CD11b⁺ Mononuclear Cells Mitigate Hyperoxia-Induced Lung Injury in Neonatal Mice

Laurie C. Eldredge^{1,2}, Piper M. Treuting^{3,4}, Anne M. Manicone^{1,5}, Steven F. Ziegler^{6,7}, William C. Parks^{1,4,5*†}, and John K. McGuire^{1,8*}

¹Center for Lung Biology, and Department of Pediatrics, Divisions of ²Pulmonary Medicine and ⁸Critical Care Medicine, ³Department of Comparative Medicine, ⁴Department of Pathology, ⁵Department of Medicine, Division of Pulmonary and Critical Care Medicine, ⁶Department of Immunology, University of Washington, Seattle, Washington; and ⁷Immunology Program, Benaroya Research Institute, Seattle, Washington

Abstract

Bronchopulmonary dysplasia (BPD) is a common consequence of life-saving interventions for infants born with immature lungs. Resident tissue myeloid cells regulate lung pathology, but their role in BPD is poorly understood. To determine the role of lung interstitial myeloid cells in neonatal responses to lung injury, we exposed newborn mice to hyperoxia, a neonatal mouse lung injury model with features of human BPD. In newborn mice raised in normoxia, we identified a CD45⁺ F4/80⁺ CD11b⁺, Ly6G^{low-int} CD71⁺ population of cells in lungs of neonatal mice present in significantly greater percentages than in adult mice. In response to hyperoxia, surface marker and gene expression in whole lung macrophages/monocytes was biased to an alternatively activated phenotype. Partial depletion of these CD11b⁺ mononuclear cells using CD11b–diphtheria toxin (DT) receptor transgenic mice resulted in 60% mortality by 40 hours of hyperoxia exposure with more severe lung injury, perivascular edema, and alveolar hemorrhage compared with DT-treated CD11b–DT receptor–negative controls, which displayed no mortality. These results identify an antiinflammatory population of CD11b⁺ mononuclear cells that are protective in hyperoxia-induced neonatal lung injury in mice, and suggest that enhancing their beneficial functions may be a treatment strategy in infants at risk for BPD.

Keywords: interstitial macrophage; hyperoxia; lung injury; lung development; macrophage polarization

Clinical Relevance

Bronchopulmonary dysplasia (BPD) is a common and prevalent lung disease of premature infants. These infants spend many months in the hospital and can require years of respiratory support. Nearly 70% of the most premature infants will develop BPD. We do not have evidence-based therapies to alter BPD progression. Many factors contribute to BPD, including inflammation and exposure to oxygen. This study investigates the role of murine mononuclear cell populations in neonatal lung injury. These findings identify an alternatively activated monocyte/macrophage population that is critical to the neonatal response to hyperoxia-induced lung injury. These results may inform clinical studies of the role of alternatively activated macrophages in human BPD. These mononuclear cells are plastic and may be important therapeutic targets.

Bronchopulmonary dysplasia (BPD) remains a common consequence of life-saving respiratory support for severely premature infants. First described in 1967

(1), BPD comprises clinical, radiographic, and pathologic features seen in premature infants surviving severe respiratory distress syndrome and treated with prolonged

mechanical ventilation and supplemental oxygen (2). Pathology specimens from BPD lungs exhibited diffuse airway damage, smooth muscle hypertrophy, severe

(Received in original form October 16, 2014; accepted in final form June 29, 2015)

*These authors contributed equally to this article.

†Current Address: Women's Guild Lung Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048.

This work was supported by grants from the Firland Foundation (L.C.E.) and the National Institutes of Health (J.K.M., W.C.P., and S.F.Z.).

Author Contributions: Conception and design—L.C.E., P.M.T., A.M.M., S.F.Z., W.C.P., and J.K.M. Analysis and interpretation—L.C.E., P.M.T., A.M.M., S.F.Z., W.C.P., and J.K.M.; drafting the manuscript for important intellectual content—L.C.E., W.C.P., and J.K.M.

Correspondence and requests for reprints should be addressed to Laurie C. Eldredge, M.D., Ph.D., Department of Pediatrics University of Washington, Seattle Children's Hospital, Point Way NE, Seattle, WA 98105. E-mail: lauriech@uw.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 54, Iss 2, pp 273–283, Feb 2016

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Originally Published in Press as DOI: 10.1165/rcmb.2014-0395OC on July 20, 2015

Internet address: www.atsjournals.org

inflammation, and parenchymal lung fibrosis. Surfactant-based therapies have enabled increased survival of infants born extremely premature (24–29 week gestation). However, BPD has become a more complex condition, with lung injury superimposed on arrested lung development. Histologically, lungs from patients with “modern” BPD have mild airway injury, inflammation, fibrosis, vascular maldevelopment, and, most strikingly, alveolar simplification (2–4).

BPD pathogenesis is multifactorial. Pre- and postnatal inflammation is a risk factor for BPD development (5), and evidence suggests that unmitigated, excessive proinflammatory stimulation or the lack of antiinflammatory suppression contributes to BPD (6, 7). For example, levels of inflammatory markers, such as, IL-1 α , IL-1 β , IL-1 receptor antagonist, IL-6, IL-8, IFN- γ , TNF- α , monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor- β 1, can be used to predict which low-birth weight infants will develop BPD (8–10). Moreover, IL-10, an immunosuppressive cytokine secreted by both myeloid and lymphoid cells, is reduced in the placenta and tracheal aspirates of patients who develop BPD compared with those without BPD (11, 12). Thus, improved understanding of how inflammation is regulated in neonatal lung injury may identify therapeutic targets with which to alter BPD disease progression.

