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New mouse model of pulmonary hypertension induced by respiratory syncytial virus bronchiolitis

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INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of bronchiolitis in young children (30). Severe RSV bronchiolitis is known to cause mild pulmonary hypertension (PH) in previously healthy infants (1, 15, 39). In infants with congenital heart disease (CHD) and preexisting PH, severe RSV bronchiolitis can result in the worsening of PH and has been associated with increased risk of the patient requiring intensive care unit (ICU) stay, mechanical ventilator support, and increased mortality (5, 25, 28, 31, 45). Improvements in ICU management, active immunoprophylaxis with palivizumab, and advances in pulmonary vasodilators have contributed to improvements in clinical outcomes compared with 30 yr ago (24, 28, 29, 31). Further investigation into the pathogenesis of RSV is still needed to ease the suffering of infants affected by RSV bronchiolitis, particularly those with underlying CHD/PH, as ICU admission and death from PH crisis in severe cases still occurs (5). In infants with preexisting PH from CHD or bronchopulmonary dysplasia, novel therapies for severe RSV bronchiolitis must consider various mechanisms responsible for the substantial pulmonary vascular component of this condition (e.g., nonuniformly elevated pulmonary artery pressure because of hyperinflation/atelectasis or hypoxic vasoconstriction, ventilation-perfusion mismatch, secondary right heart failure, etc.) (4).

Other respiratory pathogens and diseases are known to cause secondary PH by inducing host inflammatory responses (18, 42). PH secondary to *Pneumocystis pneumonia* is largely a result of the T helper (Th)1 immune response and its main cytokine, interferon (IFN)-γ (43). It has been established that type I IFN causes PH (16), and PH is a known side effect of IFN-α or IFN-β therapy for diseases such as hepatitis (35). On the other hand, PH associated with schistosomiasis involves Th2 immune responses (19). Transgenic mice overexpressing IL-13 (a Th2 cytokine) spontaneously develop PH by 2 mo of age (7). Both acute RSV bronchiolitis and asthma cause inflammation in the airways and lower respiratory tract obstruct...
tion through induction of Th2 immune responses, and children with asthma have been shown to develop PH with recurrent hypoxia and chronic inflammation (12, 14, 26, 32).

Currently, no mouse model exists to study the pathogenesis of PH secondary to RSV bronchiolitis. A mouse model is a useful tool to understand the pathophysiology of human diseases for many reasons, including its small size, short breeding times, and, most importantly, the ability to genetically engineer genes to identify their roles in disease. Severe RSV infection in human infants involves Th2-biased immune responses (3, 27). Therefore, to model the unique pathophysiological effects that severe RSV infection has on human infants, we used a neonatal mouse model of RSV infection and reinfection that has been shown to recapitulate human severe RSV disease. RSV-infected mice develop PH as demonstrated by echocardiogram and morphometry and confirmed by right ventricular (RV) systolic pressure (RVSP) measurement. The PH induced by RSV bronchiolitis adequately mimics what is observed clinically (9, 21, 34, 50). This model will be a critical tool to delineate the pathogenesis of PH secondary to RSV bronchiolitis, discover therapeutic targets, and evaluate therapeutic interventions.

METHODS

Mice. BALB/c breeder pairs and female adult mice (6–8 wk old) were purchased from Harlan Laboratories/Envigo (Indianapolis, IN) and maintained under a specific, pathogen-free condition in the Laboratory Animal Care Unit at the University of Tennessee Health Science Center. Pups born from time-mated mice within 24 h of one another were used for experiments. All animal protocols were prepared in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee and Institutional Biosafety Committee of the University of Tennessee Health Science Center, an American Association for the Accreditation of Laboratory Animal Care-accredited institution.

Overall protocol. Neonatal mice were infected and reinfected with RSV as per established protocols (9, 49, 50) and analyzed for evidence of PH (Fig. 1). The experiment was conducted using pups (n = 4–5) born on the same date and repeated with four sets of pups. We measured peripheral capillary oxygen saturation (SpO2) daily 2–4 days after infection, before reinfection, and 2–7 days after reinfection using a SpO2 monitoring system (Mouseox Plus, Starr Life), which was applied to the groin area. Echocardiography was conducted at 5 days after RSV infection and before reinfection. RVSP was measured using anesthesia at 6 days after RSV reinfection (Fig. 1). In some sets of the animals, muscularization and medial wall thickness.

Measurement of muscularization and medial wall thickness. Lung sections were immunostained with rabbit anti-α-smooth muscle actin (α-SMA) antibody (1:100, catalog no. ab5694, Abcam, Cambridge, MA) and anti-rabbit IgG reagent (ImmunPRESS, Vector Laboratories). Slides were developed with diaminobenzidine peroxidase substrate and counterstained with hematoxylin.

