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ORIGINAL ARTICLE

Toll-like Receptor 3 Is a Therapeutic Target for Pulmonary Hypertension

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Abstract

Rationale: Pulmonary arterial hypertension (PAH) is characterized by vascular cell proliferation and endothelial cell apoptosis. TLR3 (Toll-like receptor 3) is a receptor for double-stranded RNA and has been recently implicated in vascular protection.

Objectives: To study the expression and role of TLR3 in PAH and to determine whether a TLR3 agonist reduces pulmonary hypertension in preclinical models.

Methods: Lung tissue and endothelial cells from patients with PAH were investigated by polymerase chain reaction, immunofluorescence, and apoptosis assays. $TLR3^{-/-}$ and $TLR3^{+/+}$ mice were exposed to chronic hypoxia and SU5416. Chronic hypoxia or chronic hypoxia/SU5416 rats were treated with the TLR3 agonist polyinosinic/polycytidylic acid (Poly[I:C]).

Measurements and Main Results: TLR3 expression was reduced in PAH patient lung tissue and endothelial cells, and $TLR3^{-/-}$ mice exhibited more severe pulmonary hypertension following exposure to chronic hypoxia/SU5416. TLR3 knockdown promoted double-stranded RNA signaling via other intracellular RNA receptors in endothelial cells. This was associated with greater susceptibility to apoptosis, a known driver of pulmonary vascular remodeling. Poly(I:C) increased TLR3 expression via IL-10 in rat endothelial cells. *In vivo*, high-dose Poly(I:C) reduced pulmonary hypertension in both rat models in proof-of-principle experiments. In addition, Poly(I:C) also reduced right ventricular failure in established pulmonary hypertension.

Conclusions: Our work identifies a novel role for TLR3 in PAH based on the findings that reduced expression of TLR3 contributes to endothelial apoptosis and pulmonary vascular remodeling.

Keywords: pulmonary hypertension; toll-like receptor 3; endothelial cell; double-stranded RNA; apoptosis

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At a Glance Commentary

Scientific Knowledge on the Subject: Endothelial cell apoptosis contributes to vascular remodeling in pulmonary hypertension. But the pathways causing endothelial cell apoptosis are only partially understood. Current concepts indicate a role for chronic inflammation in pulmonary hypertension, yet innate RNA recognition may also be a protective mechanism in the vasculature.

What This Study Adds to the Field: Here we demonstrate, for the first time, that TLR3 (Toll-like receptor 3) expression is reduced in lung tissue and endothelial cells from patients with pulmonary hypertension and show *in vitro* and *in vivo* that TLR3 deficiency increases susceptibility to apoptosis and pulmonary hypertension. We further show that the TLR3 agonist and double-stranded RNA polyinosinic/polycytidylic acid increases TLR3 expression and reduces established pulmonary hypertension. Our data suggest that this protective upregulation of TLR3 involves induction of IL-10. We propose that TLR3 expression is required for endothelial cell homeostasis and that restoring TLR3 signaling may be a novel avenue to complement existing treatment strategies in pulmonary hypertension.

Pulmonary arterial hypertension (PAH) is a severe and progressive disease of the pulmonary arterial bed and is characterized by occlusive remodeling of pulmonary arteries (1). Although current therapies improve quality of life and prognosis, PAH remains a life-limiting condition (2). One reason is that current treatment options do little to change the chronic pathology in the pulmonary arteries (3). Apoptosis of endothelial cells (EC) contributes to lung vascular remodeling by promoting selection of apoptosis-resistant EC, initiating proliferation of vascular smooth muscle cells, and directly, via vascular pruning (4–11). Multiple pathogenic mechanisms likely contribute to EC apoptosis (7–10) and identifying targetable mechanisms remains an important goal (11).

Recently, a protective role in blood vessels was shown for TLR3 (Toll-like receptor 3), a member of the TLR family of innate immune receptors (12). TLR3 responds to viral and synthetic double-stranded RNA (dsRNA), and RNA released from healthy and apoptotic cells (13, 14). TLR3 not only regulates RNA signaling by its primary localization at the endosomal membrane, but TLR3 can also influence RNA signaling through additional cytosolic RNA receptors. These RNA receptors include RIG-I (retinoic acid-inducible gene-I) and MDA-5 (melanoma differentiation–associated protein-5) (15, 16).

In addition to the role of TLR3 in the intracellular response to RNA, a protective effect has been shown for the synthetic dsRNA and TLR3 agonist polyinosinic/polycytidylic acid (Poly[I:C]). This

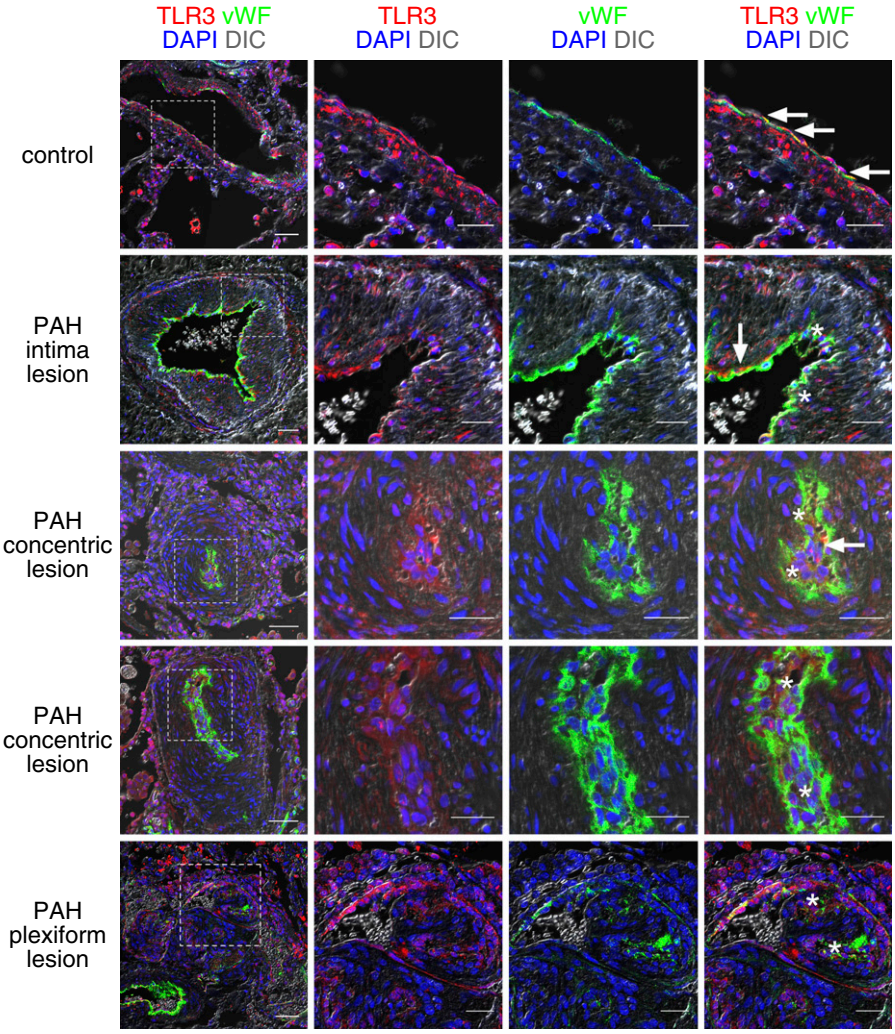

Figure 1. Reduced endothelial TLR3 (Toll-like receptor 3) in lung vascular lesions of patients with pulmonary arterial hypertension. Double immunofluorescence shows strong immunostaining of TLR3 in von Willebrand factor–positive endothelium in pulmonary artery of control subject (arrows). TLR3 staining is partially lost in endothelium of pulmonary arterial hypertension pulmonary arteries with increased smooth muscle layer and intima thickening. The arrows indicate endothelium with preserved TLR3 expression, whereas asterisks indicate endothelium that is TLR3 deficient. In concentric and plexiform lesions, TLR3 staining is largely lost in von Willebrand factor–positive endothelium (asterisks). Scale bars: 50 μm (overview on the left), 25 μm (higher detail images). The dashed boxes in the overview images on the left indicate the area that is shown in more detail on the right. Nuclear counterstaining with DAPI. DIC = differential interference contrast; PAH = pulmonary arterial hypertension; vWF = von Willebrand factor.