Resident lung macrophages play key regulatory roles in lung responses to injury (13), yet little is known about the role these immune cells play in normal or aberrant lung development. Macrophages have distinct roles during different phases of wound repair, and these functions are associated with distinct states of macrophage activation (14, 15). For example, macrophages responding in the early phase of injury are typically classically activated proinflammatory cells (i.e., M1 biased or polarized), whereas the resolution-phase cells tend to be alternatively activated, remodeling-competent macrophages (M2 polarized) (14, 16, 17). M1 macrophages are polarized by Th1 effectors, such as bacterial products and IFN- γ , and express inducible nitric oxide synthase (NOS2), IL-1, TNF- α , and IL-6. In contrast, M2 macrophages are considered to be antiinflammatory and reparative and, hence, beneficial in the restoration of homeostasis after an inciting inflammatory injury. M2 macrophages

typically express immunosuppressive factors, such as IL-10, transforming growth factor- β 1, and IL-1 receptor antagonist (18, 19).

Mice are born during the late canalicular/early saccular stages of lung development, the phase in human lung development (24–36 wk gestation) when premature infants are susceptible to BPD (2, 3). Thus, newborn mice provide a model for humans born with premature lungs (20). To characterize monocytes and macrophages in normal mouse lung development, and to determine their role in the neonatal lung response to injury, we studied mononuclear cells in hyperoxia-induced lung injury, a well described model of neonatal lung injury and BPD pathogenesis (20, 21). Exposure of newborn mice to hyperoxia results in an acute lung injury in which the oxidative injury also involves activation of inflammatory cells, especially neutrophils, and, concomitantly, increased vascular permeability with pulmonary hemorrhage and interstitial, alveolar, and airway edema (22). A chronic lung injury ensues, which includes fibroblast proliferation, extracellular matrix deposition, and alveolar simplification similar to that of human BPD (20). Hyperoxia also increases airspace inflammatory cytokine levels and stimulates macrophage influx into the alveoli and lung parenchyma (23). Because of the increasingly recognized importance of macrophages in other forms of lung injury, we hypothesized that macrophages may play an important role in regulating acute neonatal lung injury. Some of the results of these studies have been previously reported in abstract form (24–26).

Materials and Methods

Animals

Mouse studies were approved by the Office of Animal Welfare at the University of Washington. C57BL/6N and FVB/NJ timed-pregnant females were ordered from Charles River Laboratories (Wilmington, MA). CD11b diphtheria toxin (DT) receptor (DTR) transgenic mice are on an FVB/NJ background (27); homozygotes and heterozygotes had similar phenotypes, and were all considered functionally DTR⁺. No significant differences in baseline or stimulated cell populations were identified between C57BL/6 and FVB/NJ wild-type mice. Transgene-negative (DTR[−]) littermates were used as controls for

CD11bDTR⁺ mice. Mice were housed in a specific-pathogen-free facility in polycarbonate microisolator cages.

Hyperoxia-Induced Lung Injury

Mice were placed in a nonairtight Plexiglas chamber located in an approved out-of-facility laminar flow hood. FI_O₂ within the chamber was titrated to greater than 90% with an oxygen concentrator; a back-up vacuum port instilled room air into the chamber and removed excess CO₂ in the event of a concentrator failure. Mouse litters were placed in the chamber within 12 hours of birth; dams were rotated between control and hyperoxia conditions every 24–36 hours to avoid oxygen toxicity (21).

Flow Cytometry Cell Analysis

Lungs were treated with Liberase TM (Roche Life Science, Pleasanton, CA) and DNase (Sigma-Aldrich, St. Louis, MO), followed by mechanical dissociation with GentleMacs (Miltenyi Biotec, San Diego, CA). After red blood cell (RBC) lysis (eBioscience, San Diego, CA), digests were incubated with an Fc-receptor-blocking antibody, then incubated with fluorescent-conjugated antibodies before analysis with a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Antibodies used are listed in the online supplement. In some experiments, cells were sorted with a BD FACSaria II Cell Sorter (BD Biosciences) into Trizol reagent for RNA isolation. Total RNA was isolated and processed for quantitative RT-PCR, as previously described (28). Control histograms include fluorescence-minus-one samples (29, 30).

Histology

Neonatal mice were humanely killed and then immersion fixed in 10% neutral-buffered formalin and embedded in paraffin. Serial 4- μ m sections were stained with hematoxylin and eosin or Movat's pentachrome or processed for immunohistochemistry. Antibodies used are listed in the online supplement. A certified veterinary pathologist (P.M.T.) assessed lung injury with attention to degree of hemorrhage, atelectasis, aspiration pneumonia, and inflammation.

DT Depletion of CD11b⁺ Cells

Newborn mice within 12 hours of birth were injected intraperitoneally with 500 ng DT (List Biological Laboratories, Campbell, CA) diluted in sterile PBS (50 μ l total). Injected

mice were returned to their cage and placed in hyperoxia or normoxia conditions.

Ly6G-Antibody Depletion of Neutrophils

Newborn mice within 12 hours of birth were injected intraperitoneally with 500 ng DT and anti-mouse Ly6G antibody (catalog no. 127632; BioLegend, San Diego, CA) or an isotype control diluted in sterile PBS (50 μ l total). Injected mice were returned to their cage and placed in hyperoxia conditions.