RVSP was measured by inserting a 1.1-Fr microtip pressure catheter (AD Instruments, Colorado Springs, CO) via the right jugular vein in spontaneously breathing anesthetized mice [Avertin (250 mg/kg ip)] (6). Data were recorded via the PowerLab system and analyzed with the Chart Pro software (AD Instruments). Lung and heart samples were removed after euthanasia. RV hypertrophy was assessed by calculating the ratio of RV weight to LV + septum (S) weight.

Fig. 1. Respiratory syncytial virus (RSV) infection protocol. Neonatal mice were infected with RSV at 5 days of age and then reinfected at 4 wk of age. Oxygen saturation (SpO2) monitoring was performed after RSV infection and before and after RSV reinfection. Echocardiography (ECHO) was performed 5 days after reinfection, and right ventricular systolic pressure (RVSP) was measured at 6 days after reinfection. Mice in the control group received serum-free medium on the same schedule.
were measured, and MWT determined using the following formula $\{(a + b + c + d)/4\}/(e + f/2)$ (7). These muscularized arteries were further categorized into fully muscularized (>75% of the circumference) and partially muscularized (50–75% of the circumference).

**Protein concentration in BALF.** BALF samples were kept frozen at –80°C until used. Total protein concentrations in BALF were measured using Pierce BCA Protein Assay (ThermoFisher). Samples were duplicated and measured as per the manufacturer’s protocol.

**Statistics.** All data are plotted as means ± SE. Student’s t-tests (unpaired, two-tailed) were conducted to compare between the groups for hemodynamic data, RVSP, RV/LV + S, echocardiographic and histological measurements, and total protein concentration in BALF using JMP Pro (SAS, Heidelberg, Germany). Differences were considered significant if $P < 0.05$.

## RESULTS

**\(\text{SpO}_2\) in mice after RSV reinfection does not indicate hypoxia in mice.** To establish the mouse model of PH secondary to RSV infection, we infected 5-day-old pups with RSV and reinfected at 4 wk after the first infection (Fig. 1). RSV infection in neonates followed by reinfection results in increased airway resistance after methacholine challenge, indicating airway hyper-reactivity similar to bronchiolitis (50). Because hypoxia has been shown to induce PH (46), we first measured \(\text{SpO}_2\) daily during both infection and reinfection. (Fig. 2). \(\text{SpO}_2\) levels remained higher than 95% across all days, suggesting that RSV-infected mice were not hypoxic. The body weight of RSV-infected mice was significantly lower than control mice (18.6 ± 0.7 vs. 21.7 ± 0.7 g, $P < 0.01$).

**Pulmonary vascular changes after RSV reinfection.** Histological analysis of the lungs from RSV-reinfected mice revealed significant peribronchial and perivascular inflammation and fibrosis compared with those of the sham-infected control mice (Fig. 3). To evaluate muscularization and the medial thickness of the pulmonary arteries, lung sections were immunostained to localize α-SMA (Fig. 4). RSV infection induced prominent medial thickness of pulmonary arteries and caused an increase in the number of muscularized peripheral pulmonary arteries (Fig. 4, C and F). The MWT ratio of the peribronchial pulmonary arteries in RSV-infected mice was significantly increased compared with uninfected mice (9.0 ± 1.6 vs. 15.2 ± 3.1, $P < 0.0001$; Fig. 4H). RSV also increased muscularized peripheral pulmonary arteries, which is an indicator of PH development (full muscularization, 2.7 ± 0.8 vs. 4.0 ± 1.0, $P = 0.002$, Fig. 4F; partial muscularization, 1.6 ± 0.7 vs. 2.6 ± 1.0, $P = 0.002$; Fig. 4J).

**Echocardiographic changes indicative of PH.** Echocardiography was performed under general anesthesia at 5 days after reinfection. We evaluated both PAAT and ET, because shortened PAAT and decreased PAAT/ET was previously shown by Thibault et al. (44) to correlate linearly with increased RVSP by RV catheterization. To minimize the duration of anesthesia, whole echocardiographic images were acquired and used to evaluate PH and RV function. Pulsed-wave Doppler of pulmonary artery flow was obtained at the short-axis view in all animals (Fig. 5A) and average values of three consecutive pulses were used. RSV-reinfected mice demonstrated significantly shortened PAAT and decreased PAAT/ET compared with control mice (Fig. 5, D and E). Additionally, elevated RV pressure caused flattening of the interventricular septum in the end-systolic period resulting in a “D-shaped” LV (Fig. 5G) in one mouse, and another mouse developed small pericardial effusion. There were no significant differences in heart rate, LV ejection fraction (52.5 ± 1.6 vs. 58.7 ± 8.1%, $n = 4$–$5$, $P = 0.50$), fractional shortening (26.5 ± 1.0 vs. 31.3 ± 5.4%, $P = 0.44$), cardiac output (11.7 ± 1.2 vs. 13.0 ± 2.3 ml/min, $P = 0.63$), aortic peak velocity, and aortic valve peak pressure between the control and RSV groups (Table 1).