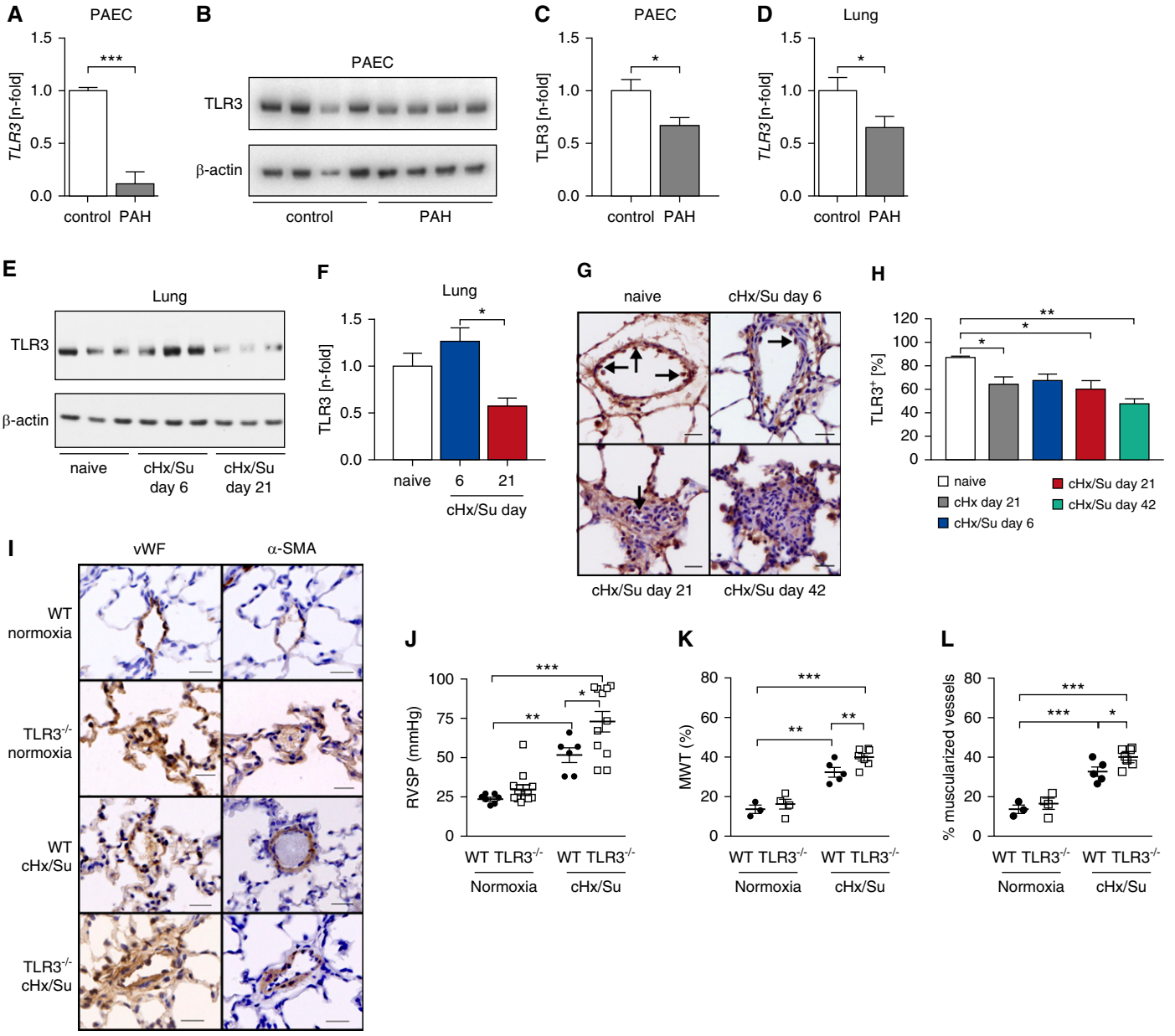

Figure 2. Reduced TLR3 (Toll-like receptor 3) expression in human pulmonary arterial hypertension (PAH) and experimental severe pulmonary hypertension. (*A*) Lower expression of *TLR3* mRNA in pulmonary artery endothelial cells (PAEC) (*A*, $n = 4$ control subjects vs. $n = 3$ PAH) from human patients with PAH as compared with control subjects without pulmonary vascular disease. (*B*) Reduced TLR3 protein expression in PAEC from patients with PAH by Western blot. β-Actin was used to ensure equal loading of lanes. (*C*) Semiquantitative densitometry of TLR3 versus β-actin confirms reduced TLR3 protein expression in PAEC from patients with PAH. $n = 4$ different cell lines per group. (*D*) Reduced mRNA expression of TLR3 in the lung tissue from patients with PAH ($n = 5$ control subjects vs. $n = 6$ PAH). Representative Western blot (*E*) and semiquantitative densitometry (*F*) show reduction of TLR3 protein expression in lung tissue protein lysate from chronic hypoxia and SU5416 (cHx/Su) rats at Day 21. β-Actin was used as loading control. $n = 3$ per group. (*G*) Representative immunohistochemistry images for TLR3 show less $TLR3^+$ cells (arrows) in endothelial/intima cells in cHx and cHx/Su rats. Counterstaining: Mayer's hematoxylin. Scale bars: 20 μm. (*H*) Quantification of $TLR3^+$ intima cells in pulmonary arteries of cHx and cHx/Su rats. $n = 3$ per group. (*I*) Representative images of von Willebrand factor and α-smooth muscle actin immunohistochemistry show increased pulmonary artery muscularization in $TLR3^{-/-}$ mice exposed to the cHx/Su protocol compared with $TLR3^{+/+}$ wild-type mice. In contrast, no substantial change occurred in $TLR3^{-/-}$ mice housed in normoxia. Counterstaining with hematoxylin. Scale bars: 20 μm. $TLR3^{-/-}$ mice have higher right ventricular systolic pressure (*J*), media wall thickness (*K*), and fraction of muscularized pulmonary arteries (*L*) than TLR3 wild-type mice when exposed to the cHx/Su protocol but not when exposed to normoxia. $n = 6$–12 (*J*), $n = 3$–7 (*K* and *L*). Bars: mean + SEM, scatter plots indicate mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ (*A*, *C*, and *D*: Student's *t* test; *F* and *H*: one-way ANOVA; *J*–*L*: two-way ANOVA). MWT = media wall thickness; RVSP = right ventricular systolic pressure; SMA = smooth muscle actin; vWF = von Willebrand factor; WT = wild type.

