The American Journal of Pathology, Vol. 191, No. 12, December 2021

The American Journal of
PATHOLOGY
ajp.amjpathol.org

CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Adrenomedullin Deficiency Potentiates Lipopolysaccharide-Induced Experimental Bronchopulmonary Dysplasia in Neonatal Mice

Amrit K. Shrestha,* Renuka T. Menon,* Chandrasekhar Yallampalli,† Roberto Barrios,‡ and Binoy Shivanna*

From the Section of Neonatology, Department of Pediatrics, and the Basic Sciences Perinatology Research Laboratories,† Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston; and the Department of Pathology and Genomic Medicine,‡ Houston Methodist Hospital, Houston, Texas*

Accepted for publication September 2, 2021.

Address correspondence to Binoy Shivanna, M.D., D.M., Ph.D., Texas Children's Hospital, 1102 Bates Ave., Houston, TX 77030. E-mail: shivanna@bcm.edu.

Lung inflammation interrupts alveolarization and causes bronchopulmonary dysplasia (BPD). Besides mechanical ventilation and hyperoxia, sepsis contributes to BPD pathogenesis. Adrenomedullin (*Adm*) is a multifunctional peptide that exerts anti-inflammatory effects in the lungs of adult rodents. Whether *Adm* mitigates sepsis-induced neonatal lung injury is unknown. The lung phenotype of mice exposed to early postnatal lipopolysaccharide (LPS) was recently shown to be similar to that in human BPD. This model was used to test the hypothesis that *Adm*-deficient neonatal mice will display increased LPS-induced lung injury than their wild-type (WT) littermates. *Adm*-deficient mice or their WT littermates were intraperitoneally administered 6 mg/kg of LPS or vehicle daily on postnatal days (PNDs) 3 to 5. The lungs were harvested at several time points to quantify inflammation, alveolarization, and vascularization. The extent of LPS-induced lung inflammation in *Adm*-deficient mice was 1.6-fold to 10-fold higher than their WT littermates. Strikingly, *Adm* deficiency induced STAT1 activation and potentiated STAT3 activation in LPS-exposed lungs. The severity of LPS-induced interruption of lung development was also greater in *Adm*-deficient mice at PND7. At PND14, LPS-exposed WT littermates displayed substantial improvement in lung development, whereas LPS-exposed *Adm*-deficient mice continued to have decreased lung development. These data indicate that *Adm* is necessary to decrease lung inflammation and injury and promote repair of the injured lungs in LPS-exposed neonatal mice. *(Am J Pathol 2021, 191: 2080–2090; https://doi.org/10.1016/j.ajpath.2021.09.001)*

Preterm infants are at increased risk of developing the chronic lung disease, bronchopulmonary dysplasia (BPD), the most common complication of preterm birth in the United States.[1] Alveolar simplification is a unique histopathologic feature of this disease.[2] This disease lacks curative therapies, and the affected infants continue to have cardiorespiratory and neurodevelopmental morbidities in later life.[3] Therefore, studies to determine the mechanisms and develop therapeutic strategies for BPD are warranted.

Balanced signaling of the innate and adaptive immune systems is needed to restore the immune homeostasis following an inflammatory insult. Failure to achieve this homeostasis leads to several inflammatory disorders, including BPD.[4,5] Inflammatory stimuli, such as infection, mechanical ventilation, and hyperoxia, disrupt growth factor signaling and cell proliferation in the developing lungs and contribute to BPD pathogenesis.[6–9] More important, postnatal infection independently increases the risk for developing BPD.[10–18] Thus, understanding the molecular mechanisms that lead to infection-mediated inflammatory response is important to develop therapeutic strategies for this disease. The current experiments were designed to meet this necessity.

Adrenomedullin (*Adm*) is a ubiquitous multifunctional peptide that is predominantly present in highly vascularized organs, including the lungs.[19] *Adm* signals via calcitonin receptor-like receptor (Calcrl) and

Supported by NIH grants HL139594 (B.S.) and P30DK056338 (Digestive Disease Center Core at Baylor College of Medicine), American Lung Association Award RG-349917 (B.S.), and Baseball for Babies (B.S.).

Disclosures: None declared.

https://doi.org/10.1016/j.ajpath.2021.09.001

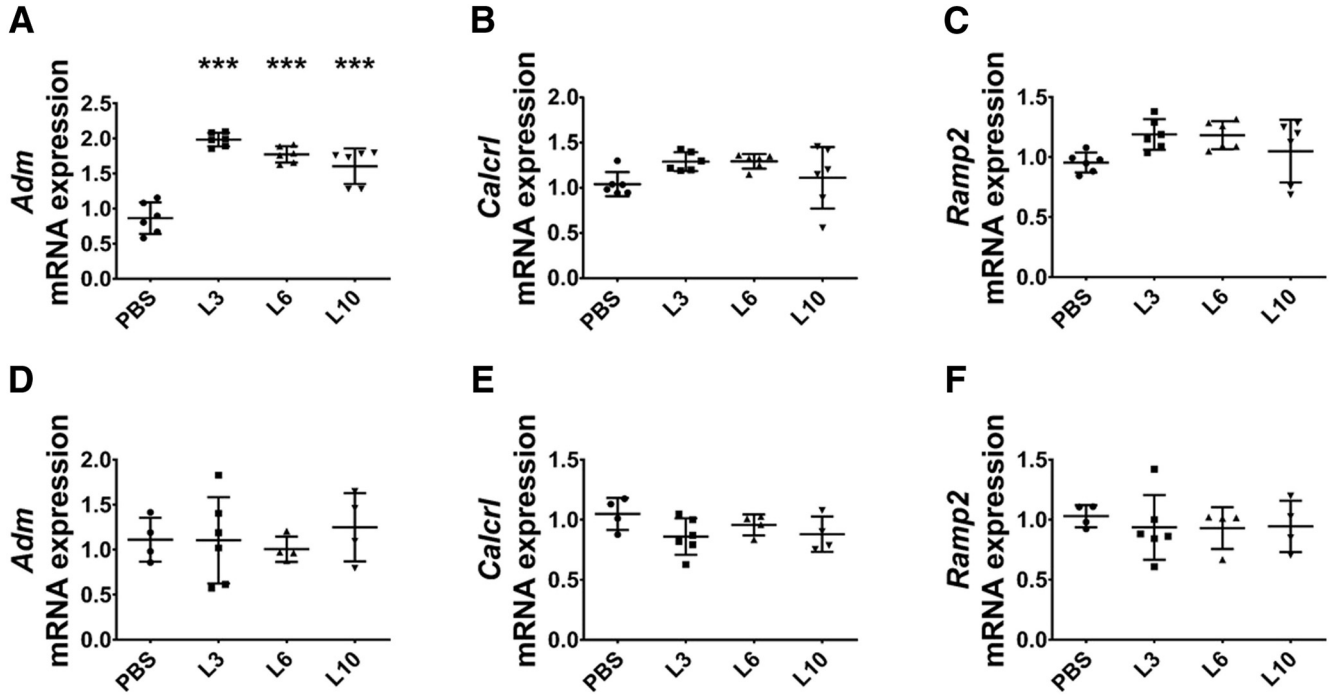

Figure 1 Lipopolysaccharide (LPS) increases pulmonary adrenomedullin (*Adm*) expression. Lung tissues of C57BL6J wild-type mice treated intraperitoneally daily with phosphate-buffered saline (PBS) or LPS at doses of 3 (L3), 6 (L6), or 10 (L10) mg/kg through postnatal days (PNDs) 3 to 5 were harvested on either PND3 (**A**–**C**) or PND5 (**D**–**F**) for gene expression assays. Real-time RT-PCR analyses-based determination of *Adm* [PND3 (**A**) and PND5 (**D**)], *Calcrl* [PND3 (**B**) and PND5 (**E**)], and *Ramp2* [PND3 (**C**) and PND5 (**F**)] mRNA levels. Significant differences between PBS- and LPS-treated animals are indicated. Values are presented as the means ± SD (**A**–**F**). $n = 4$ to 6 mice per group (**A**–**F**). $^{***}P < 0.001$ versus PBS (analysis of variance).

receptor-activity–modifying protein 2 (Ramp2).[20] In addition to its critical role in vascular development, *Adm* protects adult rodents against lung injury secondary to mechanical ventilation,[21] ischemia-reperfusion,[22] lipopolysaccharide (LPS),[23] and carrageenan.[24] However, the role of *Adm* in LPS-induced developmental lung injury is unknown. Gram-negative bacterial infection substantially increases the risk of developing BPD.[17] Consequently, LPS, a major biologically active component and primary recognition structure of Gram-negative bacteria,[25] has been widely used to model infection in animals.[26,27] A recently developed mouse model of lung injury caused by chronic LPS exposure during the saccular phase of lung development had a phenotype similar to that of human BPD.[28] Therefore, in the curernt study, this model was used to test the hypothesis that *Adm*-deficient neonatal mice will display increased LPS-induced experimental BPD than their wild-type (WT) littermates.

