ORIGINAL RESEARCH

Foxm1 Regulates Resolution of Hyperoxic Lung Injury in Newborns

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Abstract

Current treatments for inflammation associated with bronchopulmonary dysplasia (BPD) fail to show clinical efficacy. Foxm1, a transcription factor of the Forkhead box family, is a critical mediator of lung development and carcinogenesis, but its role in BPD-associated pulmonary inflammation is unknown. Immunohistochemistry and RNA analysis were used to assess Foxm1 in lung tissue from hyperoxia-treated mice and patients with BPD. *LysM-Cre/Foxm1*$^{-/-}$ mice, in which Foxm1 was deleted from myeloid-derived inflammatory cells, including macrophages, monocytes, and neutrophils, were exposed to neonatal hyperoxia, causing lung injury and remodeling. Measurements of lung function and flow cytometry were used to evaluate the effects of Foxm1 deletion on pulmonary inflammation and repair. Increased Foxm1 expression was observed in pulmonary macrophages of hyperoxia-exposed mice and in lung tissue from patients with BPD. After hyperoxia, deletion of Foxm1 from the myeloid cell lineage decreased numbers of interstitial macrophages ($CD45^+CD11b^+Ly6C^-Ly6G^-F4/80^+CD68^-$) and impaired alveologenesis and lung function. The exaggerated BPD-like phenotype observed in hyperoxia-exposed *LysM-Cre/Foxm1*$^{-/-}$ mice was associated with increased expression of neutrophil-derived myeloperoxidase, proteinase 3, and cathepsin g, all of which are critical for lung remodeling and inflammation. Our data demonstrate that Foxm1 influences pulmonary inflammatory responses to hyperoxia, inhibiting neutrophil-derived enzymes and enhancing monocytic responses that limit alveolar injury and remodeling in neonatal lungs.

Keywords: Foxm1; bronchopulmonary dysplasia; hyperoxia; inflammation; macrophage

Clinical Relevance

Our data demonstrate that Foxm1 influences pulmonary inflammatory responses to hyperoxia, inhibiting neutrophil-derived enzymes and enhancing monocytic responses that limit alveolar injury and remodeling in neonatal lungs. Discovery of pharmacological agents that can modulate Foxm1 function in postnatal lungs could be beneficial to inhibit hyperoxia-induced inflammation and stimulate lung repair in patients with bronchopulmonary dysplasia.

Bronchopulmonary dysplasia (BPD) commonly develops in premature babies born before 36 weeks after conception. With approximately 50% of very-low-birth-weight infants born before 28 weeks gestational age developing BPD, it is the most common chronic lung disease among infants in industrialized nations (1). Every year, 7,500 to 25,000 American infants develop BPD (2). BPD is characterized by lung inflammation and arrested alveolar and vascular lung development (3). Inflammation is a major contributor to the pathogenesis of BPD. Often initiated by a pulmonary fetal inflammatory response, lung inflammation is exacerbated by mechanical ventilation and exposure to supplemental oxygen (4). Persistent lung inflammation has been shown to greatly increase the severity of BPD (2). Lung inflammation is typically characterized by increased pulmonary macrophages and neutrophils (2, 5). Pulmonary inflammation has been characterized to follow a standard

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timeline, with neutrophils moving from circulation to the lungs and recruiting macrophages, which resolve the inflammatory response (2, 5).

At 28 to 36 weeks gestation, lung development, an alveologenesis in particular, is insufficient to support the respiratory needs of the neonate. Standard clinical practice is mechanical ventilation with higher than atmospheric oxygen to provide an adequate supply of oxygen to the tissues of the body. Oxygen treatment causes undesirable side effects, including extensive inflammatory responses, destruction of the alveolar–capillary barrier, and pulmonary edema (39), all contributing to delayed or permanent disruption of postnatal lung development. Inflammation induced as a result of this insult has been shown to be a major contributor to the development of BPD, yet attempts to limit inflammation have not significantly improved survival, leading to a critical need for new approaches to supplement existing BPD therapy (40).

Exposure of neonatal animals to high concentrations of oxygen causes inflammation and lung remodeling that is similar to pulmonary responses in patients with BPD (6–8). Exposure to hyperoxia causes increased generation of reactive oxygen species, inducing lung injury, which results in cell cycle arrest and/or cell death (9, 10). Hyperoxia causes chemoattractant synthesis and release of inflammatory cytokines to recruit innate immune cells to the lung. Accumulation of neutrophils and macrophages enhances lung injury by the elaboration of proteases, oxidant stress (11), and chemokine release (12), which cause further injury and growth arrest resulting in loss of alveoli. In mouse models, prevention of neutrophil accumulation reduced acute lung injury (13). The beneficial or harmful effects of various inflammatory cells in the pathogenesis of BPD are unclear. Insight into the mechanism of inflammation after prolonged hyperoxic exposure could help identify potential new therapeutic targets in patients with BPD.

Foxm1 (formerly known as HFH-11B, Trident, Win, or MPP2), a member of the Forkhead Box (Fox) family of transcription factors, is mainly expressed in proliferating cells. Foxm1 is widely expressed during embryogenesis, consistent with its role in proliferation and cell differentiation. In adult mice, Foxm1 is more restricted, being observed in intestinal crypts, testes, and thymus (14, 15). During carcinogenesis and after tissue injury, Foxm1 is present in various cell types, including neoplastic, inflammatory, and regenerating cells (16–18). Global deletion of Foxm1 in transgenic mice causes lethal embryonic malformations in multiple organ systems (19). Cell-specific roles for Foxm1 are emerging from studies in transgenic mice with conditional deletion of Foxm1. Embryonic or perinatal lethal phenotypes were observed after Foxm1 deletion from hepatoblasts (20), precursors of cerebellar granule neurons (21), and smooth muscle cells (22). Conditional deletion of Foxm1 in developing respiratory epithelium caused neonatal respiratory failure due to delayed differentiation of type I cells and impaired lung maturation (23). In hepatocytes and in epithelial and endothelial cells, Foxm1 facilitated tissue repair after injury in the lungs and liver (15, 18, 24–26). Mice with conditional deletion of Foxm1 from myeloid inflammatory cells (*LysM-Cre/Foxm1*$^{-/-}$ mice) were recently generated and exhibited delayed liver repair (27). Foxm1 deletion inhibited carcinogenesis and airway allergic responses (28, 29). Although previous studies indicate that Foxm1 plays a critical role in myeloid-derived inflammatory cells, its role in the pathogenesis of neonatal lung disorders remains unknown.

In the present study, the role of Foxm1 in regulation of pulmonary inflammation and remodeling in BPD-like lung disease was investigated. Foxm1 expression was increased in lung tissue from patients with BPD and in hyperoxia-exposed neonatal mice. Selective deletion of Foxm1 from myeloid cells exacerbated lung injury and remodeling in hyperoxia-exposed mice, disrupting formation of peripheral alveoli and reducing lung function.

