

## Tlr4 regulates pulmonary vascular homeostasis and remodeling via redox signaling

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### 1. ABSTRACT

Pulmonary arterial hypertension (PAH) contributes to morbidity and mortality of patients with lung and heart diseases. We demonstrated that hypoxia induced PAH and increased pulmonary arterial wall thickness in wild-type mice. Mice deficient in toll-like receptor 4 (TLR4<sup>-/-</sup>) spontaneously developed PAH, which was not further enhanced by hypoxia. Echocardiography determined right ventricular hypertrophy and decreased pulmonary arterial acceleration time were associated with the development of PAH in TLR4<sup>-/-</sup> mice. In pulmonary arterial smooth muscle cells (PASMC), hypoxia decreased TLR4 expression and induced reactive oxygen species (ROS)

and Nox1/Nox4. Inhibition of NADPH oxidase decreased hypoxia-induced proliferation of wild-type PASMC. PASMC derived from TLR4<sup>-/-</sup> mice exhibited increased ROS and Nox4/Nox1 expression. Our studies demonstrate an important role of TLR4 in maintaining normal pulmonary vasculature and in hypoxia-induced PAH. Inhibition of TLR4, by genetic ablation or hypoxia, increases the expression of Nox1/Nox4 and induces PASMC proliferation and vascular remodeling. These results support a novel function of TLR4 in regulating the development of PAH and reveal a new regulatory axis contributing to TLR4 deficiency-induced vascular hypertrophy and remodeling.

## 2. INTRODUCTION

Pulmonary arterial hypertension (PAH) is a disease characterized by vascular proliferation, hypertrophy, and fibrosis, leading to right ventricular hypertrophy, which contributes to the morbidity and mortality of patients with lung and heart diseases (1;2). All forms of pulmonary hypertension have similar clinical presentations, functional derangements and pathologic features regardless of their etiology. The pathogenesis of PAH includes structural remodeling of pulmonary arteries and sustained pulmonary vasoconstriction (2). Vascular remodeling represents a key step in the progression of PAH, which involves medial hypertrophy, as well as intimal and adventitial thickening. These changes are believed to result from an imbalance between proliferation and apoptosis of the various cell types forming the vascular walls (1). Hypertrophy and hyperplasia of smooth muscle cells (SMC) are critical cellular events associated with vascular remodeling of the media. Hypoxia-induced PAH is a common experimental model for PAH. Chronic hypoxia induces vascular remodeling with medial hypertrophy leading to the development of pulmonary hypertension.

Chronic hypoxia-induced PAH is mediated by increased production of reactive oxygen species (ROS). Hypoxia-induced ROS generation has been well documented (3;4). The Nox family of NADPH oxidases is the primary enzyme that transports electrons across the plasma membrane to generate superoxide and other downstream ROS in the vasculature (4). NADPH oxidases play a major role in the regulation of physiological and pathophysiological processes and contribute to a variety of vascular diseases, including hypertension (5), aortic media hypertrophy (6;7), atherosclerosis (5) and pulmonary hypertension (8;9). Increase in NADPH oxidase expression or activity has been demonstrated in hypoxic pulmonary hypertension (9;10). Nox-derived ROS have also been shown to promote cell survival/proliferation (11-15) and inhibit cell death (16). Therefore, ROS-induced increased proliferation and decreased apoptosis may contribute to the abnormal accumulation of pulmonary arterial SMC (PASMC), and thus vascular remodeling in the pathogenesis of PAH.

ROS have been linked to activation of toll-like receptor 4 (TLR4) signaling (17). The toll-like receptors are a group of type I transmembrane proteins that play a central role in specific recognition of pathogen-associated molecular patterns and are critical for the induction of innate immunity and inflammation (18). They can also cause significant immunopathology if over-activated or insufficiently controlled (19). TLRs are mainly expressed on antigen-presenting cells, such as macrophages and dendritic cells. In addition, expression of these receptors has currently been described on a variety of other cells, including the cells of the arterial wall (20,21). Expression of these innate immune receptors, especially TLR4 in