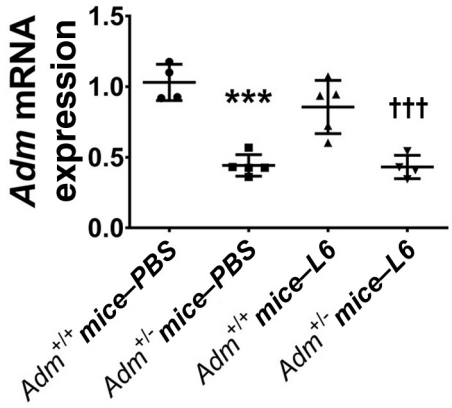

Figure 2 Lipopolysaccharide (LPS) does not affect pulmonary *Adm* expression in adrenomedullin (*Adm*) haplodeficient mice: $Adm^{+/-}$ mice and their wild-type littermates ($Adm^{+/+}$) were treated intraperitoneally with phosphate-buffered saline (PBS) or 6 mg/kg of LPS (L6) on postnatal days (PNDs) 3 to 5, and their lung tissues were harvested on PND5 to determine *Adm* mRNA levels by real-time RT-PCR analyses. Values are presented as the means ± SD. $n = 4$ to 5 mice per group. $^{***}P < 0.001$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to PBS; $^{\dagger\dagger\dagger}P < 0.001$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to L6 (analysis of variance).

Materials and Methods

Animals

This study was approved and conducted in strict accordance with the federal guidelines for the humane care and use of laboratory animals by the Institutional Animal Care and Use Committee of Baylor College of Medicine (Houston, TX). Dr. Kathleen Caron (University of North Carolina at Chapel Hill) provided us the *Adm* haplodeficient ($Adm^{+/-}$) mice on a 129/SvEv background, and the generation of these mice has been reported previously.[29] These $Adm^{+/-}$ mice were backcrossed onto C57BL/6J wild-type mice (stock number 000664; The Jackson Laboratory, Bar Harbor, ME) for 12 generations to obtain $Adm^{+/-}$ mice on a C57BL/6J

background for the current experiments. Time-pregnant mice raised in our animal facility were used for the experiments. $Adm^{-/-}$ mice are embryonically lethal; therefore, $Adm^{+/-}$ mice were used for the studies. $Adm^{+/-}$ mice underwent both genotyping and real-time RT-PCR analysis.

Pharmacologic Inhibition of *Adm* Signaling

To inhibit *Adm* signaling *in vivo*, neonatal C57BL/6J WT mice were injected intraperitoneally with 100 μg/kg of AM_{22-52} (American Peptide Company Inc., Sunnyvale, CA) or an equivalent volume of phosphate-buffered saline, once daily on postnatal days (PNDs) 1 to 7. The dose of the *Adm* receptor antagonist, AM_{22-52}, was based on its *in vivo* use in rodents, as described previously.[30,31]

LPS Treatment

Adm-sufficient and *Adm*-deficient mice were injected intraperitoneally with 6 mg/kg of *Escherichia coli* O55:B5 LPS (Sigma-Aldrich, St. Louis, MO; L2880) or an equivalent volume of phosphate-buffered saline, once daily on PNDs 3 to 5. In a separate set of experiments, neonatal WT mice were injected intraperitoneally with 3, 6, or 10 mg/kg of LPS through PNDs 3 to 5 to investigate the dose- and time-dependent effects of LPS on pulmonary *Adm*, *Calcrl*, and *Ramp2* mRNA expression.

Analysis of Alveolarization and Pulmonary Vascularization

The mice were euthanized on PND7 or PND14, and their lungs were inflated and fixed with 10% formalin at 25 cm H_2O pressure for lung morphometry studies.[28] Alveolar development was determined by radial alveolar counts (RACs) and mean linear intercepts (MLIs), as described previously.[28] Pulmonary vessel density was also determined as described before.[32] Briefly, the number of von Willebrand factor (vWF)–stained blood vessels with a diameter of <150 μm was quantified from at least 10 random nonoverlapping fields (original magnification, ×20) for each animal to determine the pulmonary vascular density.

Real-Time RT-PCR Assays

Total RNA was isolated from the lungs at PND3 or PND5 and reverse transcribed to cDNA. Real-time quantitative RT-PCR analysis was performed using gene expression master mix (Thermo Fisher Scientific, Waltham, MA; 4369016) and the following gene-specific primers: *Adm* (AP7DPHX and PN4331348), *Calcrl* (Mm00516986_m1), chemokine (C-C motif) ligand 2 (*CCL2*; Mm00441242_m1), *CCL3* (Mm00441259_g1), *CXCL1* (Mm04207460_m1), intercellular adhesion molecule 1 (*ICAM1*; Mm00516023_m1), *IL1B* (Mm00434228_m1), *Ramp2* (Mm00490256_g1), tumor necrosis factor-α (*TNF-α*; Mm00443258_m1), and glyceraldehyde 3-phosphate

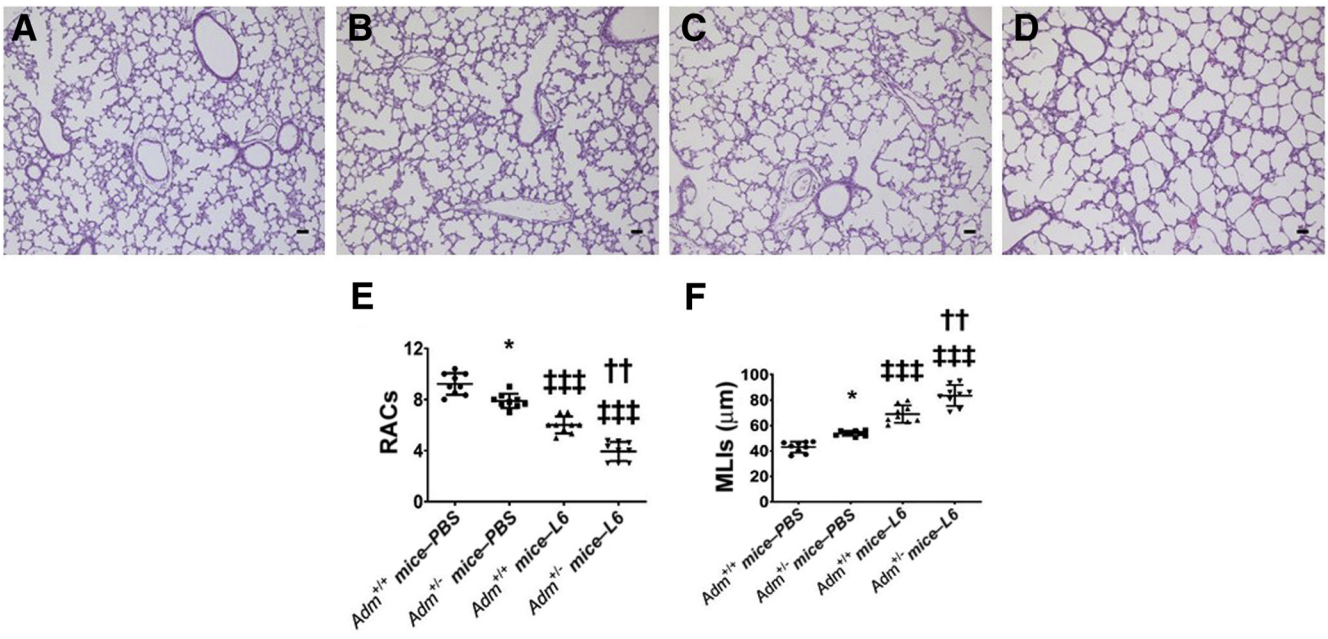

Figure 3 Adrenomedullin (*Adm*) deficiency potentiates lipopolysaccharide (LPS)–induced alveolar simplification. $Adm^{+/-}$ mice and their wild-type littermates ($Adm^{+/+}$) were treated intraperitoneally with phosphate-buffered saline (PBS) or 6 mg/kg of LPS (L6) on postnatal days (PNDs) 3 to 5, and their lung development was quantified on PND7. Representative hematoxylin and eosin–stained lung sections from $Adm^{+/+}$ (**A** and **C**) and $Adm^{+/-}$ (**B** and **D**) mice exposed to PBS (**A** and **B**) or L6 (**C** and **D**). Alveolarization was determined by radial alveolar counts (RACs; **E**) and mean linear intercepts (MLIs; **F**). Values are presented as the means ± SD (**E** and **F**). $n = 9$ mice per group (**E** and **F**). *$P < 0.05$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to PBS; $^{\dagger\dagger}P < 0.01$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to L6; $^{\ddagger\ddagger\ddagger}P < 0.001$ PBS versus L6 (analysis of variance). Scale bars = 100 μm (**A**–**D**).

dehydrogenase (*GAPDH*; Mm99999915_g1). *GAPDH* was detected as the reference gene. The ΔΔ cycle threshold (C_t) method was used to calculate the fold change in mRNA expression: $\Delta C_t = C_t$ (target gene) − C_t (reference gene), $\Delta\Delta C_t = \Delta C_t$ (treatment) − ΔC_t (control), and fold change = $2^{(-\Delta\Delta Ct)}$.

Immunoblot Assays

The lung protein lysates were obtained on PND5 using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnologies, Dallas, TX; sc-24948), separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with primary antibodies against: β-actin (Santa Cruz Biotechnologies; sc-47778; dilution 1:5000), STAT1 (Cell Signaling Technology, Danvers, MA; 9172; dilution 1:1000), phosphorylated (p)-STAT1 [STAT1(Tyr701); Cell Signaling Technology; 7649; dilution 1:1000], STAT3 (Cell Signaling Technology; 12640; dilution 1:1000), and p-STAT3 [STAT3(Ser727); Cell Signaling Technology; 9134; dilution 1:1000]. The primary antibodies were detected by incubation with appropriate horseradish peroxidase−conjugated secondary antibodies. The immunoreactive bands were detected by chemiluminescence methods, and the band densities were quantified using Image Lab software version 1.80 (Chemidoc touch imaging system; Bio-Rad Laboratories, Inc., Hercules, CA).[28]

Statistical Analysis

GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) was used to analyze the results, and the data are expressed as means ± SD. At least two separate experiments were performed to determine alveolarization and pulmonary vascularization on PND7. $P < 0.05$ was considered significant. The effects of the gene, exposure, and their associated interactions on outcome variables were analyzed using analysis of variance. Multiple comparison testing by the post hoc Bonferroni test was performed if the statistical significance of either variable or interaction was noted by analysis of variance.