Materials and Methods

Mouse Strains

Generation of *Foxm1*$^{flox/flox}$ (*Foxm1*$^{fl/fl}$) mice was previously described (20). *Foxm1*$^{fl/fl}$ female mice were bred with *LysM-Cre*$^{tg/-}$ male mice (Jackson Laboratories, Bar Harbor, ME) to generate *LysM-Cre*$^{tg/-}$ *Foxm1*$^{fl/fl}$ double transgenic mice (m*Foxm1*$^{-/-}$). *LysM*-Cre deletes Foxm1 in all myeloid cells, including macrophages, monocytes, and granulocytes (30). *Foxm1*$^{fl/fl}$ littermates were used as controls. The following primers were used to genotype mouse genomic DNA: *Foxm1* sense, 5′-TGGCTTCCCAGCAGTACAAATC-3′; *Foxm1* antisense intron, 5′-TGCTT ACAAAAGACACACTTGGACG-3′; *Foxm1* antisense 3′-UTR, 5′-TCTC GCTCAATTCCAAGACCAG-3′; *LysM-Cre* sense, 5′-CTTGGGCTGCCA GAATTTCTC-3′; *LysM-Cre* antisense, 5′-CCCAGAAATGCCAGATTACG-3′. BPD responses were similar between control *Foxm1*$^{fl/fl}$ and wild-type mice. The experimental protocol was approved by Cincinnati Children's Hospital Medical Center Animal Care and Use Committee.

Exposure to Hyperoxia

Pups (≤12 h) were placed in hyperoxic chambers (85% oxygen) or room air for up to 3 weeks. Nursing mothers were rotated between hyperoxia and room air litters daily to avoid maternal oxygen toxicity and maternal effects between groups. Oxygen levels were monitored with a Miniox II monitor (Catalyst Research, Owings Mills, MD). Survival was recorded daily. Baseline lung function was determined by a computer-controlled small animal ventilator (Flexivent, Scireq, VA) as previously described (31).

Morphometric Analysis

Morphometric measurements were performed using Image-1/Metamorph Imaging System (Universal Imaging, West Chester, PA). Radial alveolar counts were performed as previously described (32).

Immunohistochemical Staining and Flow Cytometry

Paraffin lung sections were used for immunohistochemical staining as described previously (31, 33). Human lung samples were collected as part of Cincinnati Children's Hospital Medical Center Institutional Review Board Study 2008–0844. The study includes autopsy samples from infants with BPD and from infants who did not have BPD. The following antibodies were used for immunohistochemistry: Mac-3 (1:2,000 [IHC]) (BD Pharmingen, San Jose, CA), myeloperoxidase (MPO) (1:1,000) (R&D Systems, Minneapolis, MN), and Foxm1 (1:750 [IHC]) (C-20; Santa Cruz Biotech, Dallas, TX). Immunofluorescent staining was performed as previously described (16, 28, 34). Diff-Quik (Siemens, Malvern, PA) staining was performed according to the manufacturer's instructions.

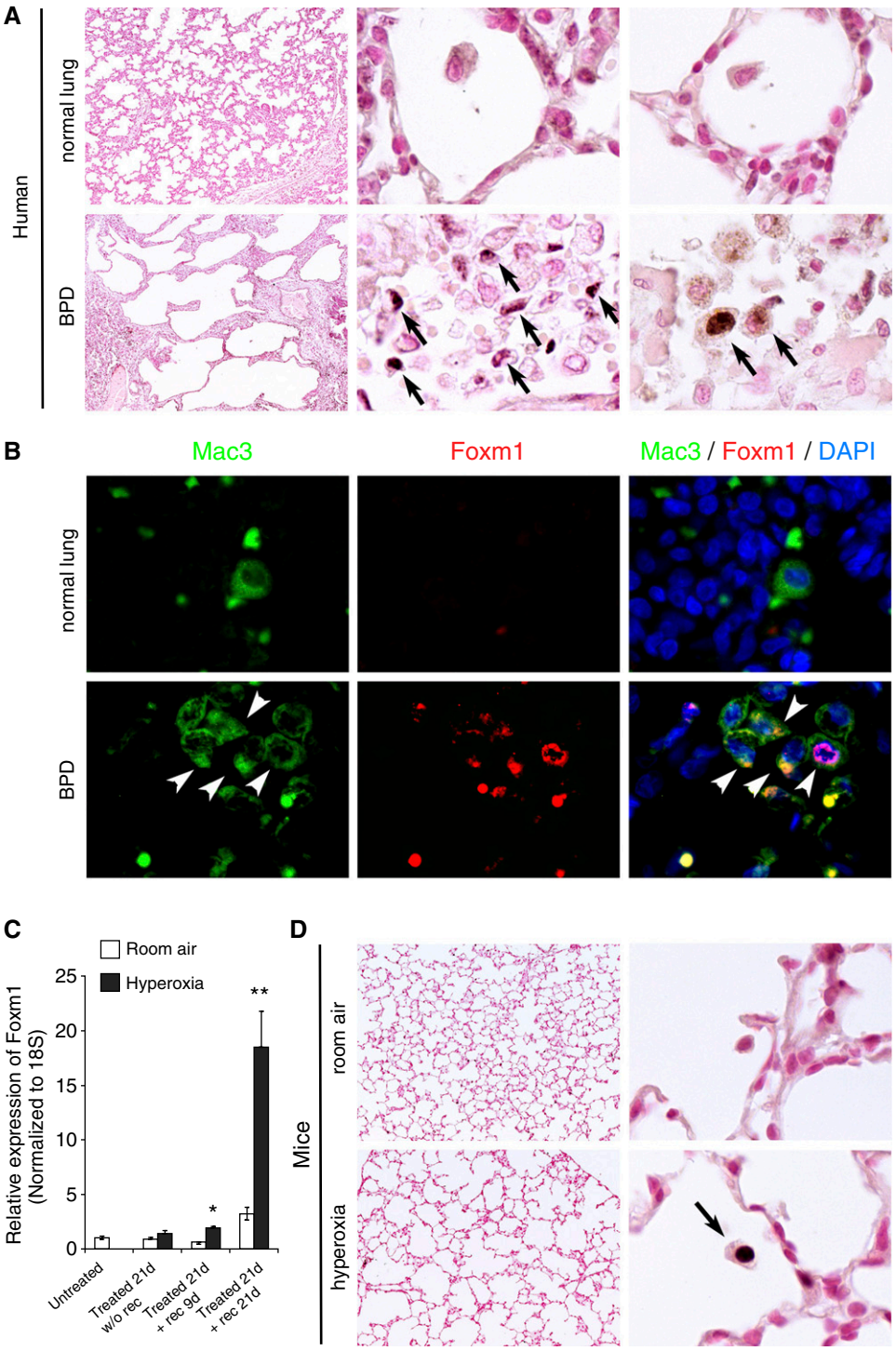

Figure 1. Increased expression of Foxm1 in lung tissue of patients with bronchopulmonary dysplasia (BPD) and hyperoxia-exposed mice. (*A*) Foxm1 expression is increased in lungs of patients with BPD compared with normal lungs. Alveolar macrophages were rare in normal human lung, but Foxm1-positive macrophages (*arrows*) were prevalent in lung autopsies from patients with BPD ($n = 7$). Original magnification: ×50 and ×1,000. (*B*) Colocalization experiments confirmed that Foxm1-positive cells in patients with BPD were macrophages as Foxm1 colocalized with the macrophage marker Mac3 (*arrowheads*). Original magnification: ×1,000. (*C*) Foxm1 expression is induced in a mouse model of BPD-like lung injury. Wild-type mice were housed in hyperoxic conditions (HO) for 3 weeks after birth followed by room air recovery (RA). Foxm1 mRNA was increased in mouse lungs after HO and 9-day RA recovery, and this increase was greater at 21-day recovery ($n = 5$ mice per group). $^{*}P < 0.05$; $^{**}P < 0.01$. (*D*) Foxm1 staining was found in pulmonary macrophages from HO-exposed mice but not RA-exposed mice. Original magnification: ×100 and ×1,000. DAPI, 4′,6-diamidino-2-phenylindole.