Statistical Analysis

Data analysis was performed with Prism 6 (GraphPad Software, Inc., La Jolla, CA).

Results are reported as means ($\pm$ SD). We performed unpaired two-tailed *t* tests with significance defined as *P* less than 0.05. Survival data are presented as Kaplan-Meier curves and analyzed for significance with the Log-rank (Mantel-Cox) test.

Results

Mononuclear Phagocyte Populations Evolve in the Early Postnatal Period

We characterized murine monocyte and macrophage populations at birth and during the first week of life. Whole lungs from Postnatal Day (PN) 0, PN1, PN5, and adult (6- to 8-wk-old) mice were digested, and

lung leukocytes were analyzed by flow cytometry. No single cell surface marker defines individual subpopulations of mononuclear cells in the mouse lung. Therefore, we used a panel of CD45, CD11b, CD11c, F4/80, CD64, and Ly6G (31). Monocytes/macrophages in the neonatal C57BL/6N lung differed significantly from those in adult lungs (Figure 1). Neonatal lungs contained more CD45⁺ F4/80⁺ CD11b^{int-hi} Ly6G^{lo-int} monocytes/macrophages than older neonatal mice and adults (Figure 1A). CD64 did not distinguish cell populations in the early postnatal period (data not shown). Adult lungs contained three distinct populations:

Figure 1. Evolution of mononuclear cell populations in the neonatal period. Flow cytometry analysis was performed on whole-lung homogenates from neonatal and adult mice at Postnatal Day (PN) 0, PN1, PN5, and adult (6–8 weeks). Flow plots are representative of three to four mice per time point. (A) Mononuclear cell populations were defined as CD45⁺ F4/80⁺ CD11b^{int-hi} Ly6G^{lo-int} ($*P < 0.001$ compared with PN0). (B) Alveolar macrophages were defined as CD45⁺ CD11b^{int} CD11c⁺ and are not present until age PN3 ($*P < 0.01$ compared with PN0). Red boxes indicate quantified population. (C) Alveolar macrophage populations were confirmed using Siglec F. CD45⁺ CD11b^{int} CD11c⁺ alveolar macrophages (population 1) were also Siglec F⁺. CD45⁺ CD11b^{hi} CD11c^{lo-int} cells (population 2) were Siglec F negative, representing dendritic cells, interstitial macrophages, and neutrophils. Control histograms represent samples incubated with the complete panel of antibodies except for Siglec F. Blue box indicates relevant population in center panel (1). Green box indicates relevant population in right panel (2).

CD11b^{int} Ly6G^{lo}; CD11b^{hi} Ly6G^{int}; and CD11b^{hi} Ly6G^{lo}. CD11b⁺ Ly6G^{lo-int} cells contained a mixture of monocytes and macrophages based on their relative size and granularity. Neonatal lungs also lacked alveolar macrophages (defined as CD45⁺ F4/80⁺ CD11b^{lo-int} Ly6G^{lo-int} CD11c^{hi}). Alveolar macrophages were first identifiable at PN1, peaked in numbers at PN5, and accounted for roughly 30% of CD45⁺ F4/80⁺ cells in the adult lung (Figure 1B). Because collection of bronchoalveolar lavage fluid is not practical in newborn mice, all of these analyses represent alveolar and interstitial compartments. We used Siglec F, a cell surface sialic acid-binding Ig-like lectin, to distinguish between airspace/alveolar and tissue macrophages (Figure 1C) (31). Consistent with findings reported by others (32), we did not identify alveolar macrophages (CD45⁺ CD11b^{lo} Siglec F⁺ CD11c⁺) in lungs of PN0 pups. Thus, the acquisition of alveolar macrophages and evolution of interstitial mononuclear cell surface marker phenotypes signify changes in myeloid cell populations in the perinatal mouse lung early after birth.

Alternatively Activated Macrophages Are More Prevalent in the Lung at Birth

We next assessed if neonatal macrophage activation markers changed with age. Defining monocyte/macrophage myeloid populations as FSC^{lo-int} SSC^{lo-int} CD45⁺ F4/80⁺ CD11b^{int-hi} and Ly6G^{lo-int} (Figure 1; see also Figure E1 in the online supplement), we classified cells as alternatively activated (M2-like) if positive for CD71 (transferrin receptor). CD71 was

previously identified as an M2 marker in a membrane proteomic analysis of IL-4/IL-13 polarized bone marrow-derived macrophages and validated *in vivo* using a mouse model of *Pseudomonas aeruginosa* infection-mediated acute lung injury (28, 33). In mice, CD45⁺ F4/80⁺ CD11b^{hi} Ly6G^{hi} cells are neutrophils, and were excluded from analysis (34). In the PN0 lung, we identified elevated proportions of M2-like myeloid cells (CD45⁺ F4/80⁺ LY6G[−] CD11b⁺ CD71⁺, Figures 2A and 2B) that comprised roughly 55% of CD45⁺ F4/80⁺ LY6G[−] cells. In adult lung, by comparison, these cells accounted for less than 20% of CD45⁺ F4/80⁺ LY6G[−] mononuclear cells (Figures 2A and 2B). These data show that alternatively activated mononuclear cells are more prevalent in the newborn lung and decrease in the postnatal period.