healthy and pathological arteries has implicated its role in the homeostasis of vasculature. Hypoxia has been shown to decrease TLR4 expression in endothelial cells via increased ROS (22). On the other hand, TLR4 deficiency increased ROS production in lung endothelial cells (23). However, the function of TLR4 in regulating ROS production and proliferation of PASMC is unknown. Using a hypoxia-induced PAH mouse model, the present studies investigated the role of TLR4 in the pathogenesis of PAH and the underlying mechanisms. We found that TLR4 deficient mice spontaneously developed PAH, which was not enhanced by hypoxia. Our studies suggest that TLR4 plays an important role in maintaining normal pulmonary vasculature, and that hypoxia induces PAH via TLR4. Mechanistic studies further demonstrated increased expression of Nox1 and 4 in primary PASMC from TLR4<sup>-/-</sup> mice. Furthermore, hypoxia induced the expression of Nox1 and Nox4, whereas inhibition of Nox activity blocked hypoxia-induced proliferation of PASMC. Taken together, these studies support a novel connection between TLR4 innate immune signal and redox signaling in PASMC in regulating vascular remodeling during pathogenesis of PAH.

## 3. MATERIALS AND METHODS

### 3.1. Experimental animals

TLR2 and TLR4 deficient mice, originally provided by S. Akira (24) (Osaka University, Osaka, Japan), are maintained and bred under specific pathogen-free conditions at the animal facility at University of Alabama at Birmingham (UAB). All knockout mice used were back-crossed with wild type C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) for more than 8 generations. All animal experiments were performed according to protocols approved by UAB Institutional Animal Care and Use Committee.

#### 3.1.1. Hypoxic exposure of animals

Mice at 10 weeks old were exposed to hypoxia (12% oxygen) or normal air (20% oxygen) in a Plexiglas chamber for 6 weeks as previously described (25,26). The O<sub>2</sub> concentration (OM-100 oxygen analyzer, Newport Medical Instruments), humidity, temperature, and barometric pressure (Fisherbrand Digital Barometer, Fisher Scientific) within the chamber were monitored continuously. Daily animal maintenance was carried out with exposure of the animals to room air for less than 10 minutes per day. Control animals were caged similarly and exposed to filtered room air for the same periods.

#### 3.1.2. Right ventricular pressure and mass measurement

At the end of six weeks, mice were weighed and anesthetized with a mixture of ketamine (80 mg/kg ip) and xylazine (12 mg/kg ip). Right ventricles (RV) were cannulated *in situ* by a closed-chest technique for RV pressure (26). After measurement of RV pressure, mice

were sacrificed and hearts were isolated for determination of the weights of RV and left ventricle plus septum (LV+S). RV pressure was used as an index of PAH and the development of RV hypertrophy was identified by the ratio of RV/(LV+S).

### 3.1.3. Tissue processing and immunohistochemical analysis

For histological and morphometric analysis, the trachea was cannulated using a 24-G angiocath and the lungs were fixed using 10% formalin and then placed in 4% paraformaldehyde for 24 hours before being placed in a tissue cassette for paraffin embedding (25,26). Hematoxylin and eosin (H&E) staining was used for histological analysis. Quantitative morphometric analysis of pulmonary vessels was performed with serial five-micron lung sections by computer assisted image analysis (Bioquant Image Analysis software, R & M Biometrics). The pulmonary artery wall thickness (WT) and vessel diameter (D) were determined along two axes perpendicular to each other in at least 20 consecutive pulmonary arteries cut transversely (longer axis <50% greater than shorter axis). Pulmonary arteries are defined as vessels that accompanied airways (veins are interlobular). Vessels <25  $\mu\text{m}$  in external diameter were not considered for analysis, as wall thickness is not uniform in these vessels. External vessel diameter (distance within external elastic lamella) and medial thickness (distance between external and internal elastic lamellae) were measured; and the wall thickness index of the pulmonary arteries was determined by the percentage of wall thickness to the vessel diameter ( $2 \times \text{WT}/\text{D}$ ) (25). Morphometric analysis was carried out by two independent examiners who were blinded with respect to the treatment assignment of the tissue samples examined.