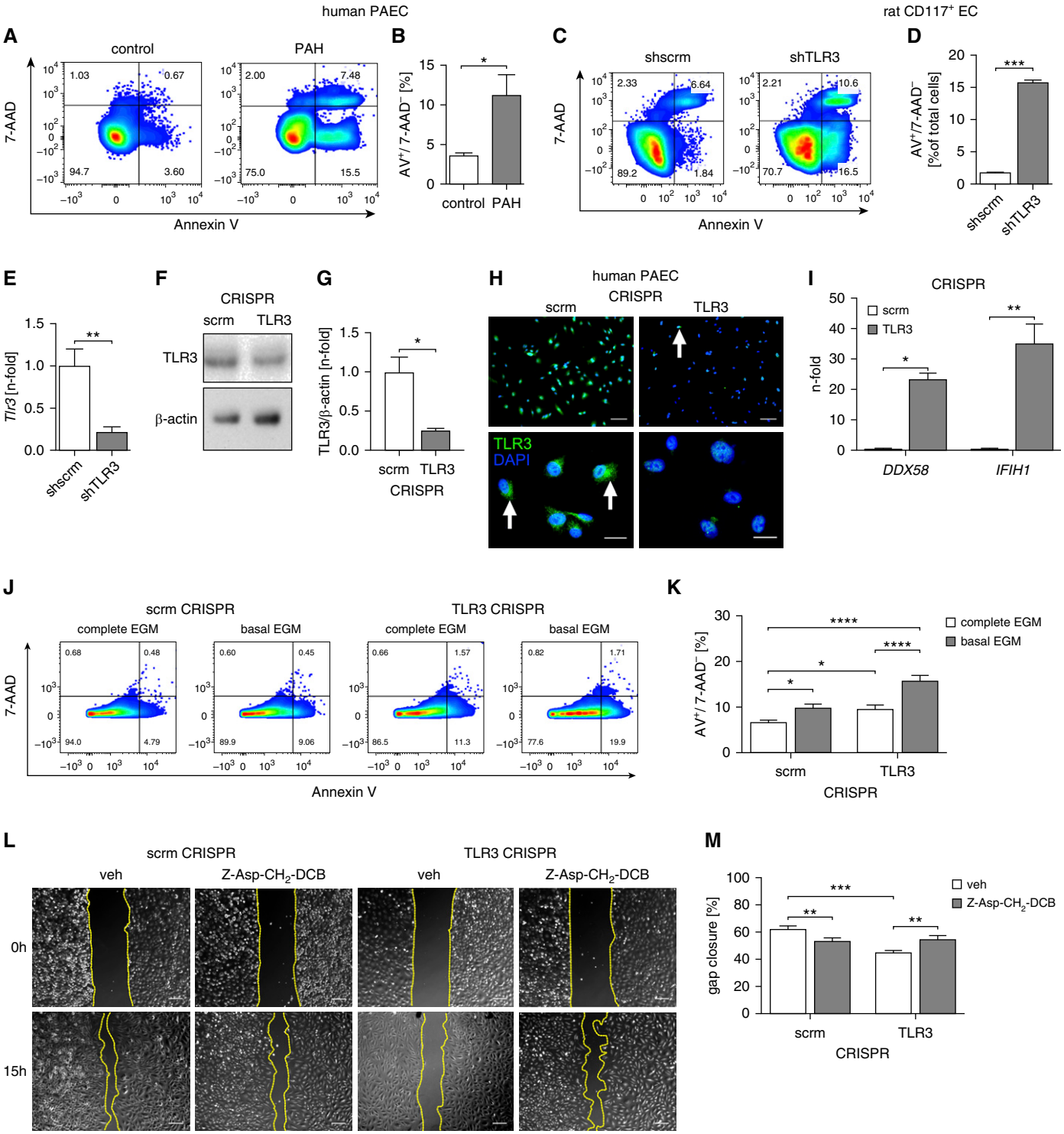

Figure 3. Endothelial TLR3 (Toll-like receptor 3) deficiency promotes apoptosis and impairs migration *in vitro*. (*A*) Representative dot plots show increased fraction of annexin V^+ (AV^+) 7-AAD^- (apoptotic) cells in pulmonary artery endothelial cells (PAEC) from patients with pulmonary arterial hypertension. (*B*) Quantification of AV^+ 7-AAD^- PAEC according to group ($n = 6$ each group). (*C*) Representative dot plots indicate increased fraction of AV^+ 7-AAD^- $CD117^+$ rat lung EC after short hairpin RNA–mediated knockdown of TLR3. (*D*) Quantification of AV^+ 7-AAD^- $CD117^+$ rat lung EC 72 hours after adenovirus-mediated overexpression of scrambled [scrm] short hairpin RNA or shTlr3 ($n = 3$ per group). (*E*) Gene knockdown was confirmed by qRT-PCR of rat *Tlr3* gene mRNA expression 72 hours after the beginning of the transfection. (*F*) Representative Western blot shows reduction of TLR3 expression in control PAEC 48 hours after overexpression of cas9 and TLR3 sgRNA (or scrm sgRNA, as control). β-actin was used as loading control. (*G*) Semiquantitative densitometry ($n = 3$). (*H*) Immunofluorescence staining for TLR3 shows strong TLR3 expression in scrm CRISPR PAEC, but loss of TLR3 expression in most TLR3 CRISPR PAEC after

protection was in part mediated via the antiinflammatory cytokine IL-10 (12, 17).

Based on this evidence, we hypothesized that TLR3 signaling protects lung vasculature, and that promoting TLR3 signaling would reduce pulmonary hypertension (PH). We therefore examined TLR3 expression and show that endothelial expression of TLR3 is reduced in occlusive vascular lesions, pulmonary artery EC (PAEC), and lung tissue from patients with PAH. TLR3 knockout exaggerated PH and endothelial apoptosis in mice exposed to chronic hypoxia and SU5416 (cHx/Su). *In vitro*, exposure to high, but not low, concentrations of dsRNA promoted TLR3 expression by increasing IL-10 expression in $CD117^+$ lung EC. *In vivo*, treatment with high-dose Poly(I:C) prevented and reduced experimental PH in rats in proof-of-principle experiments, ameliorating vascular remodeling, apoptosis, and proliferation in pulmonary arteries. Some of the results of these studies have been previously reported in the form of abstracts (18–20).

Methods

Human Tissue Samples and ECs

Deidentified human lung tissue was obtained from the Department of Pathology, University of Colorado Denver and the tissue repository of the Pulmonary Hypertension Breakthrough Initiative. PAEC were isolated by the University of Pittsburgh Cell Processing Core and the Pulmonary Hypertension Breakthrough Initiative in a deidentified manner under Pulmonary Hypertension Breakthrough Initiative–approved protocols and in compliance with the institutional review boards. Control samples and cells were from failed donor lungs and lung tissue resections for other diseases (e.g., cancer). The institutional review boards have approved the tissue collection at the respective institutions. Because of the use of deidentified tissues and cells, the study was deemed nonhuman subjects research by the Office of Research Subjects Protection at Virginia Commonwealth University.