Elevated Numbers of Alternatively Activated Mononuclear Cells Are Present in the Lung after Hyperoxia-Induced Lung Injury

We exposed PN0 mice to greater than 90% oxygen (hyperoxia) or room air (normoxia) controls for 3–5 days to assess the mononuclear cell response to hyperoxic lung injury. As expected, we observed inflammatory cell influx, interstitial thickening, mild pulmonary hemorrhage, and alveolar simplification after 5 days of hyperoxia exposure (Figure 3A). In addition, after 3–5 days of hyperoxia, there was an increase in CD45⁺ F4/80⁺ CD11b^{hi} Ly6G^{lo-int} inflammatory monocytes and macrophages (data not shown). We also evaluated the effect of hyperoxia on the polarization state of neonatal mononuclear

cells. Compared with normoxia controls, alternatively activated, M2-like (CD45⁺ F4/80⁺ CD11b⁺ LY6G^{lo-int} CD71⁺) cells were increased in the lungs of neonatal mice exposed to hyperoxia for the first 5 days of life (Figures 3B and 3C). We further distinguished polarized mononuclear cells by gating on the presence or absence of CD71. Total RNA was prepared from sorted cells, and gene expression was assessed by quantitative real-time RT-PCR (Figure 3D). CD45⁺ F4/80⁺ LY6G^{lo-int} CD11b⁺ CD71^{hi} M2-like cells from mice in hyperoxic conditions expressed more arginase 1 (*Arg1*) and *Fizz1* mRNA, widely used mouse M2 markers, than CD71^{lo} cells. CD71^{hi} cells also expressed less *IL6*, an M1 marker (35). No differences were seen in *Nos2* expression in CD71^{hi} or CD71^{lo} cells. In response to hyperoxia, *Arg1*, *Fizz1*, and *IL6* were up-regulated in both CD71^{hi} and CD71^{lo} cells compared with normoxia controls. Although *IL6* was induced to a greater extent in the CD71^{lo} cells, differences did not achieve statistical significance due to the high variability in expression levels.

Conditional Ablation of CD11b⁺ Monocytes and Interstitial Macrophages Increases Hyperoxia-Induced Lung Injury and Mortality

Given that mononuclear cell polarization states change during normal postnatal development and after hyperoxia treatment, we determined if these cells function in the response to hyperoxia-induced lung injury. We used transgenic mice expressing the human DTR under control of the *CD11b* promoter (27) to conditionally deplete

Figure 2. M2 antiinflammatory mononuclear cells are more prevalent at birth. Flow cytometry was performed on whole-lung homogenates from PN0, PN1, PN5, or adult (6–8 weeks) mice. (A) M2-like mononuclear cells were defined as CD45⁺ F4/80⁺ LY6G^{lo-int} CD11b^{int-hi} CD71⁺. Red boxes indicate quantified population. (B) Quantification of M2-like mononuclear cells at PN0, PN1, PN5, and adult. Quantification performed with three to six mice per time point, averaged across three experiments. **P* < 0.001, Student's unpaired *t* test. Data are represented as mean ± SD. Macs, macrophages; monos, mononuclear cells.

Figure 3. Hyperoxia-induced lung injury promotes M2 mononuclear cell polarization. (A) PN0 mice were placed in a chamber containing 95% FiO_2 within 12 hours of birth. Representative hematoxylin and eosin (H&E)-stained sections of lungs of normoxia control and hyperoxia-treated mice at PN5. (B) Representative flow cytometry plots from whole lungs of normoxic and hyperoxia-treated mice at PN5. “M2-like cells” (green boxes) are identified as $\text{CD45}^+ \text{F4/80}^+ \text{CD11b}^{\text{int-hi}} \text{LY6G}^{\text{lo-int}} \text{CD71}^+$. (C) CD71^+ M2-like populations in normoxia and hyperoxia-treated mice at PN1 and PN5 (top panel). Normoxia controls at PN0 and adult time points are also included. (D) CD71^{hi} and CD71^{lo} cells were identified as described previously here and sorted from whole lungs of normoxia and hyperoxia-treated mice. Quantitative RT-PCR for arginase 1 (*Arg1*), found in inflammatory zone 1 (*Fizz1*), *IL-6*, and nitric oxide synthase 2 (*Nos2*) was performed for all four conditions. Representative flow plots are pictured. Flow cytometry quantification was performed with five to six PN0 mice per time point per condition; quantitative real-time RT-PCR data analysis was performed with three to five mice per time point per condition. Results are representative of at least three independent experiments. $*P < 0.01$, Student’s unpaired *t* test. Data are represented as mean $\pm$ SD. Scale bar = 100 μm . RQ, relative quantity.

CD11b^+ monocytes and interstitial macrophages (27, 36).