Inflammatory cells were isolated from lungs of normoxic and hyperoxic *mFoxm1*$^{-/-}$ and *Foxm1*$^{fl/fl}$ mice by flow cytometry as described previously (35). The following antibodies were used to stain inflammatory cells: anti-F4/80 (clone BM8; eBiosciences, San Diego, CA), anti-CD11b (clone M1/70; eBiosciences), anti-Ly-6C (clone HK1.4; BioLegend, San Diego, CA), anti–Ly-6G (clone 1A8; BioLegend), anti-CD68 (clone FA-11; Biolegend), and anti-CD45 (clone 30-F11; BD Pharmingen). Dead cells were excluded using 7-aminoactinomycin stain (eBiosciences). Stained cells were separated using cell sorting (five-laser FACSAria II; BD Biosciences). Specific cell subsets were identified using the indicated surface marker phenotypes: neutrophils, CD45$^+$CD11b$^+$Ly6C$^+$Ly6G$^+$; monocytes, CD45$^+$CD11b$^+$Ly6C^{hi}Ly6G$^-$F4/80$^+$; interstitial macrophages, CD45$^+$CD11b$^+$Ly6C$^-$Ly6G$^-$F4/80$^+$CD68$^-$; and alveolar macrophages, CD45$^+$CD11b$^+$Ly6C$^-$Ly6G$^-$F4/80$^+$CD68$^+$. Purified cells were used for RNA preparation followed by quantitative RT-PCR (qRT-PCR).

Alveolar type II epithelial cells were identified using antibodies against CD324 (clone DECMA-1; eBiosciences), CD326 (clone G8.8; eBiosciences), and MHC II (clone M5/114.15.2; eBiosciences).

qRT-PCR and Western Blot

StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) and inventoried TaqMan gene expression assays were used as described previously (23, 36). Reactions were analyzed in triplicate, and expression levels were normalized to β-actin mRNA. Western blots were performed as previously described (37) with antibodies against MPO (1:1,000) (R&D Systems) and β-actin (1:3,000) (C-11; Santa Cruz Biotech).

Statistical Analysis

Student's *t* test and multivariant ANOVA were used to determine statistical significance. *P* values less than 0.05 were considered significant. All measurements are expressed as the mean ± SD.

Results

Foxm1 Expression Is Increased in BPD Lungs

Autopsy samples from infants without BPD showed little to no Foxm1 staining, whereas lungs from patients with BPD showed increased Foxm1 staining in inflammatory cells (Figure 1A). Colocalization experiments demonstrated that Mac3-positive macrophages were the primary source of Foxm1 staining in BPD lungs (Figure 1B). Because neonatal hyperoxia exposure in mice recapitulates many features of BPD (6–8), we examined Foxm1 expression in this mouse BPD model. Newborn mice were exposed to hyperoxia for 3 weeks, followed by recovery in room air for an additional 3 weeks. Although hyperoxia itself did not influence Foxm1 mRNA levels, Foxm1 was increased during the recovery period (Figure 1C). Consistent with findings in human BPD lungs, Foxm1 staining was observed in pulmonary macrophages of hyperoxia-exposed mice but not in control mice exposed to room air (Figure 1D). These results indicate increased Foxm1-positive pulmonary macrophages in lungs of patients with BPD and a mouse model of BPD-like disease.

Deletion of Foxm1 from Myeloid Cell Lineages Exacerbated Hyperoxia-Induced Alveolar Simplification in Postnatal Lungs

Foxm1 requirements in BPD were examined using mice with myeloid-specific Foxm1 deletion (*LysM-Cre/Foxm1*$^{fl/fl}$ or *mFoxm1*$^{-/-}$). We previously demonstrated that *mFoxm1*$^{-/-}$ mice had normal lung development (27). Consistent with these results, alveolar structure in *mFoxm1*$^{-/-}$ mice housed in room air was normal as indicated by measurements of terminal air space (Figures 2A and 2B) and radial alveolar count (Figure 2C). Prolonged neonatal hyperoxia increased terminal air space area (Figure 2B) and decreased radial alveolar counts (Figure 2C) in control *Foxm1*$^{fl/fl}$ mice, a finding consistent with hyperoxia-induced alveolar simplification. Alveolar simplification was exacerbated in hyperoxia-exposed *mFoxm1*$^{-/-}$ mice compared with control *Foxm1*$^{fl/fl}$ mice (Figures 2B and 2C). Consistent with impaired alveologenesis, respiratory system resistance and elasticity were increased, whereas respiratory compliance was decreased in hyperoxia-exposed *mFoxm1*$^{-/-}$ mice (Figures 3A–3C). Central airway resistance, tissue damping, and tissue elastance were not altered by deletion of Foxm1 (Figures 3D–3F). Thus, deletion of Foxm1 from myeloid cells exacerbated

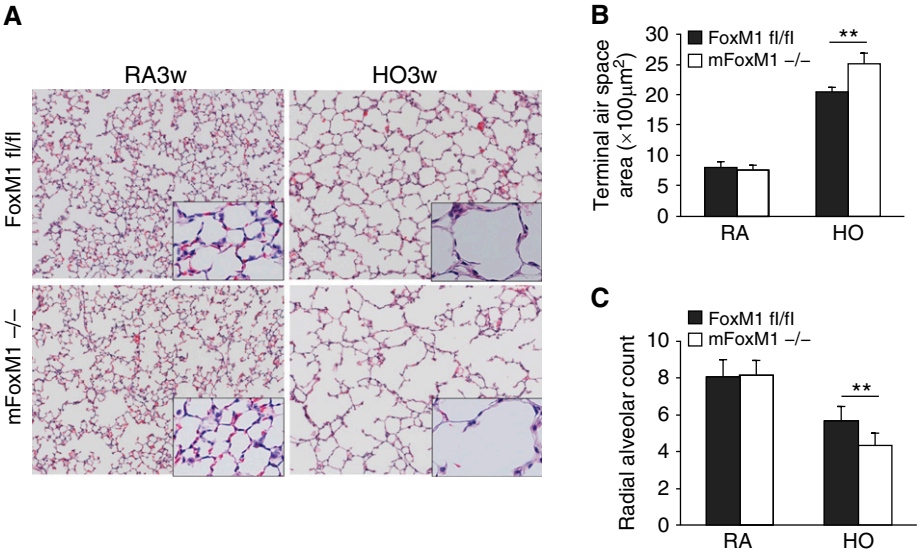

Figure 2. Impaired alveologenesis in *mFoxm1*$^{-/-}$ mice after prolonged hyperoxia. Newborn *mFoxm1*$^{-/-}$ and littermate control *Foxm1*$^{fl/fl}$ mice were exposed to HO or RA from Postnatal Day 1 until 3 weeks of age. Both groups of mice were subsequently housed in RA for an additional 3 weeks. HO disrupted alveologenesis in *mFoxm1*$^{-/-}$ and control mice, as shown by hematoxylin and eosin staining (*A*). HO also increased terminal air space area (*B*) and decreased radial alveolar count (*C*). Ten random lung sections were measured (n = 5 mice per group). Hyperoxia-induced changes were greater in *mFoxm1*$^{-/-}$ mice compared with controls. $^{**}P < 0.01$. There were no differences between *mFoxm1*$^{-/-}$ and control mice exposed to RA. Original magnification: ×100.

alveolar simplification in a mouse model of BPD-like disease.