In Vivo Treatment in Animal Models

All animal experiments were approved by the Institutional Animal Care and Utilization Committees in accordance with the Health Research Extension Act (Public Law 99–158) at Virginia Commonwealth University and the U.K. Home Office Animals (Scientific Procedures) Act of 1986 at the University of Sheffield. The procedures followed the 1996 Guide for the Care and Use of Laboratory Animals. Additional details regarding the experimental models and strains are found in the online supplement. The treatment was applied as follows. Preventive strategy (cHx and cHx/Su) consisted of 1 mg/kg (only cHx/Su), 10 mg/kg Poly(I:C), or vehicle (phosphate-buffered saline) three times a week by intraperitoneal injection from Day 1–21. Therapeutic strategy (cHx/Su) consisted of 10 mg/kg Poly(I:C) three times a week intraperitoneally from Day 29 to Day 42. Additional control subjects were naive rats treated with 10 mg/kg Poly(I:C) or vehicle for 14 days. Animals were randomly assigned to the treatment groups. At Day 21 or 42, the animals underwent echocardiographic and hemodynamic evaluation under anesthesia with ketamine, 100 mg/kg, and xylazine, 15 mg/kg intraperitoneally, followed by tissue harvest after exsanguination (10, 21). In mice, right ventricular pressure–volume measurements were collected under isoflurane anesthesia as previously described (22).

Additional detail on the methods for cell culture experiments and animal experimentation is provided in the online supplement.

Statistical Analysis

Data were compared using Student's *t* test (two groups), or one- or two-way ANOVA (more than two groups), followed by multiple comparison testing using the Holm-Sidak or Sidak tests. The calculations were performed using Prism 6.0 (GraphPad Software Inc.). *P* less than 0.05 was considered significant.

Results

Reduced TLR3 Is Associated with PAH, and Loss of TLR3 Promotes Severe PH *In Vivo*

Triple immunofluorescence showed reduced endothelial TLR3 staining in remodeled pulmonary arteries from patients with PAH. In arteries with intima lesions, a part of the endothelium has lost TLR3 expression (Figure 1). Loss of TLR3 was widespread in the endothelium of concentric and plexiform lesions. In contrast, pulmonary artery endothelium of control subjects had strong TLR3 expression (Figure 1). We further confirmed reduced endothelial TLR3 expression in cultured PAEC and PAH lung tissue (Figures 2A–2D). We further studied the timing of TLR3 expression in the cHx and cHx/Su PH models. TLR3 protein expression was lower in cHx/Su lung tissue at Day 21 (Figures 2E and 2F), and cHx and cHx/Su animals had reduced fraction of $TLR3^+$ EC (Figures 2G and 2H).

Then, we evaluated TLR3 deficiency *in vivo*. Naive $TLR3^{-/-}$ mice failed to develop spontaneous PH (Figures 2I–2L). However, media wall thickness (MWT), fraction of muscularized arteries, and PH were aggravated in $TLR3^{-/-}$ mice following exposure to cHx/Su (Figures 2I–2L). These data suggest that loss of TLR3 altered vascular function, leading to increased remodeling. Because of the importance of endothelial apoptosis in PAH pathobiology, we next sought to determine the link between TLR3 deficiency, endothelial apoptosis, and PH.

Figure 3. (Continued). 72 hours. The images on the top show an overview (scale bar: 50 μm) and the images on the bottom show cells in more detail, with the scrm cells exhibiting a typical cytoplasmic TLR3 staining pattern. Arrows indicate cells that retained TLR3 expression. (*I*) qRT-PCR shows mRNA expression of *DDX58* (RIG-I) and *IFIH1* (MDA-5) in TLR3 and scrm CRISPR PAEC. (*J*) Representative dot plots show increased fraction of $AV^+/7\text{-}AAD^-$ PAEC following serum starvation (basal endothelial growth medium) and TLR3 knockdown. (*K*) Quantification of $AV^+/7\text{-}AAD^-$ PAEC. $n = 9$ per group. (*L*) Representative differential interference contrast images of gap closure assay of PAEC after TLR3-targeted or scrm CRISPR. Cells from both groups were treated with vehicle or 100 μM Z-Asp-CH_2-DCB. The images show the damage-free gap at 0 and 15 hours. The borders of the gaps are indicated by yellow dotted lines. Scale bar: 100 μm. (*M*) Quantification of percent wound closure after 15 hours ($n = 9$ per group). Bars: mean + SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (*B*, *D*, *E*, *G*, and *I*: Student's *t* test; *K* and *M*: two-way ANOVA). CRISPR = clustered, regularly interspaced short palindromic repeats; EGM = endothelial growth medium; PAH = pulmonary arterial hypertension; shscrm = scrambled short hairpin RNA; veh = vehicle.

