (Figure 4A).

Total BAL cell numbers were somewhat reduced by hyperoxia (Figure 4B), and flow cytometry of BAL (Supplemental Figure S2) showed that the proportion of CD45+CD11c+ AMs was reduced in C57BL/6 mice, but surprisingly was unchanged in G-CSF−/− deficient mice.
Hyperoxia induced a marked increase in expression of the activation marker CD11b\(^+\) on AM\(^\circ\)s from C57BL/6 mice, but it was only slightly up-regulated on AM\(^\circ\)s from G-CSF-/- mice (Figure 4D). In response to hyperoxia, proportions of CD11b\(+\)CD115\(+\) monocytes were increased significantly in the BAL of C57BL/6, but not G-CSF-/-, mice (Figure 4E). A small proportion of eosinophils was present in the BAL at steady-state, likely related to induction of type 2 responses at birth.\(^{47}\) Interestingly, hyperoxia drove a marked reduction in eosinophils in both strains, suggesting susceptibility to oxygen toxicity (Figure 4F).

Strikingly, hyperoxia induced a prominent infiltration of neutrophils into the airspaces of C57BL/6 mice, which was reduced markedly in G-CSF-/- mice (Figure 4G). To further analyze inflammation in BAL, levels of the three inflammatory proteins that were identified in the initial screen as being induced significantly in C57BL/6 mice by 14 days of hyperoxia were measured. Unlike in C57BL/6 mice, neither IL-6 nor MCP-1 were up-regulated by hyperoxia in G-CSF-/- mice (Figure 4, H and I), and
although MIP-1α was induced, this was not to the same extent in G-CSF-/- mice (Figure 4J).

G-CSF Deficiency Improves Respiratory Function in Neonatal Mice Exposed to Hyperoxia

On PN14, C57BL/6 mice exposed to hyperoxia showed no change in compliance or elastance (Figure 5, A and B), but a significant increase in dynamic respiratory resistance, tissue damping, and tissue elastance (Figure 5, C–E) compared with normoxia controls, whereas no significant changes were observed in hyperoxia-exposed G-CSF-/- mice (Figure 5, A–E). Because iatrogenic barotrauma during supportive ventilation relates directly to the force needed to overcome lung resistance, it was of great interest that C57BL/6 mice developed a marked increase in airway resistance, but this did not occur in G-CSF-/- mice (Figure 5F). This suggests that G-CSF deficiency is protective in reducing constriction in the lung induced by neonatal hyperoxia.

G-CSF Deficiency Limits Retinal Thinning Within the Neonatal Mouse Eye

To examine if G-CSF also plays a role in retinopathy, the retinas of hyperoxia-exposed mice were examined for thinning, a known index of disease (Figure 6, A and B). After hyperoxia, thinning of the inner plexiform layer in the central and midperipheral retina was observed in C57BL/6 mice, whereas only the central retina was affected in G-CSF-/- mice (Figure 6, C and D). Similarly, there was thinning of the inner nuclear layer in the central and midperipheral regions of hyperoxia-exposed C57BL/6 mice, which were unaffected in G-CSF-deficient mice (Figure 6, E and F). Thus, although hyperoxia drove the thinning of all four retinal layers in C57BL/6 mice, G-CSF-/- mice were more protected from the hyperoxia insult.

In the neonatal patient cohort examined in this study (Table 1), 8 of the 30 neonates had ROP, 6 of which had moderate or severe comorbid BPD and 1 had mild BPD. However, no significant difference was observed when comparing plasma levels of G-CSF in infants with and without ROP (Supplemental Figure S4).

G-CSF Deficiency Partly Protects the Lungs of Adult Mice Exposed to Hyperoxia in Infancy

To determine whether the protection provided by G-CSF deficiency in early life extended into adulthood, mice were examined on PN40. Young adult C57BL/6 mice exposed to hyperoxia during early life showed progressive alveolar hypoplasia, with larger and simplified alveoli consistent with emphysematous changes (Figure 7A). In contrast, adult G-CSF-/- mice showed partial protection from the grossly distorted alveolar architecture observed in C57BL/6 mice (Figure 7A). Airspace enlargement, measured as the mean linear intercept, was increased in adult mice that had neonatal hyperoxia exposure, but to a significantly lesser degree in G-CSF-/- mice (Figure 7B).
Discussion