### 3.1.4. Doppler echocardiography analysis of cardiac function

Echocardiography was performed with a Vevo770 High-Resolution *In vivo* Micro-System (VISUALSONICS, Toronto, Ontario, Canada) with a 30-MHz probe designed for examination of small rodents as previously described (27,28). The examination was performed on mice under general anesthesia with inhalation of 1-2% isoflurane as previously described (28). Left and right parasternal long axis views were used to obtain B-mode two-dimensional cinematic images at 50-70 Hz, from which measurements were made of LV and RV chamber area and cross-sectional area of the LV walls. B-mode images were used to position cursors for high speed (1 KHz) M-mode imaging and pulse wave (PW) doppler measurements. M-mode measurements of ventricular chamber diameter and wall thicknesses were made in a line perpendicular to the long axis of the chamber passing through the tip of the left posterior papillary muscle. RV chamber dilatation (RV end-diastolic dimension, RVEDD), RV hypertrophy (RV free wall thickness at end-diastole, RVFWd and RVFW thickness

at end-systole, RVFWs) and RV systolic function (RVFW thickening) were measured.

Doppler echocardiography of the pulmonary outflow was also utilized to estimate pulmonary artery (PA) pressure in mice non-invasively (27,28). Doppler recordings of the main PA were obtained in the right parasternal long axis position. The PA blood flow velocity was measured at the main PA root of the mice. The PA acceleration time (PAAT) was determined from the start to the peak of the flow signal.

## 3.2. *In vitro* characterization of pulmonary smooth muscle cells

Pulmonary arteries from wild type and TLR4<sup>-/-</sup> mice were isolated by microdissection; and PASMC were obtained by explantation and confirmed by immunohistochemical staining of  $\alpha$ -SMA as we previously described (29,30). Experiments were performed with SMC maintained in culture for 3 to 5 passages. In all experiments, PASMC were seeded at 80% confluence and starved in serum-free media (DMEM/F12) for 6 hours; subsequently cells were placed into an incubator with 2% oxygen or normal air for 24 and 48 hours.

### 3.2.1. ROS measurement

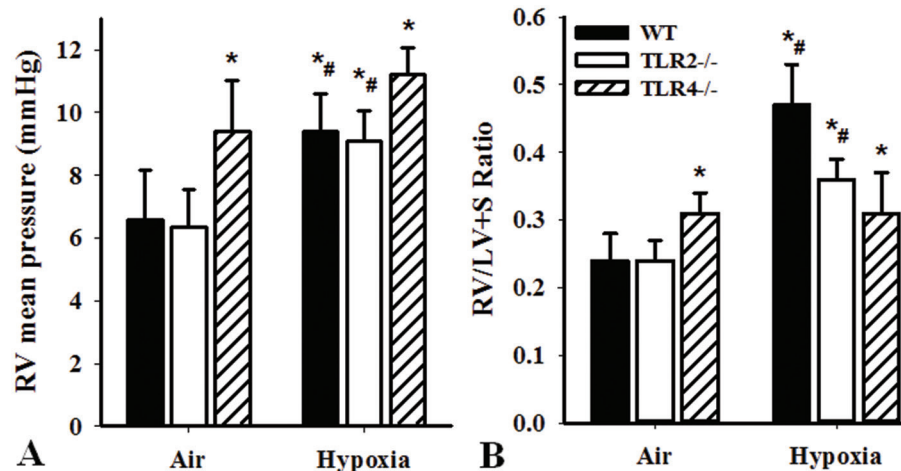
The production of reactive oxygen species (ROS) in PASMC from control and TLR4<sup>-/-</sup> mice were determined by assessing 2',7'-dichlorofluorescein diacetate (DCF-DA), an intercellular indicator for oxidative stress using flow cytometry analysis as we previously described (31).

### 3.2.2. Real-time PCR

The expressions of NOX enzymes in mouse PASMC with or without exposure to hypoxia were determined by *Real-time PCR* with specific primers: Nox1, F-5'-TGGCTAAATCCCATCCAGTC-3' and R-5'-CCCAAGCTCTCCTCTGTTTG-3'; Nox4, F-5'-ACTTTTCATTGGGCGTCCTC-3' and R-5'-AGAACTGGGTCCACAGCAGA-3'; NOXO1, F-5'-TTCCTGATGCTCATTGCTG-3' and R-5'-GGTTGGGATAAGGGCTCCTC-3'; NOXA1, F-5'-AGCTGCAGAGGTTCCAGGAG-3' and R-5'-GATGTCTTGAGCCCCCTCTG-3'. The expression of TLR4 was determined using primers: F-5'-GCTTTCACCTCTGCCTT CAC-3'; and R-5'-CGAGGCTTTTCCATCCAATA-3'. The values of each determination were normalized to those of GAPDH, assessed using primers for GAPDH: 5'-TCATCCCTGCATC CACTGGT-3' and 5'-CTGGGATGACCTTGCC CAC-3'.