Results

LPS Exposure Transiently Increases Adm mRNA Levels in Saccular Murine Lungs

The dose- and time-dependent effects of LPS on *Adm* signaling in saccular murine lungs were investigated. Because of the absence of reliable antibodies to detect *Adm* or its signaling receptors, Calcrl and Ramp2, in murine lungs, the analyses of the LPS effects on *Adm* signaling in neonatal lungs were primarily based on real-time RT-PCR analyses. One-time LPS administration increased Adm mRNA expression (3 mg/kg of LPS, 2 ± 0.1; 6 mg/kg of LPS, 1.8 ± 0.1; 10 mg/kg of LPS, 1.6 ± 0.3; and phosphate-buffered saline, 0.9 ± 0.2;

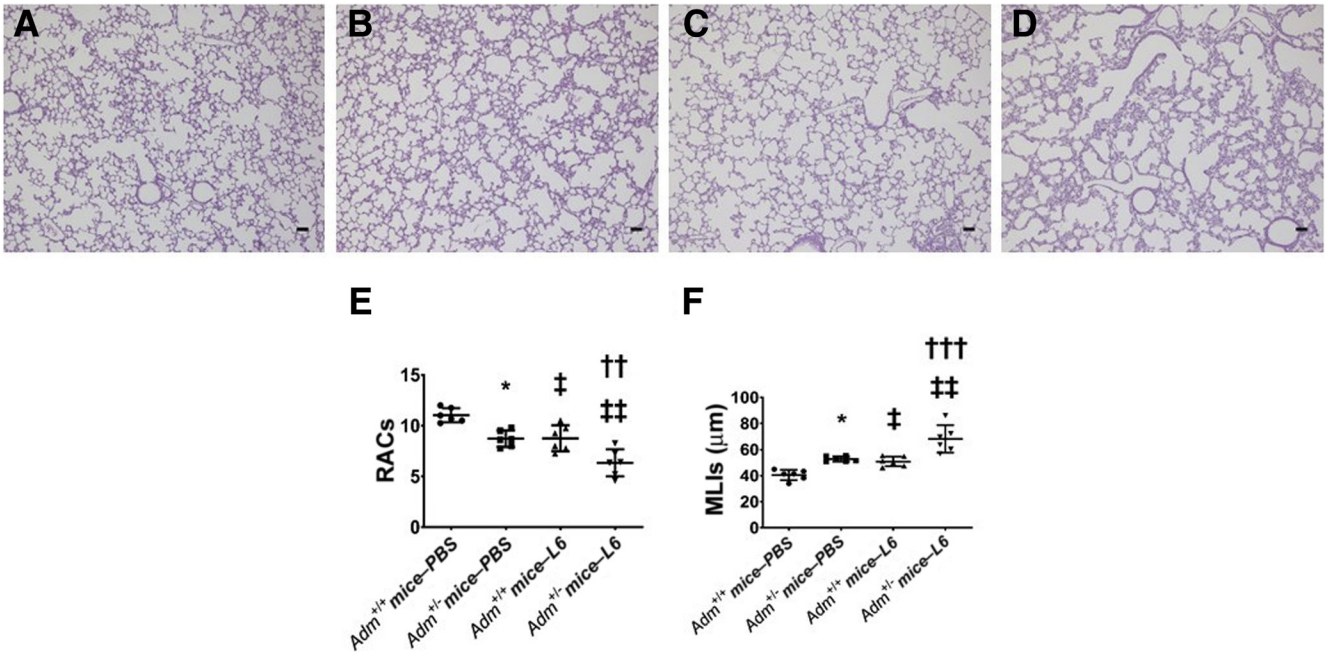

Figure 4 Adrenomedullin (*Adm*) deficiency impairs resolution of lipopolysaccharide (LPS)—induced alveolar simplification. $Adm^{+/-}$ mice and their wild-type littermates ($Adm^{+/+}$) were treated intraperitoneally with phosphate-buffered saline (PBS) or 6 mg/kg of LPS (L6) on postnatal days (PNDs) 3 to 5, and their lung development was quantified on PND14. Representative hematoxylin and eosin—stained lung sections from $Adm^{+/+}$ (**A** and **C**) and $Adm^{+/-}$ (**B** and **D**) mice exposed to PBS (**A** and **B**) or L6 (**C** and **D**). Alveolarization was determined by radial alveolar counts (RACs; **E**) and mean linear intercepts (MLIs; **F**). Values are presented as the means ± SD (**E** and **F**). $n = 6$ mice per group (**E** and **F**). $^*P < 0.05$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to PBS; $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to L6; $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$ PBS versus L6 groups (analysis of variance). Scale bars = 100 μm (**A**—**D**).

$P < 0.001$) (Figure 1A), but not Calcrl (Figure 1B) or *Ramp2* (Figure 1C) mRNA expression on PND3. No dose-dependent effect of LPS on the Adm mRNA levels was observed within the range of LPS doses used in the study (Figure 1A). Although a single dose of LPS increased Adm mRNA expression, repeated doses of LPS failed to increase either Adm mRNA levels (Figure 1D) or the mRNA levels of its signaling receptors, *Calcrl* (Figure 1E) and *Ramp2* (Figure 1F), as determined by real-time PCR analyses on PND5. On the basis of recent findings,[28] the LPS dose of 6 mg/kg was selected for the remainder of the studies to produce a robust model of moderate lung injury, which is clinically relevant and important to identify meaningful strategies for managing infants with significant BPD. Next, the effect of LPS on the pulmonary Adm mRNA levels in *Adm* haplodeficient ($Adm^{+/-}$) mice was evaluated. On LPS exposure, the pulmonary Adm mRNA levels continued to be significantly lower in $Adm^{+/-}$ than in their wild-type littermates ($Adm^{+/+}$) (Figure 2).

Adm Deficiency Potentiates LPS-Induced Alveolar Simplification in Neonatal Mice

Alveolar development was determined by RAC and MLI measurements on PND7 or PND14. Consistent with the recent report,[33] *Adm*-deficient neonatal mice had decreased alveolarization at baseline (Figures 3 and 4). LPS exposure decreased alveolar development (ie, alveolar simplification) on PND7, as evidenced by decreased RACs (Figure 3, A–E) and increased MLIs (Figure 3, A–D and F) in LPS-treated mice compared with vehicle-treated mice. However, the extent of alveolar simplification was significantly greater in *Adm*-deficient mice than *Adm*-sufficient mice (Figure 3, E and F). Alveolarization at PND14 was estimated in mice exposed to vehicle or LPS on PNDs 3 through 5, to determine whether the LPS effects on alveolarization were transient or persistent. Although LPS-exposed *Adm*-sufficient mouse lungs showed a modest increase in RACs (Figure 4, A, C, and E) and decrease in MLIs (Figure 4, A, C, and F), LPS-exposed *Adm*-deficient mouse lungs continued to have a significant decrease in RACs (Figure 4, B, D, and E) and increase in MLIs (Figure 4, B, D, and F) at PND14, indicating that *Adm* may be necessary to recover from LPS-induced inflammatory lung injury.

Pharmacologic Inhibition of *Adm* Signaling Potentiates LPS-Induced Alveolar Simplification in Neonatal Mice

AM_{22-52} is a Calcrl-Ramp2 receptor complex antagonist and is widely used to inhibit *Adm* signaling. Therefore, this compound was used to determine the effects of pharmacologic inhibition of *Adm* signaling on alveolar development at PND7. Consistent with a recent study[33] and the findings in *Adm*-deficient neonatal mice, exposure of neonatal WT mice to AM_{22-52} decreased alveolarization at baseline