Foxm1 Deletion Reduced Accumulation of Interstitial Macrophages in Response to Hyperoxia

Neonatal hyperoxia increases lung inflammation contributing to alveolar simplification in mice and humans (6–8). Therefore, we examined lung inflammation after 3 weeks of hyperoxia exposure. In *mFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice, hyperoxic exposure increased the number of Mac3-positive pulmonary macrophages (Figures 4A and 4B). However, the elevation of Mac3-positive macrophages in *mFoxm1*$^{-/-}$ lungs was significantly attenuated compared with control mice (Figures 4A and 4B). Because Mac3 antigen is present in multiple subsets of macrophages, FACS analysis was performed to determine the identity of macrophage subsets present in hyperoxia-exposed lungs. Monocytes were identified as $CD45^+CD11b^+Ly6C^{hi}Ly6G^-F4/80^+$ cells in total cell suspension from perfused lungs. Alveolar and interstitial macrophages were $CD45^+CD11b^+Ly6C^-Ly6G^-F4/80^+$ but were distinguished by the presence of CD68 antigen on their surface (Figure 4C). Consistent with airspace enlargement, total lung cell number was reduced in hyperoxia-exposed *mFoxm1*$^{-/-}$ mice compared with controls (Figure 4D). The overall percentage of $CD45^+CD11b^+$ myeloid cells was increased in both mouse lines, indicating increased inflammation in response to hyperoxia (Figure 4E). Hyperoxic exposure altered the overall balance of macrophage subsets in the lung, decreasing monocytes and alveolar macrophages but elevating the overall percentage of differentiated interstitial macrophages (Figure 4F). Compared with controls, the percentage of interstitial macrophages was decreased in hyperoxia-exposed *mFoxm1*$^{-/-}$ lungs, whereas monocyte precursors were increased. There was no change in alveolar macrophages in *mFoxm1*$^{-/-}$ mice compared with controls (Figure 4F). Thus, Foxm1 deletion from myeloid cell lineages decreased accumulation of interstitial macrophages in response to hyperoxia.

qRT-PCR analysis of FACS-sorted myeloid cell subsets indicated robust Cre mRNA expression in all myeloid cell lineages investigated, including monocytes, interstitial macrophages, alveolar macrophages, and neutrophils (Figure 5B). Foxm1 mRNA was decreased 40 to 60% in all myeloid cell lineages (Figure 5A). PCR analysis of genomic DNA confirmed the presence of the *Foxm1*-null allele in *mFoxm1*$^{-/-}$ mice (Figure 5F). Neutrophils contained very little Foxm1 mRNA, and its levels were unchanged after hyperoxia. Despite significant Foxm1 deletion in all monocytic cell lineages, only interstitial macrophages showed a significant decrease in cyclin B_1 and cdc 25B (Figures 5C–5E), both critical cell cycle regulators and known Foxm1 target genes (37). There was no difference in expression of the Foxm1-independent cell cycle regulators cMyc or cyclin D_1.

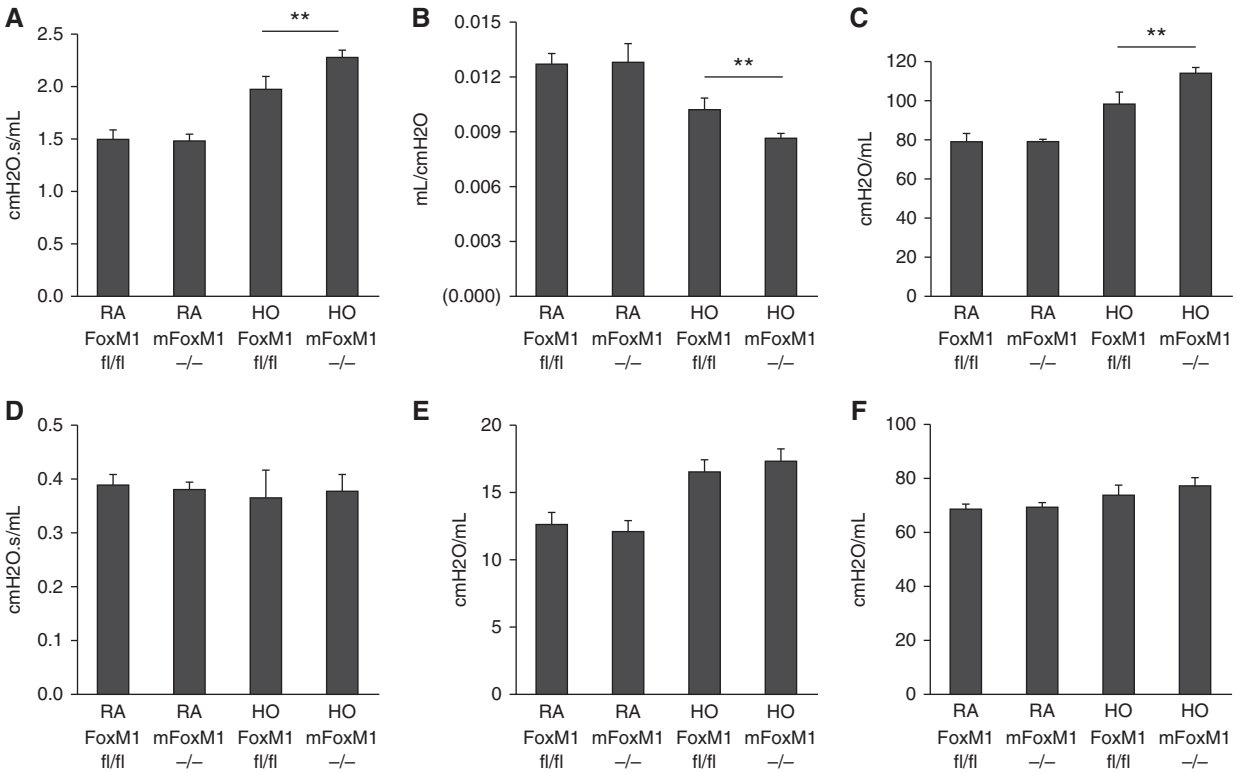

Figure 3. Impaired pulmonary function in *mFoxm1*$^{-/-}$ mice exposed to prolonged postnatal hyperoxia. Flexivent was used to measure pulmonary function in mice exposed HO or RA for 3 weeks, followed by a 3-week recovery period in RA. Exposure to HO decreased lung function in *mFoxm1*$^{-/-}$ mice to a greater extent than in control *Foxm1*$^{fl/fl}$ mice. Respiratory system resistance (*A*) and respiratory system elasticity (*C*) were increased in HO *mFoxm1*$^{-/-}$ mice, whereas respiratory system compliance (*B*) was decreased. There were no changes in central airway resistance, tissue damping, or tissue elastance (*D–F*). Five mice were used in each group. $^{**}P < 0.01$.