### 3.2.3. Proliferation and apoptosis

The effect of TLR4 signals on apoptosis and proliferation of PASMC under basal and hypoxia conditions were determined by exposure of WT and TLR4<sup>-/-</sup> PASMC to hypoxia or normal air. The NADPH oxidase inhibitor, diphenyleneiodonium (DPI, Sigma), was used to determine the effects of NADPH oxidase



**Figure 1.** Hypoxia-induced pulmonary hypertension in mice. WT, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice (10 weeks old) were exposed to hypoxia (12% oxygen) or air (20% oxygen) for 6 weeks. A). RV mean pressure was measured *in situ* by a closed-chest technique. B). The RV/(LV+S) ratio was determined. Results shown are means±SD (n=8 mice in each group, #p<0.05). One-way analysis of variance and Student-Newman-Keuls test were used to identify differences among groups (\*p<0.05 compared with WT AIR conditions, the first solid bar in Figure 1A or Figure 1B).

activity on hypoxia-stimulated PASM C proliferation. WT PASM C were incubated with or without DPI (0.1, 0.5 or 2.5  $\mu$ M) and exposed to normal air or hypoxia conditions. Cell proliferation was determined after 24 hours by BrdU incorporation (Calbiochem), and apoptosis was determined by Western blot analysis of the cleaved form of caspase-3 using specific anti-caspase-3 antibody (Enzo lifescience).

### 3.2.4. Western Blot analysis

Western blot analysis was performed as we previously described. The specific antibodies for Nox1 and Nox4 were purchased from Santa Cruz Biotechnology Inc. The expression of GAPDH (anti-GAPDH antibody, Research Diagnostics Inc.) was used for loading control.

### 3.3. Statistical analysis

All the data are expressed as means  $\pm$  SD. Differences in data between two groups were compared with Student's paired 2-tailed *t* test. For multiple groups comparison, by one-way analysis of variance followed by a Student-Newman-Keuls test was performed. A *p* value less than 0.05 was considered statistically significant.

## 4. RESULTS

### 4.1. TLR4 deficient mice develop pulmonary hypertension, which is not enhanced by hypoxia

To determine the role of TLR4 in the development of pulmonary hypertension, we compared mice with three genotypes, including TLR2 knockout (TLR2<sup>-/-</sup>), TLR4 knockout (TLR4<sup>-/-</sup>) and wild type C57BL6 (WT), in a hypoxia-induced pulmonary hypertension model as previously described (25;26). The development of pulmonary hypertension (PAH) and right ventricle (RV) hypertrophy

were determined by the mean RV pressure (Figure 1A) and the ratio of RV/(LV+S) (Figure 1B) calculated by the weights of RV to left ventricle plus septum (LV+S) in mice exposed to hypoxia or for 6 weeks. We found that hypoxia (12% oxygen) induced pulmonary hypertension in WT and TLR2<sup>-/-</sup> mice, as indicated by increased RV pressure and RV hypertrophy (Figure 1A&B), compared with air (20% oxygen). In contrast, TLR4<sup>-/-</sup> mice exposed to air exhibited signs of PAH, including increased RV pressure and RV/(LV+S) ratio compared with those of WT and TLR2<sup>-/-</sup> mice exposed to normal air (Figure 1A&B). Furthermore, hypoxia did not enhance PAH in TLR4<sup>-/-</sup> mice (Figure 1A&B, 3<sup>rd</sup> bars). These results suggested that TLR4, but not TLR2, signaling is important in the development of PAH.