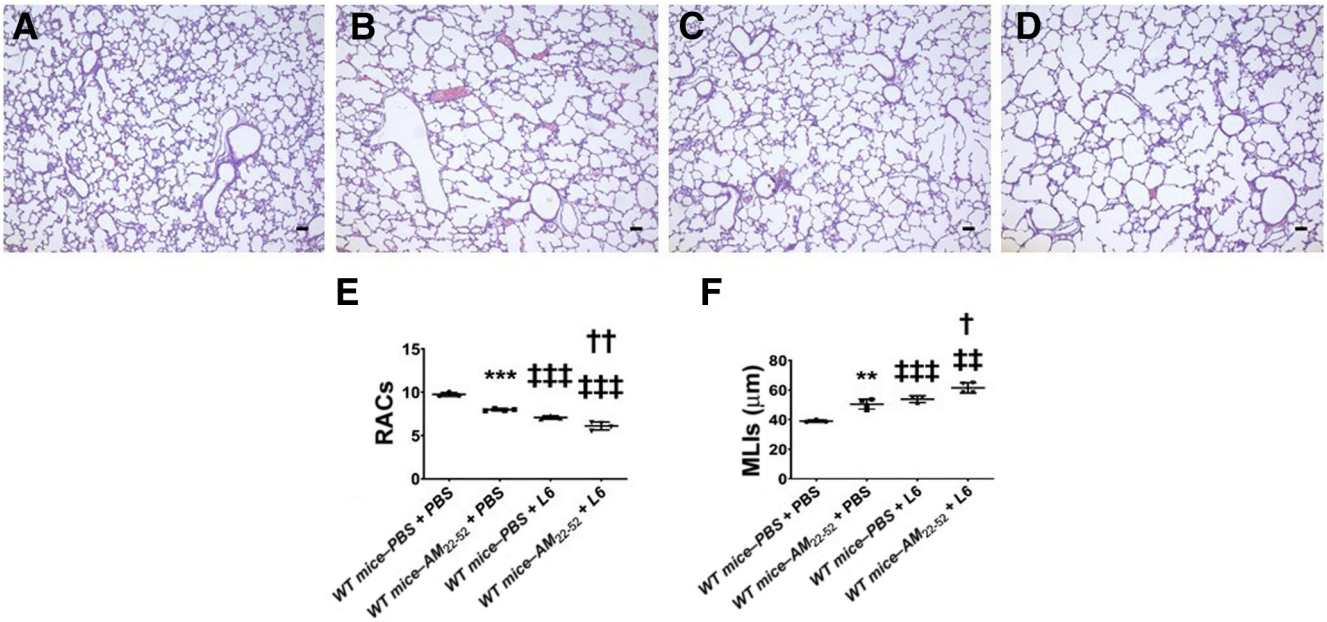

Figure 5 Adrenomedullin (*Adm*) antagonist potentiates lipopolysaccharide (LPS)–induced alveolar simplification. C57BL/6J wild-type (WT) mice were treated intraperitoneally with phosphate-buffered saline (PBS) or 100 μg/kg of the *Adm* antagonist, AM_{22-52}, once daily through postnatal days (PNDs) 1 through 7, while they were exposed to i.p. treatments with PBS or 6 mg/kg of LPS (L6) daily on PNDs 3 to 5. Lung development was quantified on PND7. Representative hematoxylin and eosin–stained lung sections from PBS (**A** and **C**) and AM_{22-52} (**B** and **D**) treated mice exposed to PBS (**A** and **B**) or L6 (**C** and **D**). Alveolarization was determined by radial alveolar counts (RACs; **E**) and mean linear intercepts (MLIs; **F**). Values are presented as the means ± SD (**E** and **F**). $n = 3$ to 4 mice per group (**E** and **F**). $^{**}P < 0.01$, $^{***}P < 0.001$ PBS versus AM_{22-52} treated mice exposed to PBS; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ PBS versus AM_{22-52} treated mice exposed to L6; $^{\ddagger\ddagger}P < 0.01$, $^{\ddagger\ddagger\ddagger}P < 0.001$ PBS versus L6 groups (analysis of variance). Scale bars = 100 μm (**A**–**D**).

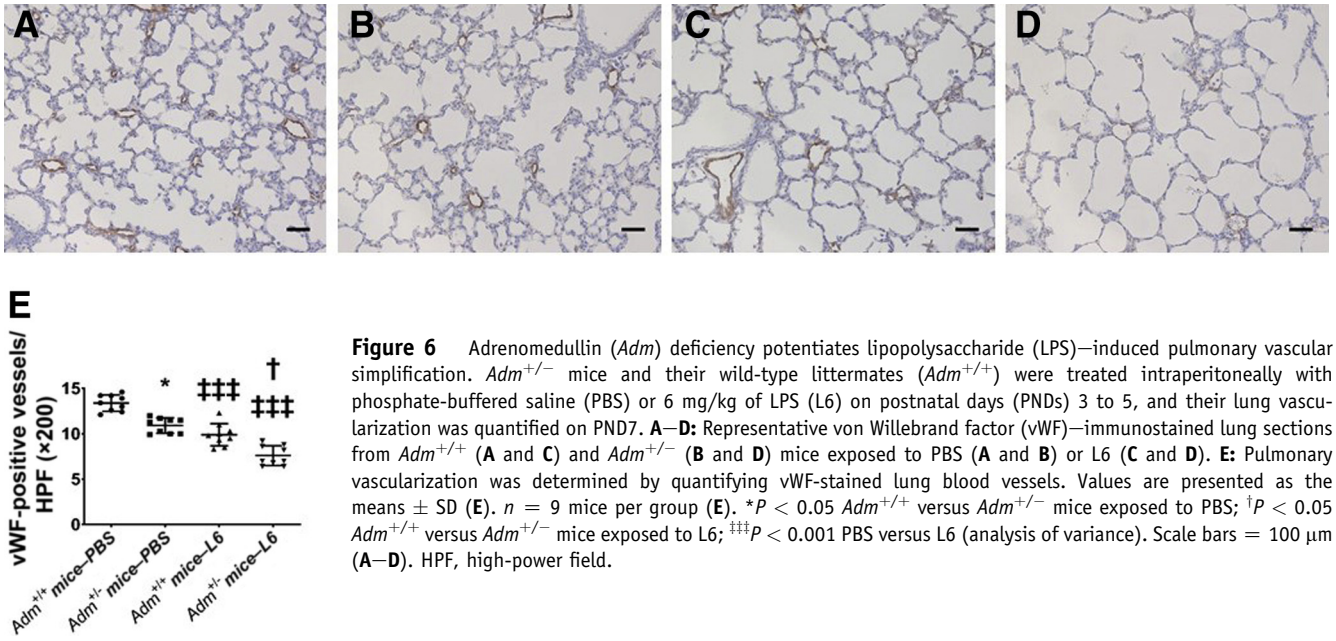

Figure 6 Adrenomedullin (*Adm*) deficiency potentiates lipopolysaccharide (LPS)—induced pulmonary vascular simplification. $Adm^{+/-}$ mice and their wild-type littermates ($Adm^{+/+}$) were treated intraperitoneally with phosphate-buffered saline (PBS) or 6 mg/kg of LPS (L6) on postnatal days (PNDs) 3 to 5, and their lung vascularization was quantified on PND7. **A**—**D:** Representative von Willebrand factor (vWF)—immunostained lung sections from $Adm^{+/+}$ (**A** and **C**) and $Adm^{+/-}$ (**B** and **D**) mice exposed to PBS (**A** and **B**) or L6 (**C** and **D**). **E:** Pulmonary vascularization was determined by quantifying vWF-stained lung blood vessels. Values are presented as the means ± SD (**E**). $n = 9$ mice per group (**E**). $^{*}P < 0.05$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to PBS; $^{\dagger}P < 0.05$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to L6; $^{\ddagger\ddagger\ddagger}P < 0.001$ PBS versus L6 (analysis of variance). Scale bars = 100 μm (**A**—**D**). HPF, high-power field.

(Figure 5). LPS exposure decreased alveolar development (ie, alveolar simplification), as evidenced by decreased RACs (Figure 5, A—E) and increased MLIs (Figure 5, A—D and F) in LPS-treated mice compared with vehicle-treated mice. However, the extent of alveolar simplification was significantly greater in mice treated with the *Adm* receptor antagonist, AM_{22-52} (Figure 5, E and F).

Adm Deficiency Potentiates LPS-Induced Pulmonary Vascular Simplification in Neonatal Mice

The observation of detrimental effects of *Adm* deficiency on LPS-induced alveolar development was followed by the evaluation of whether *Adm* deficiency caused a similar effect on LPS-induced pulmonary vascular simplification. Pulmonary vascularization was determined by quantifying vWF-stained pulmonary blood vessels on PND7 or PND14. Consistent with the recent study,[33] *Adm*-deficient neonatal mice had decreased pulmonary vascularization at baseline (Figures 6 and 7, A, B, and E). LPS exposure decreased pulmonary vascularization (ie, pulmonary vascular simplification) on PND7, as evidenced by decreased vWF-stained pulmonary blood vessels in LPS-treated mice compared with vehicle-treated mice (Figure 6). However, the extent of pulmonary vascular simplification was significantly greater in *Adm*-deficient