Importantly, CD11b^+ neutrophils are not depleted in this model, and alveolar macrophages ($\text{CD11b}^{\text{lo-int}}$), splenic macrophages, eosinophils, basophils, and lymphocytes are also preserved (27, 37). Neonatal mice were injected within 12 hours of birth with 500 ng DT intraperitoneally and the entire litter was placed into normoxia or hyperoxia ($>90\% \text{FiO}_2$) conditions for up to 40 hours, at which time mice were killed and lung digests from CD11bDTR^+ (DTR^+) mice and CD11bDTR transgene-negative (DTR^-) littermate controls were analyzed by flow cytometry. A single injection of DT depleted approximately 50% of $\text{CD45}^+ \text{CD11b}^+ \text{LY6G}^{\text{lo}}$ monocytes/macrophages (Figures 4A and 4B). At this time point, greater than 90% of $\text{CD45}^+ \text{CD11b}^+ \text{LY6G}^{\text{lo}}$ monocytes/macrophages were also positive for F4/80. The consequences of CD11b^+ cell depletion were robust: by 40 hours after DT injection, DT-treated DTR^+ animals with depletion of CD11b^+ mononuclear cells exhibited cyanosis and

respiratory distress compared with control mice that appeared healthy (Figure 4C). At 24 hours after DT treatment, most DT-injected, hyperoxia-exposed DTR^+ pups were alive and appeared healthy ($n = 16/19$ alive). However, by 36 hours after DT, all hyperoxic DTR^+ mice were moribund with labored breathing, decreased activity, and cyanosis, and, by 40 hours, nearly 60% had died ($n = 17/30$ dead). In contrast, DT-treated hyperoxic DTR^- littermate controls had 100% survival at 40 hours ($n = 9/9$ alive), indicating that DT alone did not induce mortality in neonatal mice. Non-DT-treated DTR^+ animals had 100% survival at 40 hours of hyperoxia exposure ($n = 8/8$ alive), as did C57BL/6 and FVB/NJ wild-type mice in previous experiments (Figure 3 and data not shown; $n = 40/40$ alive), indicating that mortality was due to CD11b mononuclear cell depletion. Interestingly, DT-treated normoxia DTR^+ controls also showed greater mortality at 40 hours ($n = 4/17$ dead), indicating that, even in the absence of the hyperoxic stimulus, these cells are necessary for the normal transition after birth. Similarly, DT-treated

DTR^+ mice exposed to a lower oxygen concentration (45–50% FiO_2) had a similar phenotype to those exposed to greater than 90% FiO_2 , as 40% had died by 40 hours ($n = 7/17$ dead) and the remaining pups were moribund. Moreover, 100% of DTR^- littermate controls exposed to 45–50% FiO_2 survived ($n = 3/3$ alive) and appeared healthy.

We next sorted $\text{CD45}^+ \text{CD11b}^{\text{hi}} \text{LY6G}^{\text{lo}}$ cells from DT-treated DTR^+ and DTR^- mice exposed to 36 hours of hyperoxia. We assessed the expression of the M1 and M2 genes, *Nos2* and *Arg1*, respectively, using quantitative RT-PCR (Figure 4B). In hyperoxia-exposed, DT-treated DTR^- mice, we found that the M2 marker, *Arg1*, was expressed at a higher level compared with the M1 marker, *Nos2*. Sorted $\text{CD45}^+ \text{CD11b}^{\text{hi}} \text{LY6G}^{\text{lo}}$ cells from DTR^+ mice (i.e., those cells left behind after depletion) showed a similar pattern of *Arg1* and *Nos2* expression, indicating the DTR -mediated depletion altered the numbers of these cells present in the lung, and did not alter the general gene expression pattern. These findings suggest

Figure 4. CD11b-diphtheria toxin receptor (DTR)⁺ mice exhibited severe respiratory distress and increased mortality when exposed to hyperoxia. (A) Representative flow cytometry plots from whole-lung digests of DT-treated DTR⁺ and DTR⁻ littermate controls after 40 hours of hyperoxia. The depleted population is outlined with red boxes. Approximately 50% of CD45⁺ CD11b⁺ LY6G^{lo-int} mononuclear cells are depleted in DTR⁺ mice. (B) CD45⁺, CD11b⁺, LY6G^{lo-int} cells were sorted from DT-treated DTR⁻ and DTR⁺ mice into Trizol, and *Nos2* and *Arg1* quantitative real-time RT-PCR was performed on cellular isolates. Data are expressed as 40-ΔCt using *Hprt* as a normalization control. ***P* < 0.005, Student's unpaired *t* test. (C) By 36 hours after DT treatment, DTR⁺ mice were moribund, with increased work of breathing, decreased activity, and cyanosis. DT-treated DTR⁻ mice appeared healthy and active. (D) Kaplan-Meier survival curves of DTR⁺ and DTR⁻ littermates. The *P* value for the graph between A and C is < 0.05 (**P* = 0.0182). The *P* value for the survival data in D is **P* = 0.0005, Log-rank (Mantel-Cox) test, *n* = 87 total DTR⁺ and 20 DTR⁻ mice.

that pulmonary consequences in DTR⁺ mice result from a proportional reduction of M2- and M1-like mononuclear cells, rather than alteration in the polarization phenotypes of remaining lung mononuclear cell populations. Importantly, these data demonstrate that CD11b⁺ M2-like mononuclear cells are critical components in lung protective responses to hyperoxia.

More Severe Lung Injury in Hyperoxia-Treated CD11bDTR⁺ Mice

After exposure to greater than 90% FiO₂ for 24 or 36 hours, DT-treated DTR⁺ mice had more advanced and severe pulmonary hemorrhage (Figures 5A and E2A, respectively) compared with DTR⁻ controls, which had minimal pulmonary hemorrhage similar to hyperoxic wild-type mice. Movat's pentachrome-stained sections of lung tissues of DTR⁺ mice revealed inflammation, proteinaceous material in the airspaces (Figures 5C and E2C), subacute pulmonary hemorrhage with fibrin deposition (Figures 5A, E2A, and 6B), and the presence of hemosiderin-laden

macrophages (Figure 6A, arrow). The areas of lung with most severe hemorrhage were also atelectatic. DT-treated DTR⁺ mice left in normoxic conditions had an intermediate phenotype with mild pulmonary hemorrhage at 36 hours (Figure E2E).