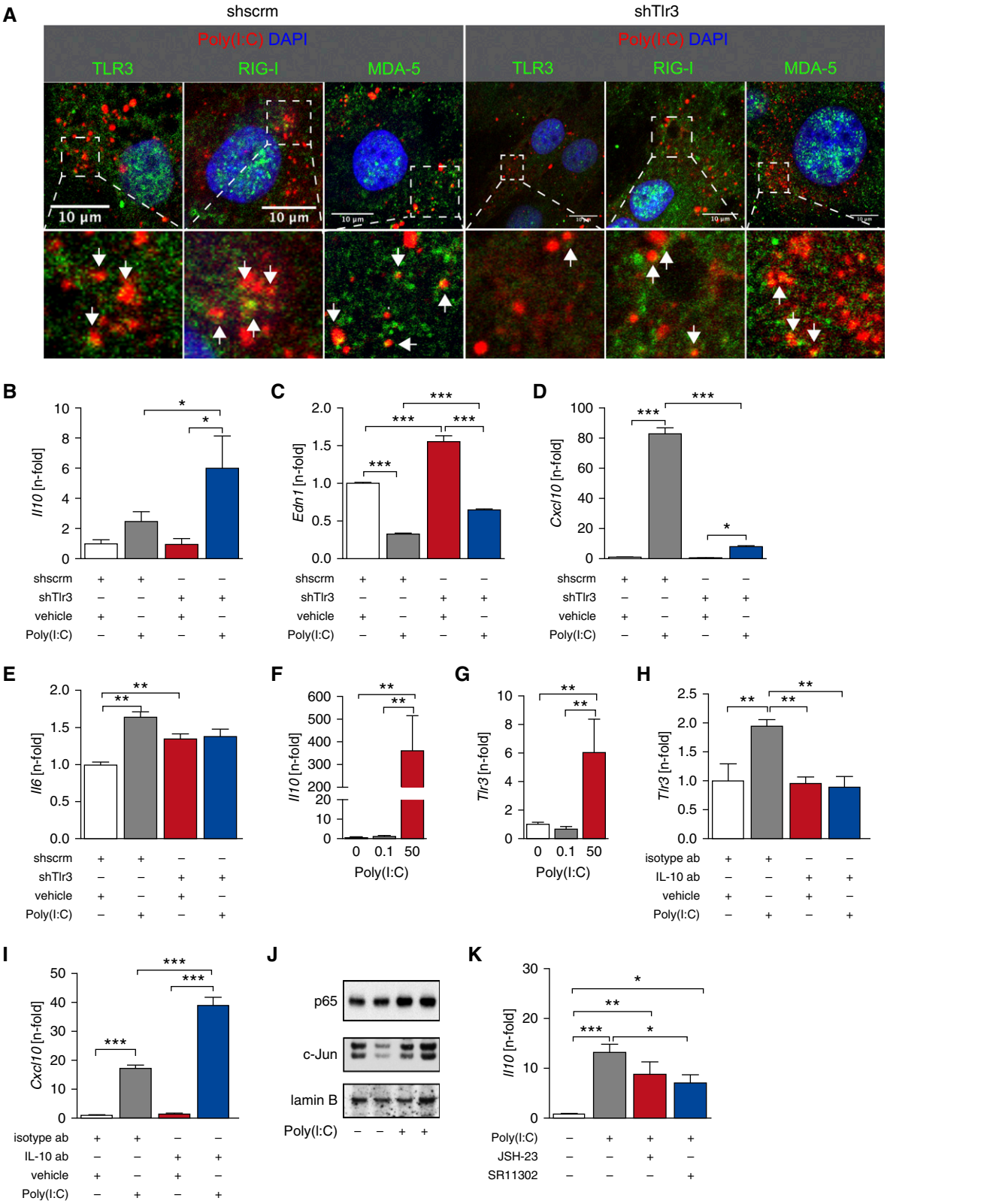

Figure 4. TLR3 (Toll-like receptor 3) deficiency channels endothelial dsRNA signaling through alternate RNA receptors RIG-I and MDA-5 and promotes IL-10 expression in rat lung CD117^{+} endothelial cells (EC). (*A*) Representative optical sections of images acquired by confocal microscopy show that rhodamine-labeled polyinosinic/polycytidylic acid (Poly[I:C]) (25 μg/ml) localized to TLR3, RIG-I, and MDA-5 in shscrm-expressing CD117^{+} EC with normal TLR3 expression. By contrast, in shTlr3-expressing cells with reduced TLR3 level, Poly(I:C) mainly interacted with RIG-I and MDA-5. Arrows: colocalization

TLR3 Deficiency Promotes Endothelial Apoptosis *In Vivo*

Then we studied a potential driver of PH, apoptosis, in relation to TLR3 expression. Although we found no apoptosis in TLR3-expressing endothelium from control subjects, we found TLR3-deficient apoptotic intima cells in PAH lesions (*see* Figure E1A in the online supplement). Further analysis revealed an increased fraction of cleaved caspase-3-positive intima cells in PAH pulmonary arteries (*see* Figures E1B and E1C). Likewise, pulmonary arteries of $TLR3^{-/-}$ mice had more apoptotic EC after 21 days of cHx/Su (*see* Figures E1D and E1E). We did not find increased apoptosis at early time points (1 or 6 d) in cHx/Su rats (*see* Figure E2).

TLR3 Deficiency Promotes Endothelial Apoptosis *In Vitro*

In vitro, PAH PAEC had elevated frequency of background apoptosis (Figures 3A and 3B) and we tested whether TLR3 deficiency promotes endothelial apoptosis using two approaches. First, we silenced TLR3 expression in rat lung $CD117^+$ EC using short hairpin RNA (shRNA). These cells were isolated from the lungs of naive rats, expressed the endothelial marker von Willebrand factor, formed angiogenic tubes, and were clonally expandable (*see* Figure E3). TLR3 knockdown increased the frequency of background apoptosis (Figures 3C–3E). As a second approach, we induced loss of TLR3 in human PAEC using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology. We found in TLR3 CRISPR cells a substantial reduction in TLR3 protein (Figures 3F–3H), which was maintained after passage (*see* Figure E4). Because PAEC were in passage 5 or higher at the start of the experiment, we did not perform a selection of TLR3-negative PAECs, retaining a small fraction of TLR3-positive cells. TLR3 CRISPR PAEC had higher baseline expression of *DDX58* (RIG-I) and *IFIH1* (MDA-5) (Figure 3I), and a higher baseline apoptosis rate with an exaggerated apoptotic response to serum starvation (Figures 3J and 3K). TLR3 CRISPR also impaired migration *in vitro*. This was partially reversed by caspase inhibition, despite a small reduction in gap closure by the caspase inhibitor in scrambled (scrm) PAEC (Figures 3L and 3M).

Reduced TLR3 Expression Promotes RNA Signaling via Alternate RNA Receptors RIG-I and MDA-5

We then evaluated the subcellular localization of dsRNA by exposing $CD117^+$ EC to rhodamine-labeled Poly(I:C) after treatment with scrambled shRNA (shscrm) or TLR3-targeted shRNA (shTLR3). In shTLR3 cells, Poly(I:C) localized to RIG-I and MDA-5 in the cytoplasm, whereas Poly(I:C) also localized to TLR3 in shscrm cells (Figure 4A). In shscrm EC, Poly(I:C) induced expression of *Cxcl10* and IL-6 (*Il6*), but not IL-10 (*Il10*), and decreased expression of endothelin-1 (*Edn1*) (Figures 4B–4E). Poly(I:C) failed to induce *Il6* in shTLR3 cells, and upregulation of *Cxcl10* was substantially lower in shTLR3 cells. Knockdown of TLR3 alone elevated expression of *Edn1* and *Il6*, but not of *Cxcl10* and *Il10*, indicating that TLR3 deficiency promotes a gene expression profile associated with inflammation and vascular remodeling. However, Poly(I:C) stimulation increased *Il10* expression substantially in shTLR3 EC.

Our supplemental data show that transfection of Poly(I:C) increased expression of *Il10*, *Tlr3*, and *Cxcl10* far more than extracellular addition (*see* Figure E5). This is relevant to our findings, because extracellular addition of Poly(I:C) targets TLR3 via endosomal uptake, whereas liposomal transfection facilitates interaction between Poly(I:C) and both cytoplasmic and endosomal receptors, increasing synergistic signaling (23).

Poly(I:C) Treatment Restores TLR3 Expression in an IL-10–Dependent Manner

We then postulated that the release of RNA from damaged cells was insufficient to upregulate TLR3. We found that only high concentration of Poly(I:C) increased expression of *Il10* and *Tlr3* (Figures 4F and 4G). Blocking IL-10 with a neutralizing antibody abolished TLR3 upregulation after Poly(I:C) treatment (Figure 4H). Consistent with effective IL-10 inhibition, we observed derepression of CXCL10 (Figure 4I) (24). Poly(I:C) increased activation of nuclear factor-κB p65 and activator protein 1 c-Jun (Figure 4J), and blocking activator protein 1 with SR11302 partially blocked Poly(I:C)–induced upregulation of IL-10 (Figure 4K).

Comparison of Poly(I:C) *In Vitro* Effects in Different Human ECs

To identify whether different vascular compartments react differently to Poly(I:C) stimulation, we compared the gene expression of human lung microvascular EC and human umbilical vein EC. In both cell lines, we found concentration-dependent upregulation of *TLR3*, *DDX58* (RIG-I), and *IFIH1* (MDA-5) (*see* Figures E6A and E6B). In human umbilical vein EC, Poly(I:C) failed to induce *IL6*, *CXCL10*, and *IL10*, but caused a more substantial induction of *DDX58* and *IFIH1*.