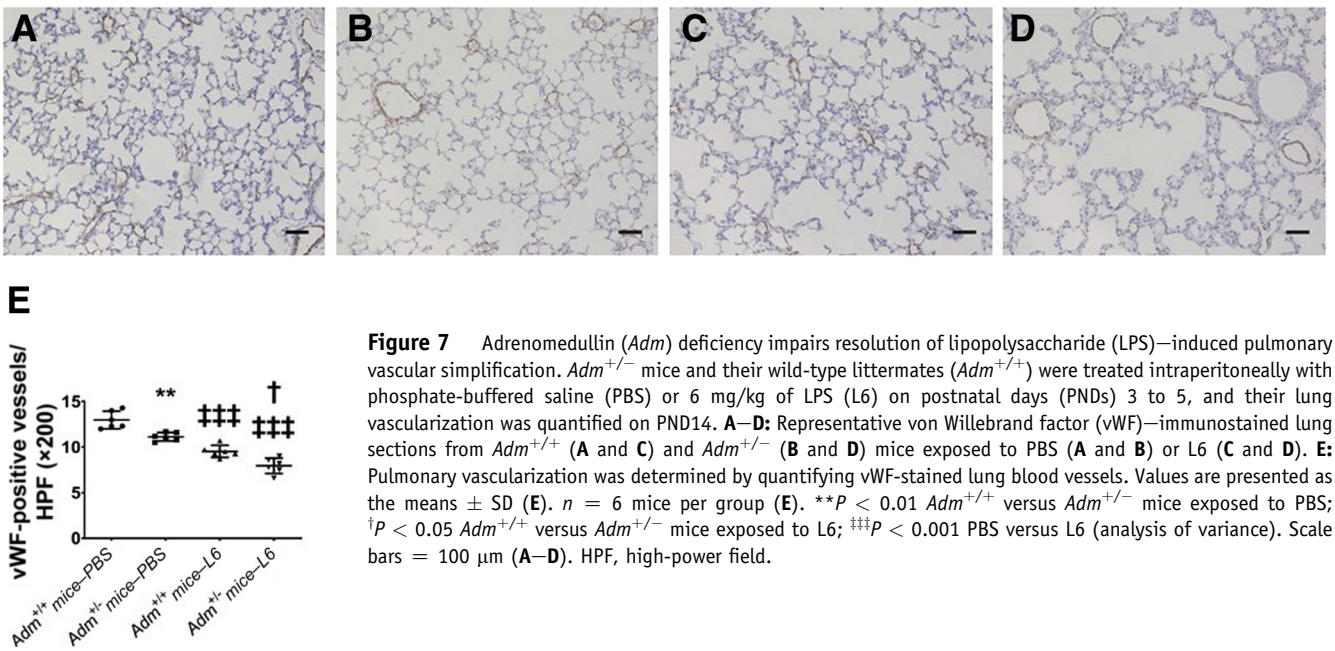

Figure 7 Adrenomedullin (*Adm*) deficiency impairs resolution of lipopolysaccharide (LPS)—induced pulmonary vascular simplification. $Adm^{+/-}$ mice and their wild-type littermates ($Adm^{+/+}$) were treated intraperitoneally with phosphate-buffered saline (PBS) or 6 mg/kg of LPS (L6) on postnatal days (PNDs) 3 to 5, and their lung vascularization was quantified on PND14. **A**—**D:** Representative von Willebrand factor (vWF)—immunostained lung sections from $Adm^{+/+}$ (**A** and **C**) and $Adm^{+/-}$ (**B** and **D**) mice exposed to PBS (**A** and **B**) or L6 (**C** and **D**). **E:** Pulmonary vascularization was determined by quantifying vWF-stained lung blood vessels. Values are presented as the means ± SD (**E**). $n = 6$ mice per group (**E**). $^{**}P < 0.01$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to PBS; $^{\dagger}P < 0.05$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to L6; $^{\ddagger\ddagger\ddagger}P < 0.001$ PBS versus L6 (analysis of variance). Scale bars = 100 μm (**A**—**D**). HPF, high-power field.

mice than in *Adm*-sufficient mice (Figure 6). To determine whether the LPS effects on pulmonary vascular simplification are transient or persistent, pulmonary vascularization was estimated at PND14 in mice exposed to vehicle or LPS on PNDs 3 through 5. Although LPS-exposed *Adm*-sufficient mouse lungs showed a modest increase in vWF-stained pulmonary blood vessels (Figure 7, A, C, and E), LPS-exposed *Adm*-deficient mouse lungs continued to have a significant decrease in vWF-stained pulmonary blood vessels at PND14 (Figure 7, B, D, and E), indicating that *Adm* may be necessary to recover from LPS-induced inflammatory lung injury.

Pharmacologic Inhibition of *Adm* Signaling Potentiates LPS-Induced Pulmonary Vascular Simplification in Neonatal Mice

The next set of experiments evaluated whether pharmacologic inhibition of *Adm* signaling caused a similar effect on LPS-induced pulmonary vascular simplification. Pulmonary vascularization was determined by quantifying vWF-stained pulmonary blood vessels on PND7. Consistent with the recent study[33] and the current findings in *Adm*-deficient neonatal mice, exposure of neonatal WT mice to AM_{22-52} decreased pulmonary vascularization at baseline (Figure 8). LPS exposure decreased pulmonary vascularization (ie, pulmonary vascular simplification) on PND7, as evidenced by decreased vWF-stained pulmonary blood vessels in LPS-treated mice compared with vehicle-treated mice (Figure 8). However, the extent of pulmonary vascular simplification was significantly greater in mice treated with the *Adm* receptor antagonist, AM_{22-52} (Figure 8).

Adm Deficiency Potentiates LPS-Induced Pulmonary Inflammation in Neonatal Mice

Lung inflammation is an important and final common mediator of lung injury, leading to the development of BPD. Therefore, the current model was used to quantify lung inflammation to determine the mechanisms through which *Adm* signaling deficiency augments neonatal lung injury. The extent of lung inflammation was determined by quantifying the production of the proinflammatory cytokines *CCL2*, *CCL3*, *CXCL1*, *ICAM-1*, *IL-1β*, and *TNF-α* in the lung tissues by real-time RT-PCR. Consistent with the prior study,[28] LPS increased the expression of *CCL2* (Figure 9A), *CCL3* (Figure 9B), *CXCL1* (Figure 9C), *IL-1β* (Figure 9E), and *TNF-α* (Figure 9F) mRNA levels between 3.4-fold and 14.3-fold, but did not affect *ICAM-1* (Figure 9D) mRNA levels in the saccular lungs of *Adm*-sufficient mice. However, the extent of LPS-induced inflammation was significantly augmented in *Adm*-deficient mice, wherein LPS not only increased the expression of *CCL2* (Figure 9A), *CCL3* (Figure 9B), *CXCL1* (Figure 9C), *IL-1β* (Figure 9E), and *TNF-α* (Figure 9F) mRNA levels between 8.8-fold and 37.6-fold (1.6-fold to 10-fold higher than *Adm*-sufficient mice), but also increased *ICAM-1* (Figure 9D) mRNA levels by 4.8-fold.

Finally, transcription factors that regulate inflammation were investigated. In alignment with the previous study,[28] LPS activated STAT3, but not STAT1, in the saccular lungs of *Adm*-sufficient mice. The p-STAT3/total STAT3 ratio increased by 18.5-fold in the LPS group (Figure 9, G and I). However, in *Adm*-deficient mice, LPS not only increased the expression of p-STAT3/total STAT3 ratio by 27.5-fold (Figure 9, G and I), but also increased p-STAT1/total STAT1 by 6.4-fold (Figure 9, G and H), suggesting that *Adm* may regulate lung inflammation via these transcription factors.

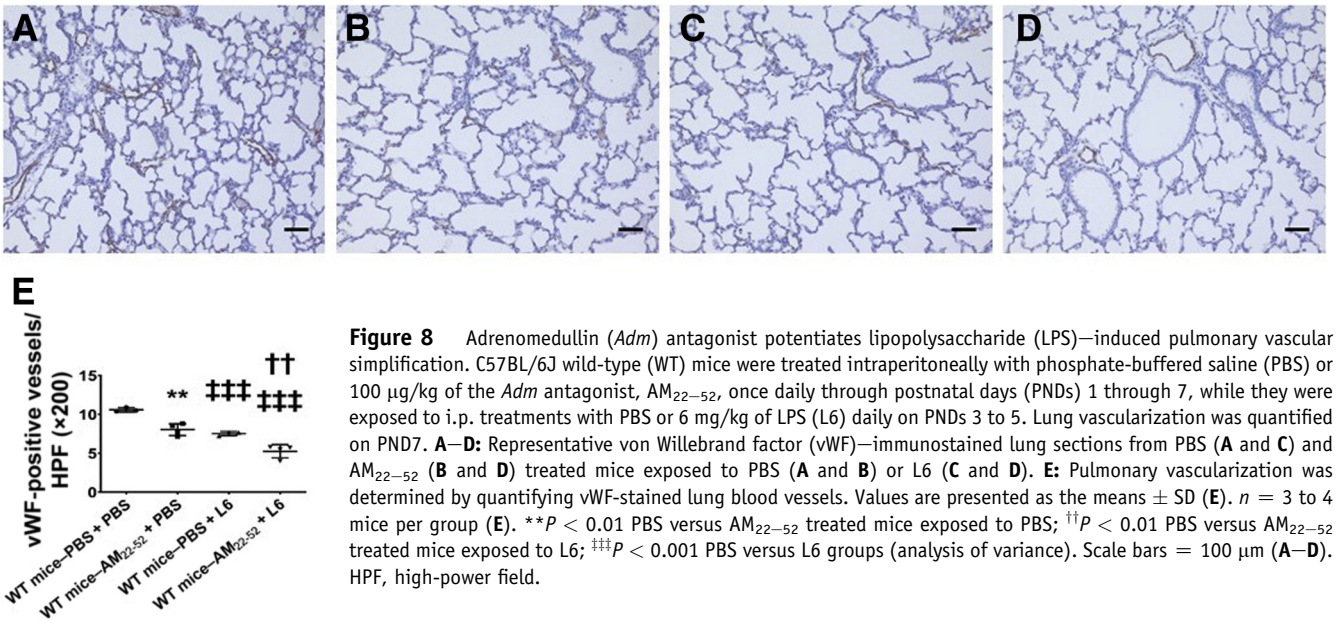

Figure 8 Adrenomedullin (*Adm*) antagonist potentiates lipopolysaccharide (LPS)—induced pulmonary vascular simplification. C57BL/6J wild-type (WT) mice were treated intraperitoneally with phosphate-buffered saline (PBS) or 100 μg/kg of the *Adm* antagonist, AM_{22-52}, once daily through postnatal days (PNDs) 1 through 7, while they were exposed to i.p. treatments with PBS or 6 mg/kg of LPS (L6) daily on PNDs 3 to 5. Lung vascularization was quantified on PND7. **A—D:** Representative von Willebrand factor (vWF)—immunostained lung sections from PBS (**A** and **C**) and AM_{22-52} (**B** and **D**) treated mice exposed to PBS (**A** and **B**) or L6 (**C** and **D**). **E:** Pulmonary vascularization was determined by quantifying vWF-stained lung blood vessels. Values are presented as the means ± SD (**E**). $n = 3$ to 4 mice per group (**E**). $^{**}P < 0.01$ PBS versus AM_{22-52} treated mice exposed to PBS; $^{\dagger\dagger}P < 0.01$ PBS versus AM_{22-52} treated mice exposed to L6; $^{\ddagger\ddagger\ddagger}P < 0.001$ PBS versus L6 groups (analysis of variance). Scale bars = 100 μm (**A—D**). HPF, high-power field.