DTR⁺ mice exposed to 45–50% FiO₂ showed similar histopathologic changes to the greater than 90% FiO₂-treated mice with severe pulmonary hemorrhage, fibrin deposition, atelectasis, and inflammation (Figure 6, 7 and data not shown). Detailed histopathologic analyses revealed similar phenotypes in hyperoxia-exposed, DT-treated DTR⁻ mice and wild-type mice not exposed to DT, further indicating that DT itself did not cause pulmonary toxicity.

Consistent with the flow cytometry analyses, in which we found that CD11b^{int} alveolar macrophages were preserved in this model, large alveolar macrophages located in airspaces were identified by morphology and expression of Galectin-3 (Mac2) (Figures 5B and E2B) and Chitinase 3-like-3 (Ym1) (data not shown), and did not appreciably differ between DT-treated

DTR⁺ and DTR⁻ mice. However, fewer MAC2⁺ macrophages were seen in the interstitium of DTR⁺ mice at 36 hours of hyperoxia treatment compared with 24 hours (Figure E2B), supporting the conclusion that DT treatment of DTR⁺ mice depleted an interstitial population.

The endothelial cell marker, CD31, was used on adjacent sections from DTR⁺ and DTR⁻ mice to identify the blood vessel endothelium. Expression of CD31 was similar in CD11bDTR⁺ and DTR⁻ controls at 24 and 36 hours of injury (arrows, Figure 5D and Figure E2D, respectively), making it less likely that the pulmonary hemorrhage was due to loss of endothelial cells. However, we did observe that hyperoxia-exposed DTR⁺ mice had changes in the morphology of cells within the media of pulmonary vessels. Pulmonary cardiomyocytes, which extend into the proximal pulmonary veins (38–40), were more injured in lungs of DTR⁺ mice, as evidenced by more abundant lacy cytoplasm, enlarged nuclei, and prominent nucleoli (Figure 6C) compared with

Figure 5. Severe hyperoxia-induced lung injury in CD11bDTR⁺ mice. Sections from DT-treated CD11bDTR⁺ and DTR⁻ mouse lungs exposed to 24 hours of hyperoxia were processed for histology and immunohistochemistry. (A) H&E-stained sections from the lungs of DT-treated DTR⁻ and DTR⁺ mice after 24 hours of hyperoxia treatment. DTR⁺ mice have increased pulmonary hemorrhage (right) compared with DTR⁻ mice (left). (B) Galectin 3 (MAC2)⁺ alveolar macrophages and fibroblasts after 24 hours of hyperoxia. Large alveolar macrophages are present in grossly similar numbers in DTR⁻ (left) and DTR⁺ mice (right). (C) Movat's pentachrome stain identifies inflammation as well as proteinaceous material within the alveolar space of DTR⁺ mice at 24 hours. (D) CD31 staining of endothelial cells (arrows) from DTR⁺ and DTR⁻ control mice is similar at 24 hours. Scale bar = 50 μ m.

DTR⁻ mice. These changes were seen in nearly 100% of DTR⁺ mice analyzed, and were most prominent at the 40-hour time point (Figure 6C and data not shown). These changes in medium to large pulmonary veins may reflect early pulmonary vascular injury in our model.

CD11b⁺ Depletion-Associated Injury Is Not Mediated by Increased Lung Neutrophilia

Hyperoxic, DT-treated CD11bDTR⁺ mice had an increase in the percentage of neutrophils that were CD45⁺, but DTR⁺ and DTR⁻ control mice had similar total numbers of pulmonary neutrophils (Figure 7A). These results indicate that

neither DT treatment alone nor DT-induced cellular death increase neutrophilic lung inflammation. Furthermore, intraperitoneal injection with an anti-Ly6G antibody resulted in an approximately 50% reduction in lung neutrophils. Neutrophil reduction did not rescue mortality of DT-treated CD11bDTR⁺ mice after 36 hours of hyperoxia exposure (Figure 7B; 6/9 died in the anti-Ly6G group, 5/8 died in the isotype control group, representative of three experiments).

Discussion

Innate immunity is critical for immunoregulation and maintaining tissue

homeostasis (17). In this study, we characterized the pattern of lung mononuclear cells in the newborn mouse lung and conclude that an alternatively activated (M2-like) pattern of macrophage/mononuclear cell polarization is present in the lungs at birth. We recognize, however, that dividing macrophages into M1 versus M2 classes oversimplifies the complex continuum of functional and reversible states in which these cells exist *in vivo* (15). Despite the caveats of nomenclature, our data are consistent with a prior report in which interstitial macrophages in the postnatal lung were considered to be M2 polarized, as gauged by expression of arginase 1 and mannose receptor on CSF-1R⁺ interstitial myeloid cells (41).