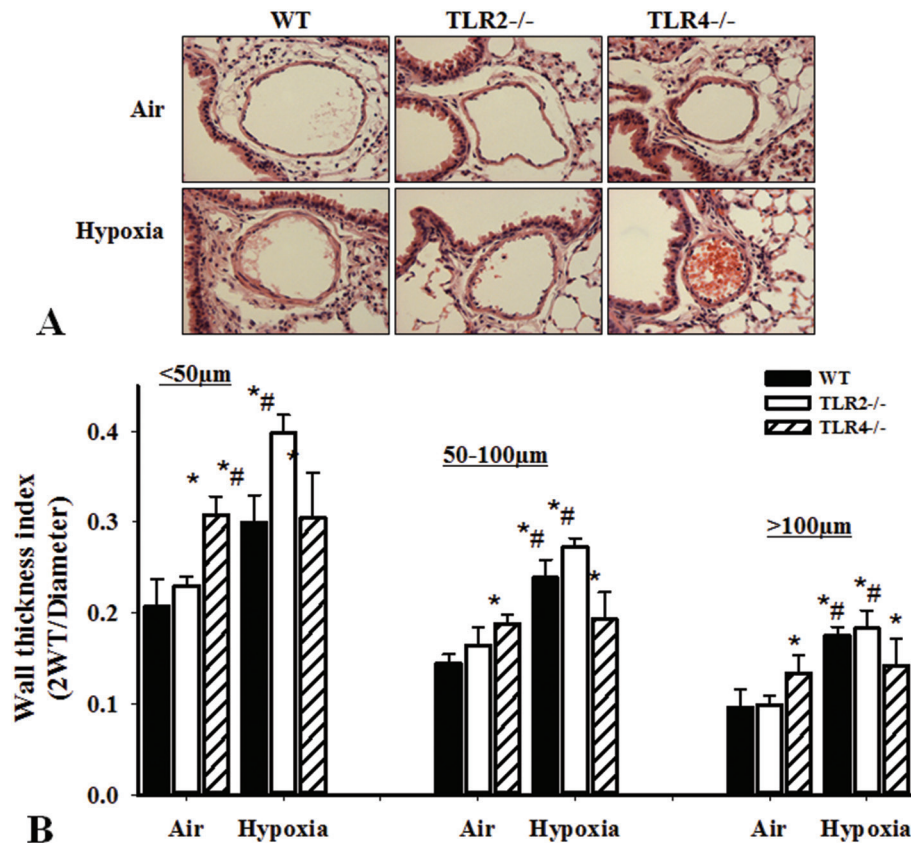
### 4.2. TLR4 deficiency increases pulmonary arterial muscularization

To assess the effect of TLR4 deficiency on hypoxia-induced vascular remodeling, we determined pulmonary arterial muscularization from WT, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice exposed to air or hypoxia for 6 weeks. Histological analysis of the hematoxylin and eosin (H&E) staining of lung sections demonstrated that TLR4<sup>-/-</sup> mice had increased pulmonary arterial wall thickness in normoxia compared to WT and TLR2<sup>-/-</sup> mice. Hypoxia increased pulmonary artery wall thickness index in WT and TLR2<sup>-/-</sup> mice (Figure 2A&B, #p<0.05, hypoxia vs. normal air) but did not further enhance thickening in TLR4<sup>-/-</sup> mice (Figure 2A&B, #p<0.05 hypoxia vs. normal air).

### 4.3. Effect of TLR4 deficiency on hemodynamics and RV function

Echocardiography was performed on WT and TLR4<sup>-/-</sup> mice to characterize the role of TLR4 deficiency on the RV function (Figure 3). Heart rate was 483 $\pm$ 14 bpm





**Figure 2.** Effect of TLR deficiency on vascular muscularization. WT, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice at 10 weeks old were exposed to hypoxia or normal air for 6 weeks. After measurements of RV pressure, lungs were isolated, fixed, and sectioned and immunohistochemical analyses were done. A). Representative pictures of hematoxylin and eosin staining of the lung sections (showing the pulmonary arteries) from the WT, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> mice exposed to air (upper) and hypoxia (lower). B). Wall thickness index of pulmonary arteries. 10 high-power lung fields were examined in each mouse. Wall thickness was analyzed in three groups of arteries based on their diameters: less than 50 µm, 50-100 µm and greater than 100 µm. Results shown are means±SD (n=8 mice for each group, #p<0.05 hypoxia vs. normal air for each genotype; \*p<0.05 compared with WT air in each group).