Comparing caspase 3/7 activity of human lung microvascular EC and PAEC, we found that Poly(I:C) caused a concentration-dependent increase in apoptosis only in serum-starved human lung microvascular EC, but not PAEC (*see* Figure E7A). Because Poly(I:C) increases

Figure 4. (Continued). of Poly(I:C) and respective receptor. Scale bars: 10 μm. The lower row shows the areas indicated by dashed boxes in the upper row in more detail. Counterstaining with DAPI. (*B–E*) Changes in the Poly(I:C)–induced mRNA expression of genes regulating inflammation and vasotonus/remodeling between shscrm- and shTlr3 EC: *Il10* (*B*), *Edn1* (Endothelin-1, *C*), *Cxcl10* (CXCL10, *D*), and *Il6* (*E*). (*F* and *G*) Whereas a low dose of Poly(I:C) (0.1 μg/ml) fails to induce *Il10* (*F*) or *Tlr3* (*G*) mRNA expression in $CD117^+$ EC, high concentration of Poly(I:C) (50 μg/ml) strongly elevates expression of *Il10* and *Tlr3*. (*H* and *I*) Poly(I:C)–induced elevation (25 μg/ml) of *Tlr3* expression depends on IL-10, because treatment with a neutralizing anti-IL-10 antibody (ab) abolishes Poly(I:C)–induced upregulation of *Tlr3* (*H*) but enhances Poly(I:C)–induced *Cxcl10* upregulation (*I*). (*J*) Representative Western blots from nuclear lysates show increased nuclear accumulation (activation) of nuclear factor-κB p65 and activator protein 1 (AP-1) c-Jun in Poly(I:C) (25 μg/ml) treated $CD117^+$ EC. Lamin B was used as loading control. (*K*) Inhibition of AP-1 with SR11302 (1 μM) significantly reduces Poly(I:C) (25 μg/ml)–induced IL-10 upregulation. Inhibitor of nuclear factor-κB nuclear translocation JSH-23 (25 μM) only resulted in a nonsignificant trend. All bars: mean + SEM, $n = 3$ per group, except $n = 3$–5 per group for *K*. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA). shscrm = scrambled short hairpin RNA.

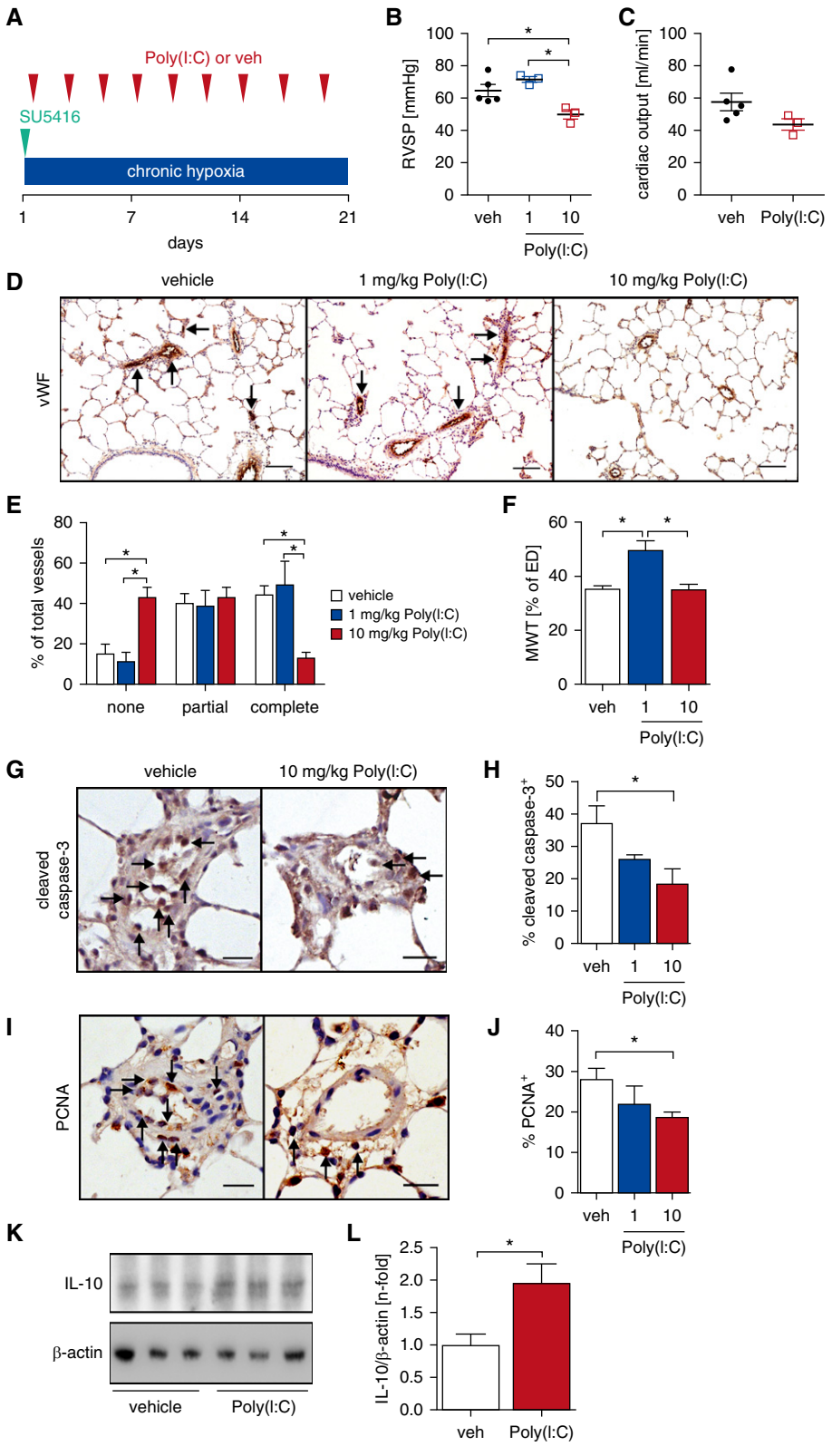

Figure 5. Preventive treatment with high-dose, but not low-dose, dsRNA polyinosinic/polycytidylic acid (Poly[I:C]) reduces severe pulmonary hypertension induced by chronic hypoxia and SU5416. (*A*) Diagram of treatment protocol. (*B*) Early high-dose (10 mg/kg), but not low-dose (1 mg/kg), Poly(I:C) treatment reduces right ventricular systolic pressure. $n = 5$ (vehicle [veh]) and $n = 3$ (Poly[I:C]). (*C*) High-dose early

production of reactive oxygen species in systemic endothelium (25), we compared reactive oxygen species production. We found no difference in Poly(I:C)–induced reactive oxygen species production between human umbilical vein EC and PAEC (*see* Figure E7B).

dsRNA Reduces Pulmonary Artery Remodeling, PH, and Right Ventricular Failure *In Vivo*

Because the loss of TLR3 signaling predisposed to more severe PH we sought to determine in proof-of-principle experiments whether activation of this pathway would prevent the development of PH. First, we tested Poly(I:C) in naive animals. We detected no increase in right ventricular systolic pressure and arterial occlusion after treatment with Poly(I:C) (*see* Figures E8A–E8C). However, we found an increase in MWT and fraction of apoptotic cells in the artery wall after Poly(I:C) treatment (*see* Figures E8A and E8D–E8F). Then, rats with PH induced by chronic hypoxia were treated with Poly(I:C). In our prophylactic treatment groups, right ventricular systolic pressure was significantly lower in rats treated with Poly(I:C). Reduced PH was associated with lower MWT and smaller fraction of fully muscularized arteries (*see* Figures E9A–E9F). Poly(I:C) treatment did not significantly compromise echocardiographic right ventricular cardiac output (*see* Figure E9D) and it ameliorated apoptosis and proliferation in the artery walls (*see* Figures E9G–E9J). Finally, to further explore whether Poly(I:C) reduces severe PH with vascular obliteration, rats with cHx/Su–induced severe PH were treated with Poly(I:C) in prophylactic and interventional strategies.