The most prominent finding was that a subpopulation of interstitial CD11b⁺ monocytes and macrophages was necessary for the response to hyperoxia-induced lung injury. Depletion of only half of the CD45⁺ CD11b^{hi} GR1^{lo} cells in CD11bDTR⁺ myeloid cells resulted in profound loss of lung tissue integrity, with pulmonary hemorrhage and more severe tissue inflammation. This degree of severe lung injury led to significant mortality, with less than 40% of DTR⁺ mice surviving 40 hours of hyperoxia exposure, an effect that was only partially mitigated by a reduction in the level of oxygen exposure to 40%. That these depleted cells expressed high levels of *Arg1* and lower levels of *Nos2* suggests that they are an antiinflammatory, reparative M2-like population. Possible explanations could also include reduction in IL-10-expressing macrophages, as IL-10 promotes resolution of inflammation through apoptosis of neutrophils (42) and/or a lack of phagocytosis of cellular debris by macrophages (43), possibilities we will evaluate in follow-up studies.

A state of immunotolerance is likely beneficial in the perinatal period. To this end, IL-10-expressing, alternately activated M2 macrophages are present in the placenta (11, 44). Consistent with published reports (41, 45), neonatal lung leukocytes evolved in the early postnatal period. Specifically, alveolar macrophages were not present until a few days after birth. In comparison to the adult, elevated numbers of monocytes and interstitial macrophages biased toward an M2-like or alternatively activated phenotype were present in the newborn lung. Hyperoxia-exposed

Figure 6. CD11b-DTR⁺ mice have severe pulmonary hemorrhage hyperoxia-induced lung and vascular injury. H&E-stained sections from the lungs of DT-treated DTR⁻ and DTR⁺ mice after hyperoxia. (A) Severe hyperoxia-induced lung injury in CD11bDTR⁺ mice. DTR⁻ mice had mild pulmonary hemorrhage after 40 hours of hyperoxia exposure (*left*). DTR⁺ mice (*right*) had severe acute and subacute pulmonary hemorrhage with evidence of hemosiderin-laden macrophages (*inset*, *arrow*). Scale bar = 100 μ m. This DTR⁺ mouse also had aspiration pneumonia and associated inflammation. (B) Mild pulmonary hemorrhage in DTR⁻ mouse (*left*) and severe pulmonary hemorrhage with fibrin deposition and atelectasis in DTR⁺ mice (*right*). (C) Pulmonary veins (*arrow*) are relatively normal in DTR⁻ mice (*left*). Cardiomyocytes from medium-to-large pulmonary veins of DTR⁺ mice have abundant lacy cytoplasm, enlarged nuclei, and prominent nucleoli. Scale bar = 50 μ m.

newborn mice had further elevations in the number of M2-like mononuclear cells in the lung, an observation that could be a result of stimulation of M2 polarization or, alternatively, a hyperoxia-induced delay in pulmonary M2 cell attrition over the first few days of postnatal life.

Whether these developmental-specific aspects of myeloid immunity afford a newborn mouse more or less protection from lung injury was not known. We hypothesized that lung injury superimposed on this relative state of immunotolerance in the neonate may be less severe in the acute phase. However, an M2-biased immunotolerant state could potentially promote pathological remodeling with chronic lung injury in BPD, as chronically activated M2 macrophages are considered to be competent at tissue remodeling (14). Our results support a role for alternatively activated mononuclear cells having a protective role against severe lung injury early after hyperoxia exposure. However, the high mortality in DT-treated DTR⁺

mice, even in the lower 40% FiO₂-treated group, did not allow us to evaluate longer-term effects on lung remodeling in these studies.

The pulmonary responses to hyperoxia-induced lung injury differ markedly between adult and newborn mice. Indeed, the high sensitivity of adult rodents to hyperoxia resulted in limited enthusiasm for hyperoxia as a model for adult lung injury. Adult mice show 100% mortality after 5 days of 90–100% hyperoxia exposure (46), whereas neonatal mice can survive for several weeks in these experimental conditions (21). This dichotomy was mirrored by differences in the leukocyte populations that we observed between adult and neonatal mice. Thus, the presence of a large pulmonary population of apparently protective CD11b⁺ cells that is not present in high numbers in lungs of adult mice may, in part, explain this differential sensitivity to hyperoxia. Moreover, the marked increase in sensitivity of newborn mice after depletion of these cells raises the

possibility that strategies to increase their numbers, function, or downstream effects might be protective against lung injury in both newborn and more mature mice, and, by extension, to lung disease in children and adult humans.

CD11c⁺ SiglecF⁺ alveolar macrophages are not present in significant numbers until PN1–PN3, a finding consistent with studies that describe the appearance of CD45⁺ F4/80⁺ CD11b^{hi} CD11c⁺ F4/80⁺ alveolar macrophages starting at PN3 (32, 41). Thus, it follows that alveolar macrophages are not sufficient to maintain pulmonary immune homeostasis when newborn mice are exposed to hyperoxia. In addition, alveolar macrophages are preserved in DT-treated CD11bDTR⁺ mice, perhaps due to lower availability of systemically administered DT in the alveolar space, but more likely to their low expression of CD11b.