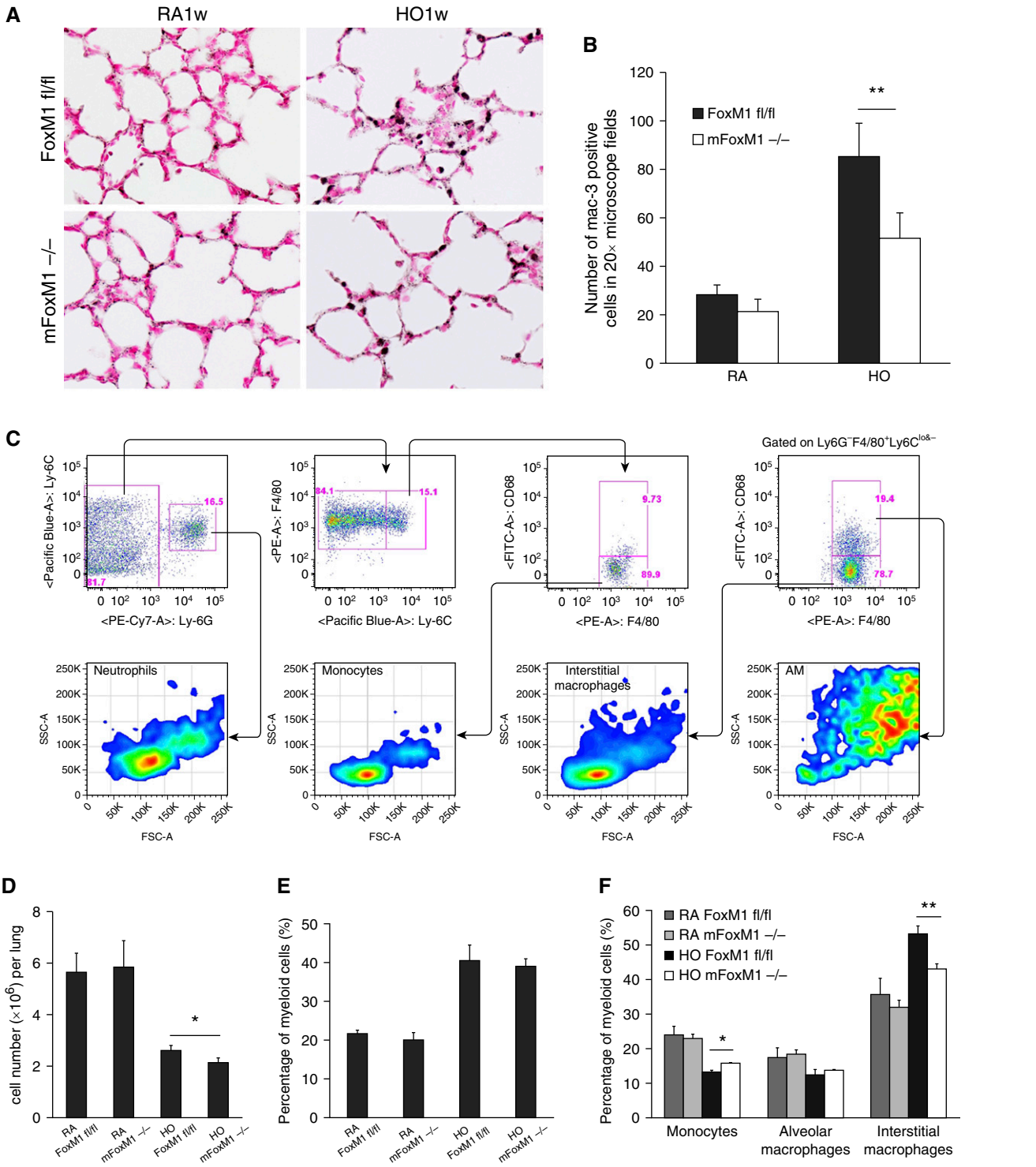

Figure 4. *Foxm1* deletion from myeloid cell lineages decreases accumulation of pulmonary interstitial macrophages after prolonged postnatal hyperoxia. Newborn *mFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice were exposed to HO or RA and harvested at 3 weeks of age. (*A* and *B*) Immunostaining for Mac-3 showed reduced numbers of macrophages in HO-exposed *mFoxm1*$^{-/-}$ lungs. The number of Mac-3–positive cells was counted in 10 random sections using five mice in each group. Original magnification: ×400. $^{**}P < 0.05$. (*C*) Inflammatory cells were isolated from lung tissue of *mFoxm1*$^{-/-}$ or *Foxm1*$^{fl/fl}$ mice after 1 week of HO-exposure. Dead cells were excluded using 7-aminoactinomycin stain. The following cell surface markers were used to identify cell types: neutrophils, CD45$^+$CD11b$^+$Ly6C$^+$Ly6G$^+$; monocytes, CD45$^+$CD11b$^+$Ly6C^{hi}Ly6G$^-$F4/80$^+$; interstitial macrophages, CD45$^+$CD11b$^+$Ly6C$^-$Ly6G$^-$F4/80$^+$CD68$^-$; alveolar macrophages, CD45$^+$CD11b$^+$Ly6C$^-$Ly6G$^-$F4/80$^+$CD68$^+$. (*D*) HO-exposed *mFoxm1*$^{-/-}$ mice had a lower total cell count than *Foxm1*$^{fl/fl}$ mice. (*E*) The percentage of myeloid cells was calculated by flow cytometry using CD45$^+$CD11b$^+$ cell surface