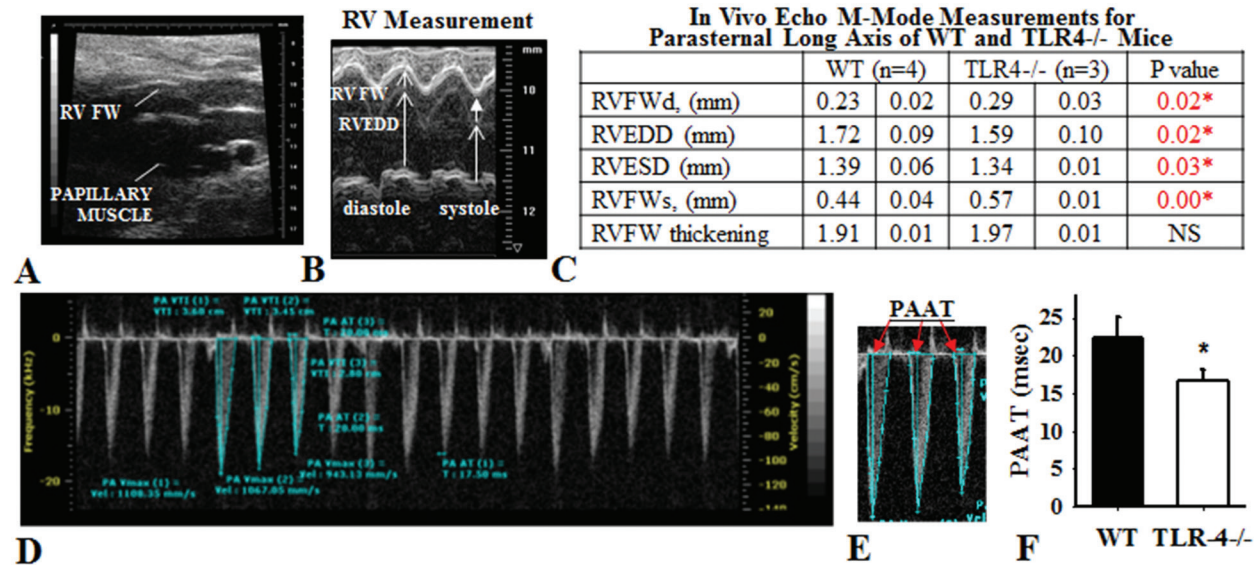
for WT mice and 454±22 bpm for TLR4<sup>-/-</sup> mice. We found that the RVFW thickness in TLR4<sup>-/-</sup> mice at both end-diastole and end-systole was significantly increased compared to WT mice (Figure 3C). RVEDD decreased slightly in the TLR4<sup>-/-</sup> mice as RVFWd increased, thereby demonstrating a concentric RV chamber hypertrophy with normal wall thickening, indicative of preserved RV systolic function. Thus, these studies demonstrated that TLR4 deficiency in mice was associated with RV hypertrophy with well preserved systolic function at this stage.

In addition, Doppler echocardiography of the pulmonary outflow was utilized to estimate pulmonary artery (PA) pressure in mice non-invasively (27,28). Doppler recordings of the main PA in the right parasternal long axis position were obtained. As indicated in Figure 3D, the PA blood flow velocity was measured at the main PA of the WT and TLR4<sup>-/-</sup> mice (Figure 3A), and the PA acceleration time (PAAT) was determined from the start to the peak of the flow signal as indicated (Figure 3E). PAAT of the TLR4<sup>-/-</sup> mice exhibited a 23% decrease compared with WT mice (Figure. 3F). The

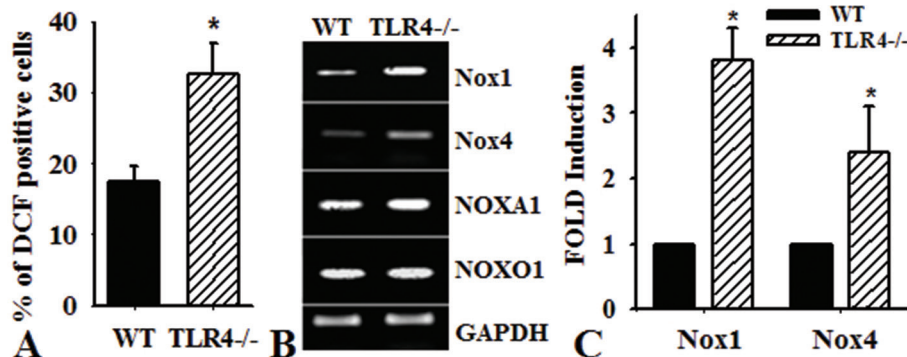
shorter PAAT in TLR4<sup>-/-</sup> is consistent with increased RV and pulmonary pressure, which has been used for estimating PAH in mouse and rat models (27,32). Taken together, the short PAAT and RV hypertrophy in the TLR4<sup>-/-</sup> mice compared to WT mice are consistent with pulmonary hypertension.