In this study, mediators that were highly induced in neonatal mouse hyperoxic lung injury were determined and a candidate, G-CSF, was validated in a human neonatal BPD cohort. Because the injured mouse pups were extremely frail and small, precluding repeat pharmacologic inhibition of G-CSF, atraumatic gene deletion was used to study its pathogenic role. The main preclinical findings of the potential clinical translation importance were a reduction of alveolar simplification associated with a marked decrease in neutrophil and mononuclear cell infiltrates and attendant oxidative injury, improvements in lung function and weight gain, and partial attenuation of coincident retinopathy. Importantly, this atraumatic method allowed pups to survive into young adulthood, demonstrating a persisting benefit. Accordingly, we propose targeting G-CSF, and by inference its receptor, signaling, and linked biology, as a therapeutic strategy in BPD and ROP.

Inflammation is prominent in human BPD, and the simplest explanation of the findings in this study is that deficiency of G-CSF reduces the inflammatory cell number, activation state, and capacity to cause oxidative injury. Ambient air constitutes a hyperoxic insult to the human immature neonatal lung, which is worsened by supportive oxygen therapy and replicated in the mouse hyperoxia model. It has been known since its original description in the 1960s that lung tissue readily releases G-CSF, which acts on bone marrow to mobilize leukocytes, principally neutrophils and mononuclear/macrophage cells, expand their hematopoietic lineages, and activate their host defense effector functions, such as oxidative capacity.

G-CSF-deficient mice are neutropenic and mobilize neutrophils less efficiently, consistent with an increased susceptibility to *Listeria* infection. Nonetheless, they are able to mount a robust pulmonary neutrophilic response to lipopolysaccharide, which is interesting given their impaired ability to induce neutrophils in response to hyperoxia. G-CSF is implicated directly in severe neutrophilic asthma and it has been demonstrated previously that G-CSF deficiency greatly reduces lung and comorbid systemic pathologies in experimental chronic obstructive pulmonary disease. Inflammatory neutrophils and monocytes/macrophages readily cause oxidative microvascular damage in the lung consistent with the pathology, oxidative biomarkers, and balance of inflammatory mediators were changed earlier in the disease process. We also measured protein, in the future, a time course to examine protein expression in the lung and circulation in response to hyperoxia would be valuable to determine if other inflammatory mediators were changed earlier in the disease process.

In addition to inflammation, BPD is characterized by disrupted lung growth, microvascular damage causing alveolar simplification, and lung function abnormalities.
G-CSF deficiency moderately protected against hyperoxia-induced endothelial destruction and protected against pulmonary function defects characteristic of lung consolidation and reduced tissue elasticity. Furthermore, loss of G-CSF led to reduced cellular proliferation in alveolar walls in response to hyperoxia. Hyperoxia expands type II AECs, which can transdifferentiate into structural type I AECs during repair, and there is emerging evidence that G-CSF may have a protective effect on this compartment to support the lung after acute lung injury, which is in keeping with its receptor being highly expressed by type II AECs (Human Protein Atlas). Although this may seem at odds with the data presented herein showing that deficiency of G-CSF is protective during hyperoxia, the BPD model involves exposing mice to hyperoxia for 14 days, not acutely, which leads to damaging effects on the endothelial compartment driven by inflammation and oxidative stress, and this may well underpin the deleterious effect on the epithelial compartment. It will be of considerable interest to apply new technologies, such as single-cell sequencing or spatial transcriptomics, to understand how blocking G-CSF affects the fate of EpCAM<sup>int</sup> cells marking both type I and type II AECs. Alveolarization, which continues postpartum in humans and mice, is slowed by injury and inflammation. G-CSF receptors are expressed by numerous cells in the lung including immune cells, type II AECs, and specialized cells such as club and ciliated cells (LungMAP), and it will be interesting to determine if blocking G-CSF promotes alveolarization, even indirectly by reducing injury. G-CSF also may affect the airways more than appreciated currently, as a marked protection from increased lung resistance, and fewer EpCAM<sup>high</sup> ciliated cells<sup>45</sup> in G-CSF-/− mice were noted, again suggesting effects on central airways, where persisting defects are of increasing interest in the long-term management of BPD.<sup>21</sup>