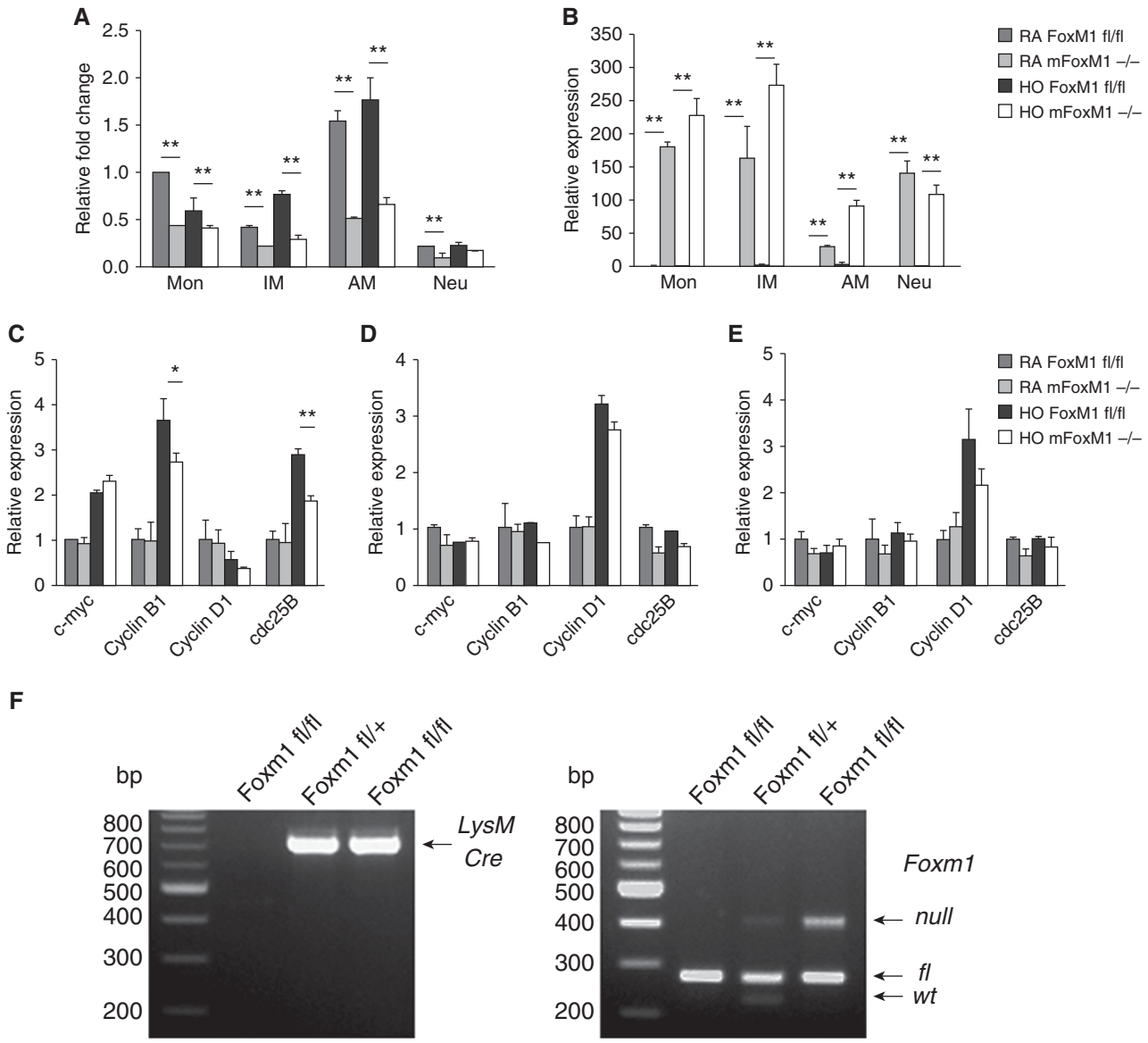

Figure 5. Decreased expression of cyclin B_1 and cdc25B in interstitial macrophages from hyperoxia-exposed *mFoxm1*$^{-/-}$ lungs. Cells were purified from lungs of *mFoxm1*$^{-/-}$ and control mice by flow cytometry (*see* Figure 4 for cell surface markers) and used for quantitative RT-PCR (qRT-PCR). (*A*) Foxm1 mRNA expression was decreased in monocytes (Mon), interstitial macrophages (IM), and alveolar macrophages (AM) from *mFoxm1*$^{-/-}$ mice. Foxm1 mRNA levels were low in neutrophils (Neu). Six mice were used in each group. (*B*) Robust Cre mRNA expression was detected in all subsets of myeloid cells from *mFoxm1*$^{-/-}$ mice. (*C*) qRT-PCR showed decreased mRNAs of cyclin B_1 and cdc25B in interstitial macrophages from hyperoxia-exposed *mFoxm1*$^{-/-}$ lungs compared with controls. (*D* and *E*) There were no statistical differences in mRNA levels of cell cycle regulatory genes in *mFoxm1*$^{-/-}$ alveolar macrophages or monocytes. $^{*}P < 0.05$; $^{**}P < 0.01$. (*F*) PCR analysis of genomic DNA was used to identify the presence of the *Foxm1*-null allele in *mFoxm1*$^{-/-}$ mice.

These results demonstrate that Foxm1 downstream signaling events were inhibited in interstitial macrophages of *mFoxm1*$^{-/-}$ mice after hyperoxia.

Unaltered Development of Alveolar Type II Cells in *mFoxm1*$^{-/-}$ Mice

Because *LysM-Cre* targets a subset of type II cells (38), FACS sorting was used to isolate alveolar type II cells (ATII) for analysis of Foxm1 expression (Figure 6A). Type II cells were identified as cells expressing $EpCam^{+}E\text{-cadherin}^{+}MHC\ II^{+}$ but were

Figure 4. (Continued). markers. Although the percentage of myeloid cells increased significantly after HO exposure, there were no significant differences between *mFoxm1*$^{-/-}$ and *Foxm1*$^{fl/fl}$ mice. (*F*) Decreased numbers of interstitial macrophages were observed in HO-exposed *mFoxm1*$^{-/-}$ lungs. Cell numbers were counted after enzymatic digestion of lung tissue (n = 5 mice per group).The percentage of interstitial macrophages was decreased in HO-exposed *mFoxm1*$^{-/-}$ lungs, whereas the number of alveolar macrophages was unaltered. The number of monocytes was increased in HO-exposed *mFoxm1*$^{-/-}$ lungs compared with controls. $^{*}P < 0.05$; $^{**}P < 0.01$. FSC-A, forward scatter defined by area; PE-A, intensity of phycoerythrin-stained cells defined by area; SSC-A, side scatter defined by area.

negative for CD45 and PECAM-1 (Figure 6A). qRT-PCR confirmed the purity of ATII cell isolation, showing high expression of ATII-specific surfactant protein (SP)-A, SP-B, and SP-C but little to no expression of aquaporin 5, Sox17, Foxj1, and smooth muscle myosin heavy chain, which are expressed in various non-ATII cell types (Figure 6B). Compared with monocytes, ATII cells from *mFoxm1*$^{-/-}$ mice contained relatively high levels of Foxm1 mRNA (Figure 6C) and low levels of Cre mRNA (Figure 6D). However, there were no significant changes in Foxm1 mRNA in normal or hyperoxia-exposed ATII cells from either group of mice. Thus, ATII cells are not targeted in *mFoxm1*$^{-/-}$ mice. These results also suggest that alveolar simplification in hyperoxia-exposed *mFoxm1*$^{-/-}$ mice occurs independently of Foxm1 deficiency in ATII cells.

Increased Expression of MPO, Proteinase 3, and Cathepsin g in *mFoxm1*$^{-/-}$ Lungs after Hyperoxia

Neutrophils produce various enzymes, such as MPO, proteinase 3, and cathepsin g, which degrade extracellular matrix and other components of the alveolar wall, directly contributing to alveolar simplification (2). To determine whether neutrophilic infiltration is associated with the *mFoxm1*$^{-/-}$ lung phenotype, peripheral blood was collected from *mFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice, and Diff-Quik staining was used to analyze circulating neutrophils. Hyperoxia increased the percentage of circulating neutrophils in both groups of mice, but these changes were exacerbated in *mFoxm1*$^{-/-}$ mice compared with controls (Figures 7A and 7B). Although FACS analysis of whole lung tissue showed an increased percentage of neutrophils ($CD45^+CD22G^+Ly6C^+Ly6G^+$) in hyperoxia-exposed *mFoxm1*$^{-/-}$ lungs versus hyperoxia-exposed *Foxm1*$^{fl/fl}$ lungs (Figure 7E), these changes were not induced by hyperoxia because there was no difference between hyperoxia-exposed and room air–housed mice. Immunostaining of lung tissue for MPO, a neutrophil-specific enzyme (39), demonstrated increased abundance of MPO-positive neutrophils in hyperoxia-exposed *mFoxm1*$^{-/-}$ mice (Figures 7C