#### 4.4. Increased ROS production in PASMC from TLR4<sup>-/-</sup> mice

PASMC from TLR4<sup>-/-</sup> mice exhibited increased intracellular ROS production compared to PASMC from WT mice (Figure 4A, n=4, \*p<0.001). To identify the NADPH oxidase that was responsible for the increased ROS, we characterized the expression of Nox subunits that are most abundant in smooth muscle cells, Nox1 and Nox4. Increased expression of Nox1 and Nox4 was demonstrated in PASMC from TLR4<sup>-/-</sup> mice compared with that in WT and TLR2<sup>-/-</sup> mice, as determined by RT-PCR (Figure 4B). The expression of Nox1 activation subunit NOXA1, an important component of NADPH oxidase in SMC (33), increased; whereas NOXO1 was not affected. Real-time PCR analysis confirmed



**Figure 3.** Echocardiography evaluation of right ventricle and pulmonary arteries of wild type and TLR4<sup>-/-</sup> mice. A). Representative WT mouse B-mode long axis of RV and LV. B). M-mode tracing of the RV captured in a line perpendicular to the long axis of the chamber passing through the tip of the left posterior papillary muscle (left). RVEDD and RVFW thickness at end-diastole (left lines) and systole (right lines). C). *In vivo* Echo M-mode RV measurements for parasternal long axis of the WT and TLR4<sup>-/-</sup> mice. D). Representative Doppler echocardiography tracings of pulmonary artery blood flow velocity in the main pulmonary artery. E). Illustration of the measurement of pulmonary arterial acceleration time (PAAT). The PAAT was determined from the start to the peak of the flow signal of three consecutive beats. Two areas were measured for each mouse. F). PAAT in WT (n=4 mice) and TLR4<sup>-/-</sup> mice (n=3, \*p<0.05)



**Figure 4.** TLR4 deficiency increases the expression of Nox1 and Nox4 in PASMC. PASMC were isolated from pulmonary arteries of mice deficient in TLR4 (TLR4<sup>-/-</sup>) or wild type (WT) mice. A). Oxidative stress in PASMC from WT and TLR4<sup>-/-</sup> mice cultured in growth media for 48 hours was determined by assessing DCF-DA by flow cytometry. Results show the percentage of cells that positively stained for DCF (n=4, \*p<0.001). B). RT-PCR analysis was performed to determine the expression of NOX enzymes in WT and TLR4<sup>-/-</sup> PASMC. The primers for mouse NADPH oxidase genes are described in materials and Methods. The expression of GAPDH was used as a control. C). Real-time PCR analysis of the expression of Nox1 and Nox4 in WT and TLR4<sup>-/-</sup> PASMC (n=3, \*p<0.001).

increased the expression of Nox1 and 4 in TLR4<sup>-/-</sup> PASMC compared with that in WT PASMC (Figure 4C).

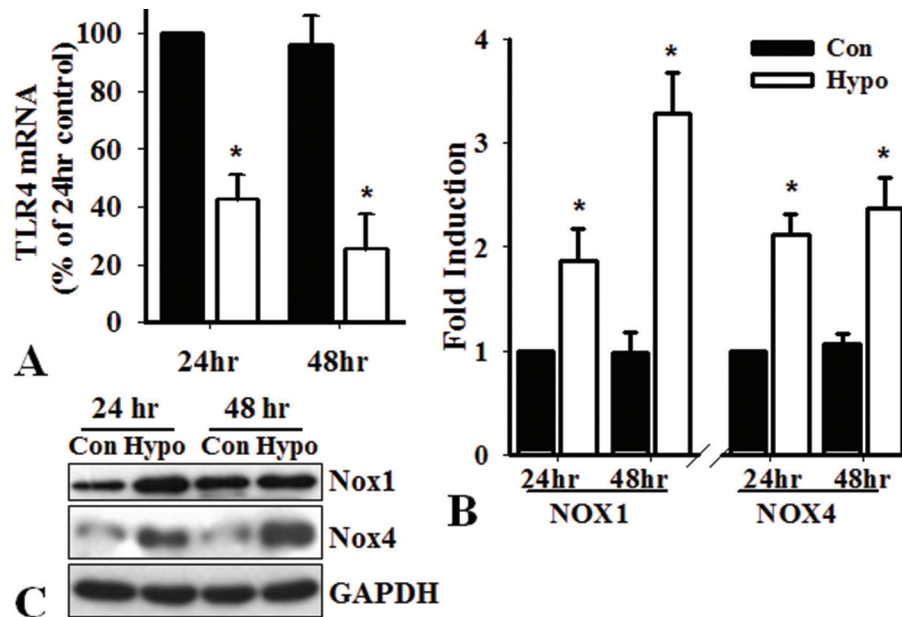
#### 4.5. Hypoxia inhibits TLR4 expression and induces NADPH expression in PASMC