**RVSP elevation after RSV reinfection.** RVSP was measured in mice following jugular vein catheterization 6 days after RSV reinfection (Fig. 6A). RVSP was significantly increased after RSV reinfection (15.9 ± 1.5 vs. 29.6 ± 6.7 mmHg, $P < 0.01$) compared with the control group (Fig. 6B). RV/LV + S was higher in RSV-infected mice than in control mice (0.20 ± 0.03 vs. 0.33 ± 0.03, $P < 0.01$; Fig. 6C).

**IL-13 immunohistochemistry.** Formerly, our group reported that this RSV reinfection protocol with primary infection as a neonate caused exacerbated Th2 responses as demonstrated by increased Th2 cells in flow cytometry and increased production of Th2 cytokines (IL-4 and IL-13) in BALF (50). To provide further evidence of the involvement of the Th2 immune response, lung sections were immunostained with anti-IL-13 antibody to localize IL-13 production (Fig. 7). IL-13-expressing Th2 cells were observed in the perivascular region. The airway epithelium showed positive stain with IL-13 in both control and RSV-infected mice. IL-13 stain was positive in the smooth muscles of both the pulmonary artery and vein in RSV-infected mice (Fig. 7).

**Protein concentration in BALF.** To investigate endothelial permeability in this mouse model, BALF samples were collected at 6 days after RSV reinfection, and total protein levels were measured. There was no significant difference between control and RSV-infected mice (0.4 ± 0.013 vs. 0.38 ± 0.02, $P = 0.2$).

## DISCUSSION

Our results demonstrate that neonatal mice infected with RSV and reinfected with RSV as adults develop PH. This was evidenced by echocardiography, histological changes, and direct RVSP measurement. Histological assessment of the lungs demonstrated infiltration of inflammatory cells around pulmo-
nary vessels and remodelling of pulmonary arteries, with increased medial thickness and muscularization of small peripheral pulmonary arteries. RVSP measured via right jugular vein catheterization in RSV-infected mice was elevated compared with that in control mice. Flattening of the interventricular septum reflecting RV pressure overload was observed in the echocardiography. As previously reported (44), shortened PAAT and decreased PAAT/ET measured by Doppler pulse wave of pulmonary outflow were consistent with elevated RVSP in our mouse model of PH secondary to RSV disease.

This is the first animal model of PH secondary to severe RSV bronchiolitis, to our knowledge. The age of the initial RSV infection has an important role in pathogenesis. Our neonatal mouse (i.e., <1 wk of the age) model resembles human infants with severe RSV infection in terms of airway remodeling and increased airway hypersensitivity, mucus production, pulmonary eosinophilia, and Th2-biased immune responses (9) and has been used by us and others to study RSV pathogenesis (10, 11, 50). Physiologically measuring the presence of PH in very young mice is challenging; therefore, these
mice were reinfected with RSV as adults. This was clinically relevant because almost all children have been infected by RSV by 2 yr of age, and >50% of infants are reported to have reinfection (17, 22). Interestingly, cardiac dysfunction has also been observed in mice infected with pneumonia virus of mice (PVM), which is similar to RSV (13). The adult PVM mouse model mimics severe human RSV bronchiolitis in clinical signs and lung histopathology with high viral replication but has less involvement of the Th2 response compared with our mouse RSV model. Although PH was not directly measured,
mice infected with PVM exhibited both right- and left-sided cardiac dysfunction as detected by cardiac MRI. There was no evidence of direct virus-induced myocardial injury, and the authors concluded that cardiac dysfunction was related to severe lung injury (2). The PH observed in our model could be also from severe lung injury or severe inflammation around pulmonary arteries and veins. In our mouse model, LV systolic function evaluated by echocardiography did not demonstrate signs of dysfunction.