It generally is thought that the endothelium is the key source of G-CSF in response to injury or infection, driving myeloid release from the bone marrow in emergency hematopoiesis. However, G-CSF receptors also have been reported to be expressed on endothelial cells, where G-CSF has been shown to regulate their proliferation, migration, and survival. These studies have suggested that G-CSF supplementation can safeguard endothelial cells from apoptosis, which may be a protective mechanism in settings of acute inflammation and high myeloid cell activity, and may falter if inflammation persists. However, these studies, although foundationally important, did not assess the effect of G-CSF on endothelial cell activity in an inflammatory in vivo setting, and although a later study assessed the response of endothelial cells to lipopolysaccharide in the presence of G-CSF, neither study assessed whether G-CSF receptor expression on endothelial cells was modulated by the presence of inflammatory mediators. Furthermore, in response to hyperoxia, oxidative stress occurs concurrently with acute inflammation, and the effect this has on G-CSF receptor expression by distinct endothelial cell subsets is pertinent. This study has shown that G-CSF deficiency provides wide-ranging protection to specific lung endothelial subsets in the context of hyperoxia, including general capillary endothelial cells as well as arterial and venous endothelial cells. However, it is not clear whether G-CSF deficiency directly protects endothelial cells or whether there is an overall protective effect as a result of reduced oxidative burden in the lungs that prevents severe damage, which will be the focus of future studies.

Systemic inflammation after high-oxygen exposure in early life also has been implicated in the development of ROP. Thinning of inner retinal layers often is associated with other inflammatory retinal conditions such as diabetic retinopathy. Thus, it is of interest that the retinas of G-CSF-/− mice are more protected from the effects of hyperoxia. However, although partial retinal protection was observed in G-CSF−/− deficient mice, the resultant effect on retinal function or vision remains unknown, and, furthermore, an association in humans between serum G-CSF and ROP was not observed. However, it is possible that local eye-specific levels of G-CSF are more important in ROP and these may not necessarily correlate with circulating levels of G-CSF in the disease. Understanding this also may provide a rationale for intravitreal therapy to preserve sight.
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in human infants. Future studies to localize G-CSF to the different retinal layers in response to hyperoxia now is important to extend upon these preliminary observations.

This study shows the feasibility of studying G-CSF in BPD by genetically deleting the G-CSF/CSF3 locus. In future studies, it should be technically feasible to delete G-CSF (or its receptor or signaling intermediates) using an inducible system under temporal control and in precisely defined cells or cell lineages. Notwithstanding the advantages of genetic deletion, the current study had pertinent limitations. To enable clinical translation, it will be essential to test inhibitors, such as monoclonal antibodies or alternative technologies. Here, the choice of a technology allowing rapid tapering of effect (eg, by renal clearance or metabolism) may be important given the central protective role of G-CSF in infection, a common complication of BPD. In humans, a correlation between G-CSF levels during infection and BPD severity was not observed. Blocking G-CSF systemically, however, is known to be safe in higher primates.60 It also will be important to establish in future studies whether there are any specific advantages of direct pulmonary or intravitreal delivery of such inhibitors. Furthermore, it is necessary to determine whether inhibiting G-CSF works simply by reducing inflammatory damage and/or if it also influences fundamental features of developmental alveolarization and repair such as vascular remodeling because this could optimize the timing and duration of therapy. Specific studies on neurocognitive impairment, a common complication of BPD,1 are warranted. Despite these limitations, targeting G-CSF (and by inference its broader biology) may be an effective new treatment for BPD andROP to ameliorate their debilitating life-long consequences.

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Disclosures

None declared.

Author Contributions

M.L.H. conceived the study; M.L.H., G.P.A., E.T., and A.H. funded the research; L.C.W., A.H., and M.L.H. designed the research; L.C.W., E.T., A.K., A.L.R., T.A.G., and J.G.B. performed the experiments in the study; L.C.W., E.T., A.K., T.A.G., J.G.B., A.H., and M.L.H. analyzed the data; A.M. provided expert clinical insight into the project; P.V.W. provided a critical piece of equipment, expertise on the retinal studies, and expert review of intellectual content; G.P.A. provided expert advice on intellectual content; L.C.W., G.P.A., and M.L.H. wrote the paper; and all other authors provided editorial comment.

Supplemental Data

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