CD11b⁺ macrophages also appear to be essential in maintaining vascular function and alveolar–capillary integrity. Despite only a 50% reduction in CD45⁺ CD11b⁺ GR1^{lo} monocytes/interstitial macrophages, these mice demonstrate overwhelming alveolar hemorrhage. Indeed, there is widespread hemorrhage as early as 24 hours after DT injection in hyperoxia-treated DTR⁺ mice, even before the increase in mortality becomes evident. Importantly, DTR⁻ littermate controls did not have significant hemorrhage, and their lungs appeared no different from hyperoxia-exposed wild-type mice. We saw no differences in any of the injury or inflammation parameters between DT-treated DTR⁻ controls and non-DT-treated wild-type C57/BL6N mice, ruling out a DT-specific pulmonary toxicity. Furthermore, DT-treated CD11bDTR⁺ mice in normoxic conditions had minimal pulmonary hemorrhage on histology. Thus, cellular necrosis alone does not explain the severe pulmonary phenotype in hyperoxia-injured CD11bDTR⁺ mice. Moreover, damage from unregulated neutrophil influx does not explain the lung injury in hyperoxia-treated CD11bDTR⁺ mice. Neutrophil numbers did not differ in CD11bDTR⁺ mice, and antibody-mediated depletion of neutrophils did not rescue mortality.

A consistent phenotype among hyperoxia-treated DTR⁺ mice was the presence of reactive cellular changes in the

Figure 7. Hyperoxia-induced lung injury in CD11bDTR⁺ is not mediated by neutrophils. (A) Neutrophils are identified in total lungs from neonatal mice as CD45⁺, CD11b^{hi}, Ly6G^{hi}. Quantification of CD45⁺, CD11b^{hi}, Ly6G^{hi} neutrophils by flow cytometry in lung homogenates from hyperoxia-treated DTR⁺ mice at 36 hours (left, % leukocytes; right, total number; $n = 3-18$ DTR⁺ and DTR⁻ animals; $*P < 0.05$, Student's unpaired t test). (B) Depletion of neutrophils in CD11bDTR⁺ mice with anti-Ly6G blocking antibody. (Left) quantification of % neutrophils in lungs of CD11bDTR⁺ mice treated with control antibody or anti-Ly6G blocking antibody. (Right) Kaplan-Meier survival curves of DTR⁺ mice treated with control anti-Ly6G blocking antibody ($n = 9$ anti-Ly6G and $n = 8$ isotype control antibody; $*P < 0.005$ Student's unpaired t test, Log-rank [Mantel-Cox] test). n.s., not significant.

walls of medium-to-large pulmonary veins, including swollen cytoplasm, enlarged nuclei, and prominent nucleoli. These changes were surprising, because, in mice and humans, pulmonary arterial vessels are traditionally those affected by hypoxic vasoconstriction and pulmonary hypertension (47). Although the DTR⁺ mice were exposed to hyperoxic conditions, they were cyanotic due to alveolar hemorrhage, which would predictably impair gas exchange. Over time, these mice are at risk for development of pulmonary hypertension. The significance and etiology

of the pulmonary venous changes is unclear at this time. It is possible that the changes in pulmonary veins reflect postcapillary obstruction due to alveolar hemorrhage in this model, but further investigation of this finding is warranted.

As seen in some humans with diffuse alveolar hemorrhage (48), DT-treated hyperoxic mice appear to have capillary leak, with alveolar hemorrhage and edema. We propose that antiinflammatory interstitial macrophages are broadly necessary for the neonatal response to hyperoxia-induced acute lung

injury in mice, with protective effects on controlling inflammation and maintaining vasculature homeostasis. Indeed, macrophage ablation in the ovary of CD11bDTR mice results in significant spontaneous ovarian hemorrhage with CD31⁺ endothelial cell disruption, suggesting a role for these cells in maintaining vascular integrity (49). Moreover, DT-treated CD11b-DTR mice with acute kidney injury-related systemic inflammation have increased capillary leak in the lung (50). Determining the specific mechanisms underlying these effects will be important in future studies.

The purpose of this study was to study the role of CD11b⁺ mononuclear cells in hyperoxia-induced lung injury. The CD11bDTR⁺ newborn mouse was exquisitely sensitive to hyperoxia, and depletion experiments had to be terminated at 36–40 hours, making it impossible to study how the absence of these important immunoregulatory cells affect later development of the BPD phenotype, including resolution of the acute injury and transition to fibrosis. We also have not differentiated macrophages/monocytes from dendritic cells in these studies. As reviewed recently by Lichtnekert and colleagues (14), macrophages and dendritic cells can both phagocytose, present antigen, and release chemokines and cytokines to influence the inflammatory milieu, processes for which potential relevance to the observed phenotypes can be explored in ongoing studies.

Conclusions

These results highlight the importance of interstitial macrophages and monocytes in the developing lung. To our knowledge, this is the first study investigating the role of pro- or antiinflammatory mononuclear cells in a model of BPD. We find that mononuclear cell populations change polarization states during normal development as well as in response to lung injury. It seems likely that any change in this balance of pro- and antiinflammatory polarization states could result in more severe lung injury. Importantly, polarization states are modifiable, with enormous clinical potential. Further work will be directed at determining the specific mechanisms by which protective CD11b⁺ cells modulate neonatal injury in mice, and validating these observations in clinical samples from human patients with BPD to understand the role of these cells in normal

development and injury in the human lung. ■

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

Acknowledgments: The authors thank Brian Johnson and Erin McCarty at the University of Washington Histology and Imaging Core for technical support; Xiaodong Zhu, Cliff Rims, Maura Newell, and Rachel Waworuntu for

additional technical assistance; Dr. Jeremy Duffield for his generous donation of CD11b–diphtheria toxin receptor mice; and Dr. Ron Gibson for his thoughtful review of the manuscript.

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