The pathogenesis of PH secondary to RSV bronchiolitis is likely multifactorial. However, our group previously published that this RSV reinfection protocol in neonatal mice induces an exaggerated pulmonary Th2 immune response upon reinfection (50). Flow cytometry of the lung cells isolated from RSV-infected mice showed an increase in Th2 (IL-4-expressing CD4+) cells (50). BALF from RSV-infected mice revealed eosinophil recruitment to the lung and increased Th2 cytokine (IL-4 and IL-13) production (50). IL-13-positive stains in the pulmonary artery and vein would strongly indicate the involvement of the Th2 response in the development of PH. Furthermore, RSV-induced lower respiratory tract obstruction can cause air trapping in the lungs, and a hyperinflated lung is associated with increased pulmonary vascular resistance, especially in small pulmonary arteries, which can contribute to the development of PH (47). Increased mucus production with RSV bronchiolitis would obstruct the lower airway, and the resulting atelectasis and decreased lung volume is also associated with an increase in pulmonary vascular resistance (47). Mild hypoxia secondary to interstitial lung edema and low ventilation will cause hypoxic vasoconstriction (47). Chronic hypoxia will induce pulmonary vasoconstriction and vascular remodeling and is widely used to induce PH in rodent models (36, 41). Our model did not cause hypoxia as evidenced by SpO2 monitoring after RSV primary infection and secondary infection (Fig. 2). Hypoxia-inducible factor-1α is activated in

<table>
<thead>
<tr>
<th>Hemodynamic data of the LV during echocardiogram</th>
<th>Control</th>
<th>Respiratory Syncytial Virus Infection</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice/group</td>
<td>5</td>
<td>4</td>
<td>0.96</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>381.8 ± 17.6</td>
<td>380.5 ± 19.5</td>
<td>0.96</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>52.5 ± 1.6</td>
<td>58.7 ± 8.1</td>
<td>0.50</td>
</tr>
<tr>
<td>LV fractional shortening, %</td>
<td>26.5 ± 1.0</td>
<td>31.3 ± 5.4</td>
<td>0.44</td>
</tr>
<tr>
<td>Cardiac output, ml/min</td>
<td>11.7 ± 1.2</td>
<td>13.0 ± 2.3</td>
<td>0.63</td>
</tr>
<tr>
<td>Aorta peak velocity, mm/s</td>
<td>997.8 ± 97.7</td>
<td>1,000.6 ± 104.8</td>
<td>0.98</td>
</tr>
<tr>
<td>Aortic valve peak velocity, mm/s</td>
<td>4.1 ± 0.8</td>
<td>4.1 ± 0.9</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Values are means ± SE. A two-tailed Student’s t-test was used. LV, left ventricle.
RSV infection in vitro and in a murine in vivo model, but in these cases, the activation of hypoxia-inducible factor-1α is independent of systemic hypoxia (20). Although other viruses have been shown to induce PH in children secondary to lower respiratory tract infection, RSV infection is the most cited (15, 33, 48). Inflammation may play a key role in the development of PH in children with RSV bronchiolitis. Evidence for this exists in lung specimens from fatal cases of human RSV, which show bronchovascular inflammation centered on the pulmonary arteries in the smallest airways (23).

There are several limitations in our experiments. First, PAAT and ET measurements were performed by only one “blinded” investigator; although this is done clinically, this could result in a single reader error. Second, in humans, it is known that RSV-induced PH resolves with bronchiolitis (39), but we did not gather long-term followup data on our mice and cannot address the reversibility of PH in our model. Third, this is a mouse model of human RSV and mice are semipermissive to RSV; thus, our data may underestimate the contribution of RSV-induced disease to PH. Endothelial injury may also play a role; however, we failed to detect endothelial injury 6 days after secondary infection. Because we only assayed for endothelial leak and only at one time point, it remains possible that this occurs earlier in disease. Finally, we know that our neonatal mouse model of RSV induces long-term airway remodeling and lung function changes consistent with asthma, similar to what is observed in humans (37, 38, 40). Thus, our model may resemble PH secondary to chronic wheezing/asthma triggered by viral infection.

In summary, PH is observed secondary to severe RSV bronchiolitis in humans and mice; our extensive data, presented elsewhere, of Th2 involvement in RSV pathogenesis in this neonatal mouse model suggest a role for Th2 immune responses in the development of PH. Studies are currently underway to elucidate the role and mechanism of Th2 immune responses in the development of PH secondary to RSV infection. Our mouse model of PH secondary to RSV infection, developed here, allows us and others to explore molecular mechanisms responsible for PH and test therapeutics and/or treatment strategies to improve patient outcomes.

In conclusion, we have established the first mouse model of PH secondary to RSV bronchiolitis. PH was confirmed in this mouse model by RV pressure measurement, histopathology, morphometry, and echocardiography. This mouse model of RSV mimics the human disease in terms of airway hypersensitivity, pulmonary eosinophilia, mucus production, and Th2-biased immune reaction. Considering the presence of PH is a prognostic factor for prolonged hospitalization in children with RSV bronchiolitis, identifying the mechanisms responsible for PH is of great importance and our mouse model will be a valuable clinically relevant research tool.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