Pharmacologic Inhibition of *Adm* Signaling Potentiates LPS-Induced Pulmonary Inflammation in Neonatal Mice

To determine if pharmacologic and genetic inhibition of *Adm* signaling have similar effects on lung inflammation, the extent of lung inflammation in AM_{22-52}—treated WT mice was determined by quantifying the production of the proinflammatory cytokines in the lung tissues by real-time RT-PCR analyses. Consistent with the findings in *Adm*-deficient neonatal mice, the extent of LPS-induced inflammation was significantly augmented in neonatal mice treated with the *Adm* receptor antagonist, AM_{22-52}. Herein, LPS-induced expression of *CCL2* (Figure 10A) and *TNF-α* (Figure 10C) mRNA levels were significantly greater in AM_{22-52}—treated mice than in vehicle-treated mice. Furthermore, the effect of *Adm* inhibition on *ICAM-1* gene expression in LPS-treated mice was consistent, wherein LPS increased *ICAM-1* mRNA levels only when *Adm* signaling was inhibited by AM_{22-52} (Figure 10B).

Discussion

The present study examined the immediate and intermediate effects of *Adm* gene and LPS exposure and the interactions between them during the saccular lung developmental stage on alveolarization, pulmonary vascularization, and lung inflammation in mice. The findings demonstrate that *Adm* deficiency in neonatal mice potentiates and impairs the resolution of LPS-induced lung injury. Furthermore, data show that *Adm* deficiency potentiates LPS-induced inflammation and specifically activates STAT1 in saccular lungs. Finally, data show that pharmacologic *Adm* signaling inhibition produces similar effects to genetic inhibition in LPS-exposed neonatal mice.

Adm attenuates tissue inflammation and injury in animal models of sepsis. Furthermore, this peptide is elevated in animal models[34–36] and humans[37] with sepsis. Therefore, initially, the effects of LPS on the expression of *Adm* and its receptors in WT mice were elucidated. Consistent with the above studies, LPS increased *Adm* expression in neonatal murine lungs. However, this effect was transient, indicating that neonatal murine lungs cannot mount a sustained protective response when exposed to inflammatory stimuli. More importantly, chronic LPS exposure did not increase *Adm* mRNA levels in these transgenic mice. LPS has been shown to decrease the expression of *Adm* signaling coreceptors, *Calcrl* and *Ramp2*.[34,38] In contrast, LPS did not decrease the *Adm* receptor levels in the current model. The discrepant findings may be due to the differences in the mouse age, tissue, or cell type, and LPS dose.

Sepsis increases the odds of developing BPD,[11,12] and microbial products, such as LPS, disrupt lung development.[28,39–41] Likewise, the RAC was decreased, and the

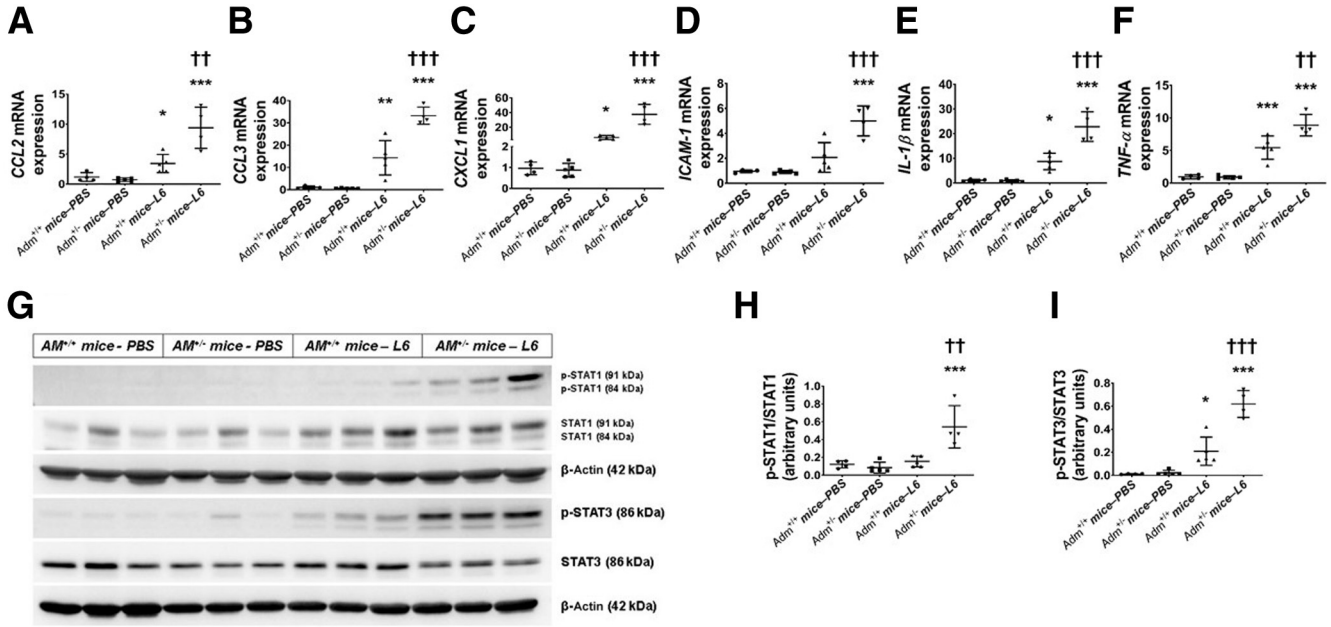

Figure 9 Adrenomedullin (*Adm*) deficiency potentiates lipopolysaccharide (LPS)—induced lung inflammation. $Adm^{+/-}$ mice and their wild-type littermates ($Adm^{+/+}$) were treated intraperitoneally with phosphate-buffered saline (PBS) or 6 mg/kg of LPS (L6) on postnatal days (PNDs) 3 to 5, and their lung inflammation was quantified on PND5. **A**—**F:** Real-time RT-PCR analyses-based determination of *CCL2* (**A**), *CCL3* (**B**), *CXCL1* (**C**), *ICAM-1* (**D**), *IL-1β* (**E**), and *TNF-α* (**F**) mRNA expression levels. **G:** Determination of phosphorylated STAT1 (p-STAT1), STAT1, phosphorylated STAT3 (p-STAT3), and STAT3 protein levels by immunoblotting. **H** and **I:** Quantification and normalization of p-STAT1 (**H**) and p-STAT3 (**I**) band intensities to those of STAT1 and STAT3, respectively. Values are presented as the means ± SD (**A**—**F**, **H**, and **I**). $n = 4$ to 5 mice per group (**A**—**F**, **H**, and **I**). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ PBS versus L6; $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ $Adm^{+/+}$ versus $Adm^{+/-}$ mice exposed to L6 (analysis of variance).

MLI was increased in LPS-exposed mice at PND7. LPS also decreased the pulmonary vasculature. Furthermore, genetic and pharmacologic inhibition of *Adm* signaling *in vivo* potentiated these LPS effects, highlighting the protective role of *Adm* in the initiation of LPS-induced alveolar and pulmonary vascular simplification. *Adm* and its receptors are co-expressed primarily in endothelial and epithelial cells of the lungs.[42,43] Increased expression of *Adm* signaling components in these cells that modulate proliferation and differentiation combined with increased *Adm* expression during alveolarization[44–46] indicate that *Adm* regulates lung development, injury, and repair. Consistent with this notion, *Adm* regenerates alveoli and vasculature in rodent models of hyperoxic lung injury[30] and pulmonary emphysema.[47] The current study provides further evidence that *Adm* also protects neonatal lungs against LPS-induced injury.

The longitudinal course of chronic LPS-induced neonatal lung injury is unknown. Consequently, the study investigated the effects of chronic LPS exposure on lung development at PND14. The RAC and pulmonary vasculature increased, and the MLI decreased, at PND14 in *Adm*-sufficient mice exposed to LPS, indicating that WT mice can substantially recover from LPS-induced developmental lung injury. By contrast, *Adm*-deficient mice exposed to LPS continued to display significant alveolar and pulmonary vascular simplification, emphasizing that *Adm* promotes lung repair, a concept supported by other investigators.[30,48] These findings indicate that low-dose LPS exposure in WT mice causes a transient perturbation in lung development without overwhelming the lung reparative homeostasis. However, when protective and/or reparative molecules, such as *Adm*, are deficient, it can cause severe and persistent lung developmental deficits.