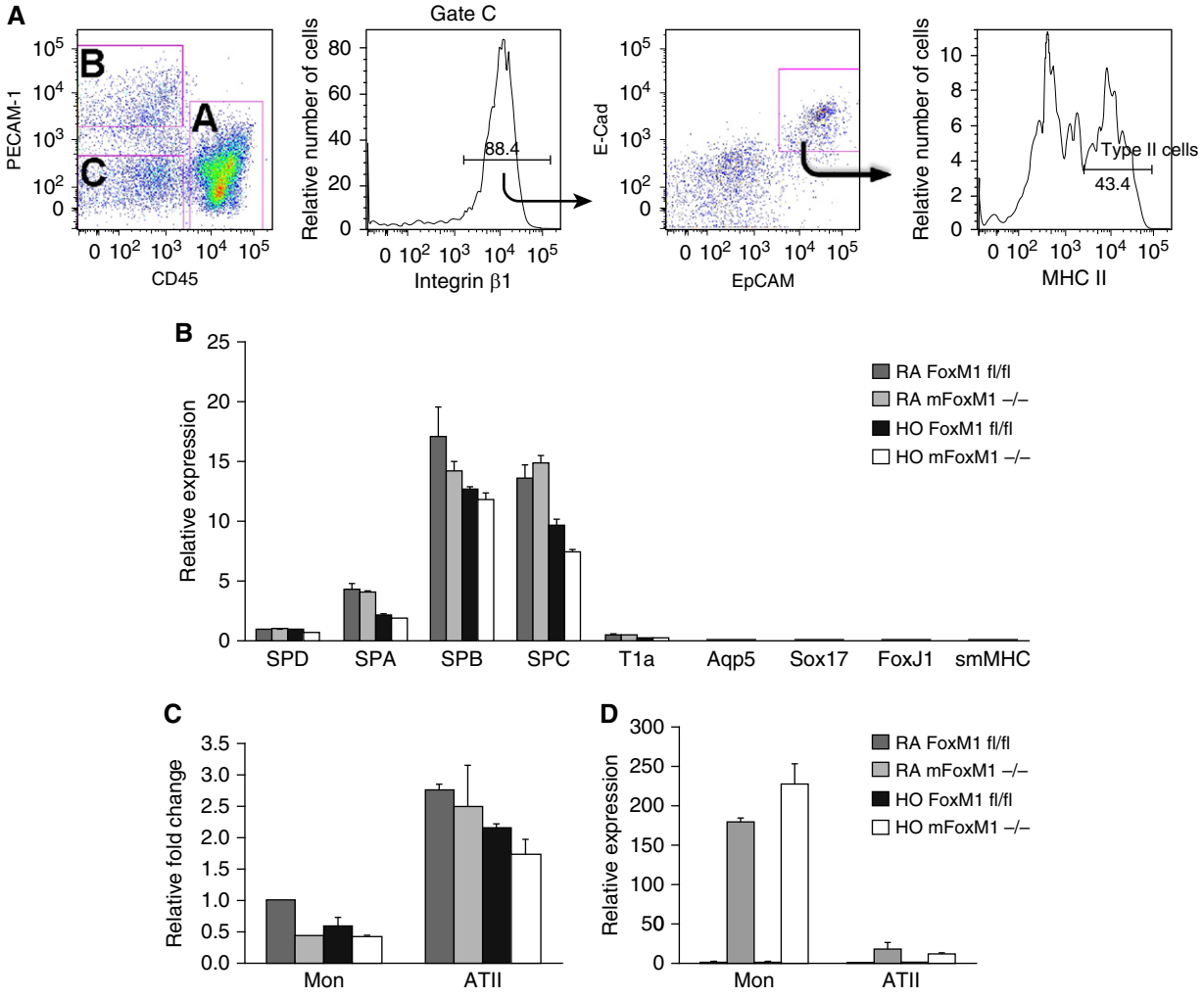

Figure 6. *Foxm1* is not deleted from alveolar type II cells of *mFoxm1*$^{-/-}$ mice. Alveolar type II epithelial cells (ATII) were isolated from either *mFoxm1*$^{-/-}$ or *Foxm1*$^{fl/fl}$ lungs by FACS. (*A*) ATII cells were identified using the following cell surface markers: $EpCAM^+$E-$cadherin^+$MHC $II^+CD45^-PECAM\text{-}1^-$. (*B*) Gene expression profile of ATII cells by qRT-PCR showed the presence of surfactant protein (SP)-A, SP-B, SP-C, and SP-D mRNAs and the absence of T1A, aqp5, Sox17, FoxJ1, and smooth muscle myosin heavy chain (smMHC) mRNAs. smMHC, smooth muscle myosin heavy chain. (*C*) There were no significant differences in Foxm1 mRNA in ATII cells from *mFoxm1*$^{-/-}$ and control *Foxm1*$^{fl/fl}$ mice. (*D*) qRT-PCR showed low levels of Cre mRNA in ATII cells from *mFoxm1*$^{-/-}$ mice. Five mice were used in each group.