We also tested whether Poly(I:C) treatment had concentration-dependent effects. Prophylactic high dose Poly(I:C) treatment reduced severe cHx/Su PH and vascular occlusion, but had no significant effect on MWT, right ventricular cardiac output, or the inflammatory cell profile in the lungs of cHx/Su rats (Figures 5A–5F; *see* Figure E10). We found that only high-dose Poly(I:C) had a protective effect (Figures 5A–5F). By contrast, low-dose Poly(I:C) treatment failed to reduce right ventricular systolic pressure and vascular obliteration, but increased MWT. Only high-dose Poly (I:C) treatment significantly reduced the fraction of cleaved caspase-3-positive

(apoptotic) and proliferating cell nuclear antigen–positive (proliferating) cells in the pulmonary arteries (Figures 5G–5J). In addition, high-dose Poly(I:C) treatment increased IL-10 expression in the lungs of cHx/Su rats (Figures 5K and 5L). Established severe PH and occlusion was ameliorated by high-dose Poly(I:C) (intervention strategy), although muscularization of pulmonary arteries was not significantly reduced (Figures 6A–6E). Right ventricular cardiac output was improved by Poly(I:C) treatment in established PH (Figure 6F) and similar to the prophylactic strategy, numbers of cleaved caspase-3-positive and proliferating cell nuclear antigen–positive pulmonary vascular cells were reduced (Figures 6G–6J). To identify the effect of Poly(I:C) treatment on systemic blood vessels, we investigated left ventricular capillary density and apoptotic index. We further found no significant change in the capillary density and apoptotic index in the left ventricle of cHx rats after preventive treatment with high-dose Poly(I:C) (*see* Figure E11). In contrast, we detected increased capillary density and reduced apoptotic index in the left ventricles of cHx/Su rats after therapeutic treatment with high-dose Poly(I:C) (*see* Figure E12).

Discussion

PAH is a devastating disease characterized by progressive narrowing and reorganization of the pulmonary arteries (1, 26). Although increased cell proliferation has been identified in pulmonary vascular lesions, a paradoxical increase in endothelial apoptosis is important for the initiation and progression of the disease (4, 27–30).

In this study, we identify the innate immune receptor TLR3 as a novel regulator of endothelial apoptosis in PAH. Our data show that loss of TLR3 leads to EC apoptosis and that a TLR3 agonist can reduce severe PH in preclinical animal models. There is a well-supported paradigm that proinflammatory signaling drives pulmonary vascular remodeling in PAH. Our data may therefore seem counterintuitive; however, there are precedents for protective effects of TLR3 expression and signaling in the systemic circulation and our data imply that this pathway also confers protection in the pulmonary vasculature (1, 12, 31).

We found reduced expression of TLR3 in the lungs and PAEC from patients with PAH and we demonstrated an association between loss of TLR3 and caspase-dependent endothelial apoptosis. Extending our *in vitro* studies, we found that TLR3$^{-/-}$ mice have more severe PH and vascular remodeling in response to the cHx/Su protocol, likely caused by increased endothelial apoptosis. Endothelial apoptosis has been implicated in pulmonary vascular remodeling (5, 30, 32) with studies suggesting that early apoptosis contributes to increased vascular cell proliferation in PAH (9, 30, 33–35). Some authors even suggest endothelial apoptosis as a direct cause of PH because of vascular pruning (4, 36). Hence, understanding the mechanism of endothelial apoptosis in PH is important to derive meaningful treatment strategies.

TLR3 is a receptor for a broad spectrum of RNAs, including mRNA released from dying cells and small interfering RNA sequences without regard of the nucleic acid sequence (14–16, 37, 38). Hence RNA is a "damage-associated molecular pattern" for TLR3 during the cellular injury response. TLR3 is mainly localized in the endosomal membrane, but additional receptors for dsRNA and RNA are found in the cytosol, and these include MDA-5 and RIG-I (23, 39, 40). In TLR3-deficient EC, we found that RNA signaling is directed toward these additional RNA receptors, which can induce apoptosis, possibly explaining the higher degree of apoptosis in TLR3-deficient EC. Furthermore, TLR3 knockdown increased expression of endothelin-1 and IL-6, which are implicated in endothelial dysfunction and vascular remodeling (41). Hence, our data indicate that endothelial RNA signaling is dysregulated as a consequence of reduced TLR3 expression. Interestingly, although we show that changes in TLR3 expression impact RNA signaling, it is also possible that loss of function polymorphisms of TLR3 could play a role in defective RNA sensing in PAH, similar to a more aggressive clinical phenotype observed in patients with TLR3 polymorphisms in idiopathic pulmonary fibrosis (42).

We then tested whether we can treat PH with dsRNA. Our approach, including dose and regimen of the dsRNA Poly(I:C), was informed by the findings of Cole and colleagues (12), who showed that Poly(I:C) promoted TLR3 expression and protected from arteriosclerotic vascular injury. Our experiments showed that Poly (I:C) treatment prevented and reduced PH, and improved right ventricular failure. Poly(I:C) treatment ameliorated apoptosis, proliferation, and remodeling in the pulmonary artery wall in two PH models. Interestingly, Poly(I:C) did not promote pulmonary inflammation in cHx/Su rats. These beneficial effects of TLR3 activation are in contrast to findings by Zimmer and coworkers (25) who showed that Poly(I:C) impaired endothelial function and systemic arterial reendothelialization after a denuding injury. Although the study by Zimmer and colleagues (25) investigated Poly(I:C) in an acute model of large-scale vascular denudation, we showed protective effects in chronic models of lung vascular remodeling without denuding injury, which

Figure 5. (Continued). Poly(I:C) treatment did not significantly alter echocardiographic right ventricular cardiac output. $n = 5$ (veh) and $n = 3$ (Poly[I:C]). (*D*) Representative von Willebrand factor immunohistochemistry demonstrates occlusion of pulmonary arteries (arrows) in veh and low Poly(I:C) (1 mg/kg), but not in high Poly(I:C) (10 mg/kg, 3×/wk) treated chronic hypoxia and SU5416 rats after early treatment (Days 1–21). (*E*) High-dose, but not low-dose, early Poly(I:C) treatment reduced the fraction of completely occluded small pulmonary arteries (external diameter >25 and <50 μm). (*F*) In contrast, media wall thickness was not reduced by high-dose Poly(I:C) treatment; instead low-dose Poly(I:C) increased media wall thickness. $n = 3$ per group. (*G*–*J*) Preventive high-dose Poly(I:C) treatment reduced the number of cleaved caspase-3–positive cells (arrows; *G* and *H*) and proliferating cell nuclear antigen–positive cells (arrows; *I* and *J*) in pulmonary arteries. Low-dose Poly(I:C) treatment only had a partial (nonsignificant) effect on apoptosis and proliferation. $n = 3$ for each group. (*K*) Representative Western blot shows increased IL-10 protein expression in the lungs of chronic hypoxia and SU5416 rats treated with high-dose Poly(I:C). β-Actin was used as loading control. (*L*) Semiquantitative densitometry calculated versus β-actin and normalized to veh. All bars: mean + SEM. Scatter plots: mean ± SEM. *$P < 0.05$. Scale bars: 100 μm (*D*), 20 μm (*G* and *I*). *B*, *E*, *F*, *H*, and *J*: one-way ANOVA; *L*: Student's *t* test. ED = external diameter; MWT = media wall thickness; PCNA = proliferating cell nuclear antigen; RVSP = right ventricular systolic pressure; vWF = von Willebrand factor.