Lung inflammation is a hallmark of BPD. Therefore, the extent of lung inflammation was next examined by quantifying the proinflammatory cytokines in the lung tissues. Consistent with the prior study,[28] LPS increased the expression of *CCL2*, *CCL3*, *CXCL1*, *IL-1β*, and *TNF-α* mRNA levels, but had no effect on *ICAM-1* mRNA levels in the saccular lungs of *Adm*-sufficient mice. However, the extent of LPS-induced inflammation was significantly augmented in *Adm*-deficient mice, wherein LPS increased the expression of all these chemokines/cytokines, including *ICAM-1*, severalfold higher than in *Adm*-sufficient mice. Furthermore, similar effects were observed in neonatal WT mice treated with the *Adm* receptor antagonist, AM_{22-52}, and exposed to LPS. These findings are congruent with those of other investigators,[23,34] indicating that *Adm* exerts potent anti-inflammatory effects in lung tissues. However, the current study differs from others in two aspects: first, it used a chronic LPS-exposure model; and second, it determined the effects of *Adm* deficiency in the developing lungs.

Transcription factors, such as STAT, modulate inflammation and play a key role in BPD pathogenesis.[49] STAT3 regulates cell proliferation during the development, injury, and repair of organs[50–52] as well as inflammation,[53,54] the biological processes that play a major role in the pathogenesis of BPD. For instance, STAT3 activation increases pulmonary vascularization,[55] which is critical for healthy lung development. Furthermore, *Adm* decreases STAT1 and STAT3 activation in animal models of inflammatory bowel disease.[56,57] Therefore, the effects of *Adm* gene on STAT signaling were investigated. In alignment with the previous study,[28] LPS activated STAT3, but not STAT1, in the saccular lungs of *Adm*-sufficient mice. However, in *Adm*-deficient mice, LPS activated both STAT1 and STAT3, severalfold higher than in *Adm*-sufficient mice, indicating that *Adm* modulates LPS-induced lung injury via STAT1 and STAT3. The molecular mechanisms responsible for

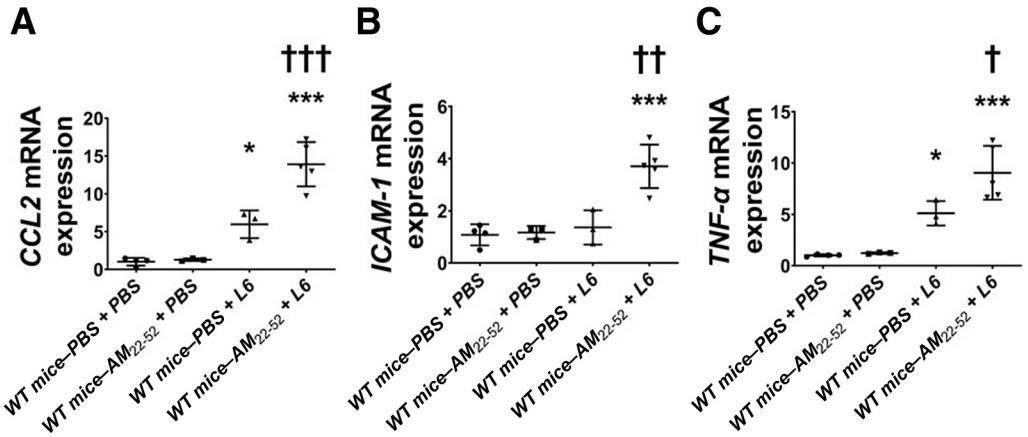

Figure 10 Adrenomedullin (*Adm*) antagonist potentiates lipopolysaccharide (LPS)–induced lung inflammation. C57BL/6J wild-type (WT) mice were treated intraperitoneally with phosphate-buffered saline (PBS) or 100 μg/kg of the *Adm* antagonist, AM_{22-52}, once daily through postnatal days (PNDs) 1 through 5, while they were exposed to i.p. treatments with PBS or 6 mg/kg of LPS (L6) daily on PNDs 3 to 5. Lung inflammation was quantified on PND5. Real-time RT-PCR analyses-based determination of *CCL2* (**A**), *ICAM-1* (**B**), and *TNF-α* (**C**) mRNA expression levels. Values are presented as the means ± SD (**A**–**C**). $n = 3$ to 4 mice per group (**A**–**C**). $^{*}P < 0.05$, $^{***}P < 0.001$ PBS versus L6; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ PBS versus AM_{22-52} treated mice exposed to L6 (analysis of variance).

these differences are currently unclear, and future mechanistic studies using transgenic mice are necessary to determine the interactions between *Adm* and these STATs and their effects on lung inflammation and development.

The strengths of the current study are that: it examined the immediate and delayed effects of chronic LPS exposure on lung development, which has a high translational significance; and it utilized a robust transgenic approach and a pharmacologic approach to identify the role of *Adm* in LPS-mediated developmental lung injury. However, this study has some limitations. It did not elucidate sex- or cell-specific effects of *Adm* and the impact of *Adm* deficiency on lung or pulmonary vascular function. It also did not elucidate the exact molecular mechanisms through which *Adm* deficiency potentiates inflammatory injury in the developing lungs. These will be addressed in future studies.

In summary, this is the first study, to our knowledge, that characterized the effects of *Adm* deficiency on LPS-exposed developing lungs and identified potential mechanisms whereby *Adm* regulates lung inflammation. Specifically, it demonstrated that *Adm* deficiency potentiates LPS-induced alveolar and pulmonary vascular simplification, and delays recovery from LPS-induced lung developmental deficits. Furthermore, it showed that the *Adm*-deficient lungs mount a robust inflammatory response by activating STAT1 and STAT3 pathways. These findings have significant implications for the development of therapeutic targets for sepsis-induced BPD in infants.

Acknowledgments

We thank Dr. Kathleen M. Caron (University of North Carolina at Chapel Hill) for providing the $Adm^{+/-}$ mice and for insightful suggestions; and Pamela Parsons for timely processing of histopathology slides.

References

1. McEvoy CT, Jain L, Schmidt B, Abman S, Bancalari E, Aschner JL: Bronchopulmonary dysplasia: NHLBI Workshop on the Primary Prevention of Chronic Lung Diseases. Ann Am Thorac Soc 2014, 11 Suppl 3:S146–S153
2. Jobe AJ: The new BPD: an arrest of lung development. Pediatr Res 1999, 46:641–643
3. Islam JY, Keller RL, Aschner JL, Hartert TV, Moore PE: Understanding the short- and long-term respiratory outcomes of prematurity and bronchopulmonary dysplasia. Am J Respir Crit Care Med 2015, 192:134–156
4. Balany J, Bhandari V: Understanding the impact of infection, inflammation, and their persistence in the pathogenesis of bronchopulmonary dysplasia. Front Med 2015, 2:90
5. Kallapur SG, Jobe AH: Contribution of inflammation to lung injury and development. Arch Dis Child Fetal Neonatal Ed 2006, 91: F132–F135
6. Stenmark KR, Abman SH: Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia. Annu Rev Physiol 2005, 67:623–661
7. Thebaud B, Abman SH: Bronchopulmonary dysplasia: where have all the vessels gone? roles of angiogenic growth factors in chronic lung disease. Am J Respir Crit Care Med 2007, 175: 978–985
8. Aslam M, Baveja R, Liang OD, Fernandez-Gonzalez A, Lee C, Mitsialis SA, Kourembanas S: Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. Am J Respir Crit Care Med 2009, 180:1122–1130
9. Chen S, Rong M, Platteau A, Hehre D, Smith H, Ruiz P, Whitsett J, Bancalari E, Wu S: CTGF disrupts alveolarization and induces pulmonary hypertension in neonatal mice: implication in the pathogenesis of severe bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 2011, 300:L330–L340
10. Marshall DD, Kotelchuck M, Young TE, Bose CL, Kruyer L, O'Shea TM; North Carolina Neonatologists Association: Risk factors for chronic lung disease in the surfactant era: a North Carolina population-based study of very low birth weight infants. Pediatrics 1999, 104:1345–1350
11. Lahra MM, Beeby PJ, Jeffery HE: Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study. Pediatrics 2009, 123:1314–1319
12. Klinger G, Levy I, Sirota L, Boyko V, Lerner-Geva L, Reichman B: Outcome of early-onset sepsis in a national cohort of very low birth weight infants. Pediatrics 2010, 125:e736–e740
13. Ballard AR, Mallett LH, Pruszynski JE, Cantey JB: Chorioamnionitis and subsequent bronchopulmonary dysplasia in very-low-birth weight infants: a 25-year cohort. J Perinatol 2016, 36: 1045–1048
14. Jensen EA, Schmidt B: Epidemiology of bronchopulmonary dysplasia. Birth Defects Res A Clin Mol Teratol 2014, 100: 145–157
15. Imamura T, Sato M, Go H, Ogasawara K, Kanai Y, Maeda H, Chishiki M, Shimizu H, Mashiyama F, Goto A, Momoi N, Hosoya M: The microbiome of the lower respiratory tract in premature infants with and without severe bronchopulmonary dysplasia. Am J Perinatol 2017, 34:80–87
16. Lee SM, Chang M, Kim KS: Blood culture proven early onset sepsis and late onset sepsis in very-low-birth-weight infants in Korea. J Korean Med Sci 2015, 30 Suppl 1:S67–S74
17. Shah J, Jefferies AL, Yoon EW, Lee SK, Shah PS: Risk factors and outcomes of late-onset bacterial sepsis in preterm neonates born at < 32 weeks' gestation. Am J Perinatol 2015, 32: 675–682
18. Novitsky A, Tuttle D, Locke RG, Saiman L, Mackley A, Paul DA: Prolonged early antibiotic use and bronchopulmonary dysplasia in very low birth weight infants. Am J Perinatol 2015, 32:43–48
19. Hinson JP, Kapas S, Smith DM: Adrenomedullin, a multifunctional regulatory peptide. Endocr Rev 2000, 21:138–167
20. McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM: RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 1998, 393:333–339
21. Muller HC, Witzenrath M, Tschernig T, Gutbier B, Hippenstiel S, Santel A, Suttorp N, Rosseau S: Adrenomedullin attenuates ventilator-induced lung injury in mice. Thorax 2010, 65:1077–1084
22. Dwivedi AJ, Wu R, Nguyen E, Higuchi S, Wang H, Krishnasastry K, Marini CP, Ravikumar TS, Wang P: Adrenomedullin and adrenomedullin binding protein-1 prevent acute lung injury after gut ischemia-reperfusion. J Am Coll Surg 2007, 205:284–293
23. Itoh T, Obata H, Murakami S, Hamada K, Kangawa K, Kimura H, Nagaya N: Adrenomedullin ameliorates lipopolysaccharide-induced acute lung injury in rats. Am J Physiol Lung Cell Mol Physiol 2007, 293:L446–L452
24. Talero E, Di Paola R, Mazzon E, Esposito E, Motilva V, Cuzzocrea S: Anti-inflammatory effects of adrenomedullin on acute lung injury induced by carrageenan in mice. Mediators Inflamm 2012, 2012:717851
25. Heine H, Rietschel ET, Ulmer AJ: The biology of endotoxin. Mol Biotechnol 2001, 19:279–296