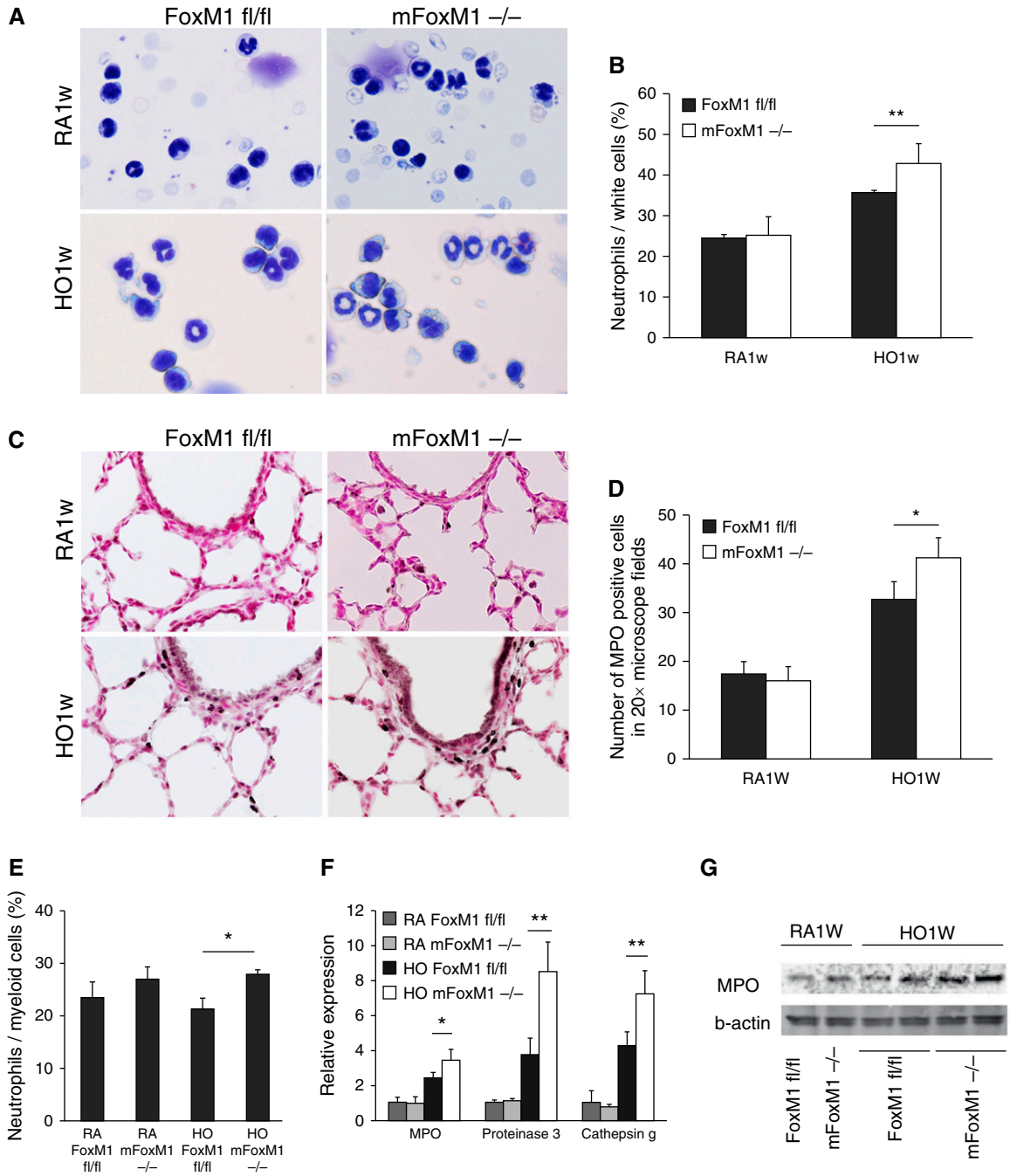

Figure 7. *Foxm1* deletion in myeloid cell lineages increases circulating neutrophils and stimulates expression of neutrophil-derived enzymes in hyperoxia-exposed lungs. (*A*) The percentage of neutrophils in peripheral blood was increased in HO *mFoxm1*$^{-/-}$ mice as shown by Diff-quik staining of blood smears. Original magnification: ×400. (*B*) The number of neutrophils was counted in 10 random sections using five mice in each group. (*C*) Lung sections from *mFoxm1*$^{-/-}$ and *Foxm1*$^{fl/fl}$ mice were stained with myeloperoxidase (MPO) antibodies (*dark brown*) and counterstained with nuclear fast red (*red nuclei*). In the HO group, the number of MPO-positive neutrophils was increased compared with controls. Original magnification: ×400. (*D*) The number of MPO-positive cells was counted in 10 random sections using five mice in each group. (*E*) Myeloid cells were isolated from either *mFoxm1*$^{-/-}$ or *Foxm1*$^{fl/fl}$ lungs by FACS. The percentage of lung myeloid cells that were neutrophils ($CD45^+CD11b^+Ly6C^+Ly6G^+$) was increased in HO-exposed *mFoxm1*$^{-/-}$ mice compared HO-exposed *Foxm1*$^{fl/fl}$ mice. (*F*) qRT-PCR showed increased mRNAs of MPO, proteinase 3, and cathepsin g in HO-exposed *mFoxm1*$^{-/-}$ lungs. mRNA levels were normalized to β-actin mRNA. (*G*) Western blot showed increased MPO protein in HO-exposed *mFoxm1*$^{-/-}$ lungs. *$P < 0.05$; **$P < 0.01$.

and 7D). Elevated MPO protein levels were confirmed by Western blot (Figure 7G). qRT-PCR analysis of whole lung RNA showed increased mRNA levels of neutrophil-derived enzymes MPO, proteinase 3, and cathepsin g in hyperoxia-exposed *mFoxm1*$^{-/-}$ mice compared with controls (Figure 7F). Altogether, our results show that Foxm1 deletion from myeloid cell lineages increased the percentage of circulating neutrophils and increased expression of neutrophil-derived enzymes in lung tissue.

Discussion

The results of this study show that Foxm1-positive macrophages are abundant in lungs of patients with BPD and in a mouse model of BPD-like disease. Myeloid cells are important mediators of pulmonary remodeling after hyperoxia exposure, with perturbations in the types of inflammatory cells present in the lung being critical to disease progression. Our data indicate that expression of Foxm1 transcription factor in myeloid inflammatory cells is critical for the recruitment and accumulation of interstitial macrophages in response to hyperoxia. Deletion of Foxm1 from the myeloid cell lineage decreased interstitial macrophage accumulation in the lung after prolonged hyperoxic exposure but increased circulating neutrophils and neutrophil-derived enzymes in lung tissue, ultimately exacerbating lung remodeling and decreasing lung function. Hyperoxia-exposed *mFoxm1*$^{-/-}$ lungs had increased expression of the neutrophilic enzymes MPO, proteinase 3, and cathepsin g, all of which are capable of disrupting alveologenesis by degrading extracellular matrix proteins critical for maintaining integrity of the alveolar wall. Thus, Foxm1 deletion from myeloid cell lineages changes the inflammatory response to hyperoxia, decreasing the accumulation of macrophages and increasing the abundance of neutrophil-derived enzymes, a phenotype that is detrimental to lung function and repair after neonatal hyperoxic exposure. Our results are consistent with clinical data showing a direct correlation between neutrophil-derived enzymes and severity of BPD in preterm infants.

Hyperoxia-induced lung injury is initiated by reactive oxygen species, followed by extensive infiltration of inflammatory leukocytes into the lung (40). After an insult, resident macrophages and epithelial cells secrete proinflammatory chemokines and cytokines, inducing migration of neutrophils out of pulmonary capillaries into the air space (41). Neutrophils migrate to the inflamed tissue, promote recruitment of inflammatory monocytes, and potentiate the proinflammatory environment, allowing appropriate resolution of the insult (42). Neutrophils undergo apoptosis after performing their action at the inflamed site, and macrophages ingest apoptotic neutrophils (43). Clearance of apoptotic neutrophils prompts a switch from a pro- to an antiinflammatory macrophage phenotype (44), which is a prerequisite for macrophage egress via the lymphatic vessels favoring return to tissue homeostasis. The increase in circulating neutrophils and neutrophil-derived enzymes in the lung observed in *mFoxm1*$^{-/-}$ mice likely exacerbates the proinflammatory response within the lung after hyperoxic insult. This in turn may prevent resolution of the inflammatory response and delay the ability of the lung to sustain proper alveologenesis, likely contributing to increased terminal air space observed in *mFoxm1*$^{-/-}$ mice after prolonged hyperoxia. Furthermore, there is accumulating evidence that macrophages reduce inflammatory responses through ingestion of dying cells or dead cell debris, thereby mitigating tissue damage (45–47). Chronic inflammation in the lung appears to be associated with delayed removal of dying cells, which may directly affect the natural ability of the injured organism to shut down inflammation and initiate tissue repair (48). Resolving lung inflammation depends on timely and adequate removal of acute inflammatory cells by macrophages. In this study, deletion of Foxm1 from myeloid cells reduced the number of interstitial macrophages after hyperoxia. This likely produces delayed resolution of the proinflammatory microenvironment, which, together with exacerbated proteolytic activities of neutrophilic enzymes, alters pulmonary remodeling after hyperoxia exposure.

In summary, the results of this study show that patients with BPD and hyperoxia-exposed mice have increased Foxm1-positive macrophages in lung tissue. Using mice with myeloid-specific Foxm1 deletion, we demonstrated that Foxm1 expression in myeloid cell lineages is required to maintain proper balance between neutrophils and interstitial macrophages in response to prolonged neonatal hyperoxia. Loss of Foxm1 increases neutrophil-derived enzymes in lung tissue, disrupting alveologenesis and exacerbating BPD-like disease. Thus, discovery of pharmacological agents that can modulate Foxm1 function in postnatal lungs could be beneficial to inhibiting hyperoxia-induced inflammation and stimulate lung repair in patients with BPD. ■

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

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