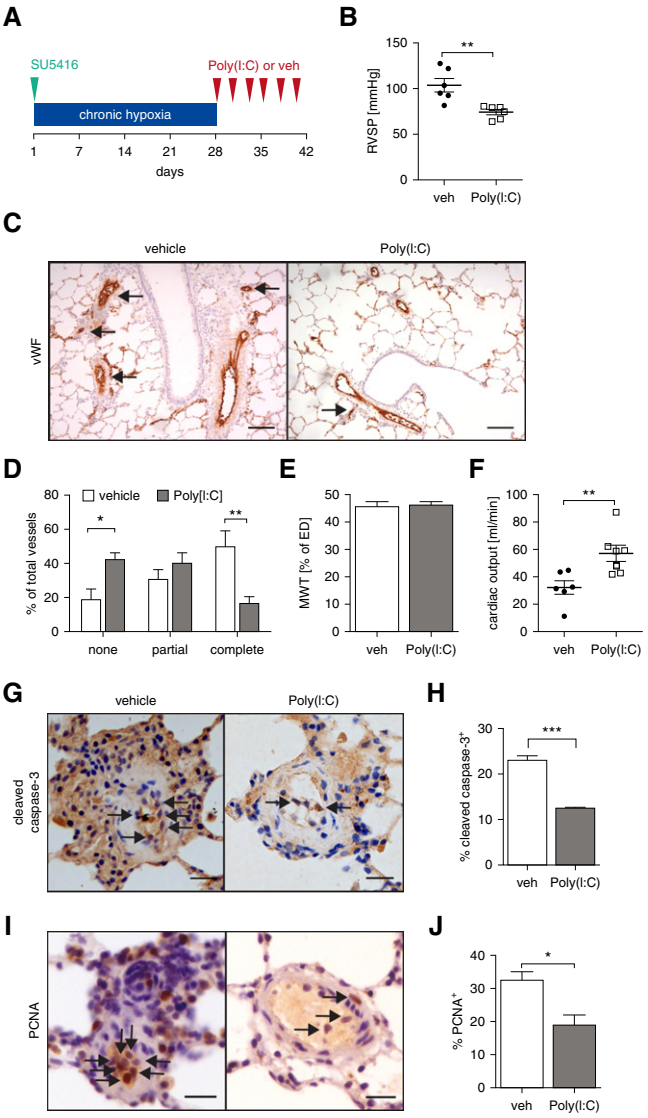

Figure 6. Therapeutic high-dose polyinosinic/polycytidylic acid (Poly[I:C]) treatment reduces pulmonary hypertension and vascular pathology in the lungs of chronic hypoxia and SU5416 rats. (*A*) Diagram of the treatment protocol. (*B*) Delayed high-dose (10 mg/kg) Poly(I:C) treatment reduced right ventricular systolic pressure in chronic hypoxia and SU5416 rats with established pulmonary hypertension ($n = 6$ each group). (*C*) Less occlusion of pulmonary arteries with von Willebrand factor–positive endothelial cells is found after treatment of chronic hypoxia and SU5416 rats with Poly(I:C) (3×/wk, 10 mg/kg) versus vehicle after pulmonary hypertension was established (Days 29–42). Arrows indicate occluded pulmonary arteries. (*D*) Histomorphometry revealed that Poly(I:C) treatment reduced the fraction of completely occluded small pulmonary arteries ($n = 3$–4 per group). (*E*) There was no change, however, in media wall thickness of small pulmonary arteries with Poly(I:C) treatment ($n = 3$ per group). (*F*) Late Poly(I:C) treatment improved right ventricular cardiac output as measured by echocardiography (vehicle, $n = 6$; Poly[I:C], $n = 7$). Therapeutic Poly(I:C) treatment decreased the fraction of cleaved caspase-3–positive cells (arrows; *G* and *H*) and proliferating cell nuclear antigen–positive cells (arrows; *I* and *J*) in the pulmonary artery wall ($n = 3$ per group, except $n = 4$ for proliferating cell nuclear antigen Poly[I:C]). Bars: mean + SEM; scatter plots: mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars: 100 μm (*C*), 20 μm (*G* and *I*). *B*, *E*, *F*, *H*, and *J*: Student's *t* test; *D*: one-way ANOVA. ED = external diameter; MWT = media wall thickness; PCNA = proliferating cell nuclear antigen; RVSP = right ventricular systolic pressure; veh = vehicle; vWF = von Willebrand factor.

could already explain the contrasting results. Further differences are Poly(I:C) dosage and administration route, and it is possible that tissue-specific differences in RNA sensor signaling contribute to the divergent findings. For example, we found that healthy extrapulmonary EC failed to upregulate IL-10 and CXCL10 in response to Poly(I:C), but had a higher induction of RIG-I and MDA-5 than pulmonary EC.

When we observed detrimental effects of Poly(I:C), this was only in the context of an uninjured pulmonary vasculature. This concept is further supported by the results of a study by Huang and colleagues (43) who found that Poly(I:C) induces *in vitro* barrier dysfunction and cytokine production in healthy lung endothelium. The results by George and colleagues (44) also back our notion by showing Poly(I:C)–induced upregulation of endothelin-1 in healthy lung vascular smooth muscle cells. Taken together, these data imply that endothelial TLR3 expression has a homeostatic role in the context of chronic lung vascular remodeling. Our data further indicate that Poly(I:C) caused no adverse effect on left ventricular capillary density and apoptotic index *in vivo* in both PH models and therapeutic Poly(I:C) treatment was even protective for the left ventricle of cHx/Su rats. Agents to manipulate TLR3 signaling, such as the dsRNA rintatalimod, are available and may exert therapeutic effects on vascular cells in the context of TLR3 deficiency, as we have observed in PH (45).

Our *in vitro* data demonstrated that induction of IL-10 and TLR3 by Poly(I:C) occurred only after high-dose treatment. These data imply that high concentrations are therefore required to restore TLR3 levels and reduce apoptosis. This offers a potential explanation of why constitutive RNA signaling fails to offer protection from vascular remodeling; the concentration of RNA internalized following cell injury may simply be too low. Although induction of TLR3 and IL-10 expression by dsRNA has been demonstrated before, induction of TLR3 via IL-10 represents a novel mechanism (12, 17). IL-10 may also explain why Poly(I:C) fails to increase inflammation in cHx/Su rats, because IL-10 reduces inflammation and thereby cardiac remodeling or PH (19, 46, 47). In addition, IL-10 is also produced by circulating endothelial progenitor cells and

can stimulate protective natural killer cells (48). Two potential drawbacks exist with our hypothesis that IL-10 mediates protective effects in response to Poly(I:C). First, although Poly(I:C) consistently induced IL-10 in rat lung CD117$^+$ EC and in cHx/Su rat lungs, human lung EC showed a less consistent upregulation of IL-10, indicating that Poly(I:C)–induced upregulation of IL-10 may be restricted to subpopulations, such as CD117$^+$ EC, or that some of the effects of Poly(I:C) have been mediated through nearby inflammatory cells, which we have not studied in detail in the current work. Second, elevated systemic levels of IL-10 have been reported in patients with PAH (49). This may represent the results of insufficient IL-10 signaling in the lung vascular lesions (46, 50).

Further potential limitations of our study are that the use of lung tissue samples from patients with end-stage PAH may underestimate the level of apoptosis in active PAH vascular lesions; and that loss of TLR3 may affect other vascular cells and immune cells.

In conclusion, we provide evidence for loss of TLR3 in pulmonary artery endothelium from patients with advanced PAH. We further show that TLR3 deficiency promotes endothelial apoptosis and exaggerates severe PH in mice. Treatment with dsRNA reduced severe PH in cHx/Su rats, which could depend on induction of TLR3 via IL-10 in EC. These surprising findings indicate that careful manipulation of TLR3 expression and signaling could supplement existing therapeutic approaches for the treatment of PAH. ■

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