26. Mannel DN: Advances in sepsis research derived from animal models. Int J Med Microbiol 2007, 297:393–400
27. Fink MP: Animal models of sepsis. Virulence 2014, 5:143–153
28. Shrestha AK, Bettini ML, Menon RT, Gopal VYN, Huang S, Edwards DP, Pammi M, Barrios R, Shivanna B: Consequences of early postnatal lipopolysaccharide exposure on developing lungs in mice. Am J Physiol Lung Cell Mol Physiol 2019, 316:L229–L244
29. Caron KM, Smithies O: Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional adrenomedullin gene. Proc Natl Acad Sci U S A 2001, 98:615–619
30. Vadivel A, Abozaid S, van Haaften T, Sawicka M, Eaton F, Chen M, Thebaud B: Adrenomedullin promotes lung angiogenesis, alveolar development, and repair. Am J Respir Cell Mol Biol 2010, 43: 152–160
31. Hussain S, Miyazawa R, Tomomasa T, Kaneko H, Takahashi A, Watanabe T, Arakawa H, Morikawa A: Possible involvement of adrenomedullin in lipopolysaccharide-induced small-intestinal motility changes in conscious rats. J Gastroenterol 2005, 40: 1123–1129
32. Reynolds CL, Zhang S, Shrestha AK, Barrios R, Shivanna B: Phenotypic assessment of pulmonary hypertension using high-resolution echocardiography is feasible in neonatal mice with experimental bronchopulmonary dysplasia and pulmonary hypertension: a step toward preventing chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 2016, 11:1597–1605
33. Menon RT, Shrestha AK, Reynolds CL, Barrios R, Caron KM, Shivanna B: Adrenomedullin is necessary to resolve hyperoxia-induced experimental bronchopulmonary dysplasia and pulmonary hypertension in mice. Am J Pathol 2020, 190:711–722
34. Dackor R, Caron K: Mice heterozygous for adrenomedullin exhibit a more extreme inflammatory response to endotoxin-induced septic shock. Peptides 2007, 28:2164–2170
35. Agorreta J, Zulueta JJ, Montuenga LM, Garayoa M: Adrenomedullin expression in a rat model of acute lung injury induced by hypoxia and LPS. Am J Physiol Lung Cell Mol Physiol 2005, 288: L536–L545
36. Cheung BMY, Hwang ISS, Li CYY, O W-S, Tsang KWT, Leung RYH, Kumana CR, Tang F: Increased adrenomedullin expression in lungs in endotoxaemia. J Endocrinol 2004, 181: 339–345
37. Chen YX, Li CS: The predictive value of adrenomedullin for development of severe sepsis and septic shock in emergency department. Biomed Res Int 2013, 2013:960101
38. Ono Y, Okano I, Kojima M, Okada K, Kangawa K: Decreased gene expression of adrenomedullin receptor in mouse lungs during sepsis. Biochem Biophys Res Commun 2000, 271:197–202
39. Hou Y, Liu M, Husted C, Chen C, Thiagarajan K, Johns JL, Rao SP, Alvira CM: Activation of the nuclear factor-kappaB pathway during postnatal lung inflammation preserves alveolarization by suppressing macrophage inflammatory protein-2. Am J Physiol Lung Cell Mol Physiol 2015, 309:L593–L604
40. Menden HL, Xia S, Mabry SM, Navarro A, Nyp MF, Sampath V: Nicotinamide adenine dinucleotide phosphate oxidase 2 regulates LPS-induced inflammation and alveolar remodeling in the developing lung. Am J Respir Cell Mol Biol 2016, 55:767–778
41. Collins JJ, Kuypers E, Nitsos I, Jane Pillow J, Polglase GR, Kemp MW, Newnham JP, Cleutjens JP, Frints SG, Kallapur SG, Jobe AH, Kramer BW: LPS-induced chorioamnionitis and antenatal corticosteroids modulate Shh signaling in the ovine fetal lung. Am J Physiol Lung Cell Mol Physiol 2012, 303:L778–L787
42. Marinoni E, Di Iorio R, Alo P, Villaccio B, Alberini A, Cosmi EV: Immunohistochemical localization of adrenomedullin in fetal and neonatal lung. Pediatr Res 1999, 45:282–285
43. Hagner S, Stahl U, Knoblauch B, McGregor GP, Lang RE: Calcitonin receptor-like receptor: identification and distribution in human peripheral tissues. Cell Tissue Res 2002, 310:41–50
44. Wong PF, O WS, Tang F: An ontogenic study of adrenomedullin gene expression in the rat lung, adrenal, kidney, and heart. Endocrine 2012, 41:256–265
45. Ramos CG, Sun X, Johnson EB, Nelson HE, Gonzalez Bosc LV: Adrenomedullin expression in the developing human fetal lung. J Investig Med 2014, 62:49–55
46. Franco-Montoya ML, Boucherat O, Thibault C, Chailley-Heu B, Incitti R, Delacourt C, Bourbon JR: Profiling target genes of FGF18 in the postnatal mouse lung: possible relevance for alveolar development. Physiol Genomics 2011, 43:1226–1240
47. Murakami S, Nagaya N, Itoh T, Iwase T, Fujisato T, Nishioka K, Hamada K, Kangawa K, Kimura H: Adrenomedullin regenerates alveoli and vasculature in elastase-induced pulmonary emphysema in mice. Am J Respir Crit Care Med 2005, 172:581–589
48. Hagner S, Welz H, Kicic A, Alrifai M, Marsh LM, Sutanto EN, Ling KM, Stick SM, Muller B, Weissmann N, Renz H: Suppression of adrenomedullin contributes to vascular leakage and altered epithelial repair during asthma. Allergy 2012, 67:998–1006
49. Park J, Hescott BJ, Slonim DK: Pathway centrality in protein interaction networks identifies putative functional mediating pathways in pulmonary disease. Sci Rep 2019, 9:5863
50. Dutzmann J, Daniel JM, Bauersachs J, Hilfiker-Kleiner D, Sedding DG: Emerging translational approaches to target STAT3 signalling and its impact on vascular disease. Cardiovasc Res 2015, 106:365–374
51. Takeda K, Akira S: Multi-functional roles of Stat3 revealed by conditional gene targeting. Arch Immunol Ther Exp (Warsz) 2001, 49:279–283
52. Thangaratnarajah C, Dinger K, Vohlen C, Klaudt C, Nawabi J, Lopez Garcia E, Kwapiszewska G, Dobner J, Nüsken KD, van Koningsbruggen-Rietschel S, von Hörsten S, Dötsch J, Alejandre Alcázar MA: Novel role of NPY in neuroimmune interaction and lung growth after intrauterine growth restriction. Am J Physiol Lung Cell Mol Physiol 2017, 313:L491–L506
53. Welte T, Zhang SS, Wang T, Zhang Z, Hesslein DG, Yin Z, Kano A, Iwamoto Y, Li E, Craft JE, Bothwell AL, Fikrig E, Koni PA, Flavell RA, Fu XY: STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. Proc Natl Acad Sci U S A 2003, 100: 1879–1884
54. Ikegami M, Whitsett JA, Martis PC, Weaver TE: Reversibility of lung inflammation caused by SP-B deficiency. Am J Physiol Lung Cell Mol Physiol 2005, 289:L962–L970
55. Pradhan A, Dunn A, Ustiyan V, Bolte C, Wang G, Whitsett JA, Zhang Y, Porollo A, Hu YC, Xiao R, Szafranski P, Shi D, Stankiewicz P, Kalin TV, Kalinichenko VV: The S52F FOXF1 mutation inhibits STAT3 signaling and causes alveolar capillary dysplasia. Am J Respir Crit Care Med 2019, 200:1045–1056
56. Ashizuka S, Inagaki-Ohara K, Kuwasako K, Kato J, Inatsu H, Kitamura K: Adrenomedullin treatment reduces intestinal inflammation and maintains epithelial barrier function in mice administered dextran sulphate sodium. Microbiol Immunol 2009, 53:573–581
57. Kinoshita Y, Arita S, Murazoe H, Kitamura K, Ashizuka S, Inagaki-Ohara K: Subcutaneously administered adrenomedullin exerts a potent therapeutic effect in a murine model of ulcerative colitis. Hum Cell 2019, 32:12–21