To determine whether hypoxia affects the expression of TLR4, PASMC from WT mice were incubated in a hypoxia chamber (2% oxygen) for 24 and 48 hours. Real-time PCR analysis showed a 58% and 75% decrease of TLR4 mRNA after PASMC were exposed to hypoxia for 24 or 48 hours, respectively (Figure 5A). These results are consistent with the previous observation that

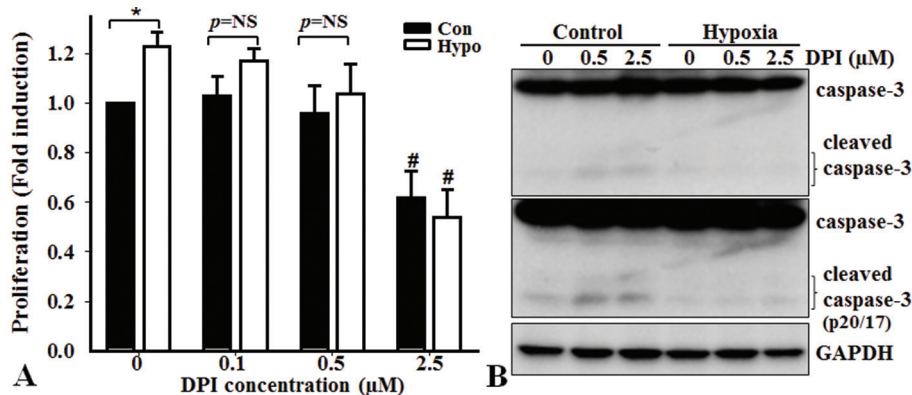
hypoxia diminishes the expression of TLR4 through ROS in endothelial cells (22). By contrast, hypoxia induced the expression of Nox1 and Nox4, as determined by real-time PCR as well as Western blot analysis (Figure 5B&C). Therefore, decreased expression of TLR4, genetically or hypoxia-induced, and increased expression of Nox1 and 4 in PASMC are likely to contribute to the development of PAH.

#### 4.6. Hypoxia-induced PASMC proliferation is decreased by an NADPH inhibitor

As ROS-induced abnormal PASMC proliferation contributes to vascular remodeling, we determined the



**Figure 5.** Hypoxia decreases the expression of TLR4 and induces Nox1 and Nox4. PASM from WT mice were seeded at 80% confluence and exposed to control (20% oxygen, Con) or hypoxia (2% oxygen, Hypo) condition for 24 and 48 hours. Real-time PCR analysis of the expression of A). TLR4 using primers for mouse TLR4: F-5'-GCTTTCACCTCTGCCTTCAC; R-5'-CGAGGCTTTTCCATCCAATA; B). Nox1 and Nox4 using specific primers shown in Figure 4. Results shown are the expressions of TLR4, Nox1 and Nox4 mRNA (normalized by GAPDH mRNA) as percentage of that in cells exposed to condition for 24 hours, defined as 100% (n=3, \* $p < 0.05$ ). C). Western blot analysis of the expression of Nox1 and Nox4 proteins. The expression of GAPDH was used as a loading control.



**Figure 6.** Inhibition of NADPH oxidase activity decreases hypoxia-induced proliferation of PASM. PASM from WT mice were seeded at 80% confluence and exposed to control (Con) or hypoxia (Hypo) condition with or without NADPH oxidase inhibitor DPI for 24 hours. A). Proliferation was determined by BrdU incorporation (n=3, \* $p < 0.05$ ;  $p = NS$ , no significant difference; # $p < 0.05$  compared with their related control conditions without DPI). B). Western blot analysis of caspase-3. The blot in the middle is a longer exposure of the upper one, to show the cleaved form of caspase-3. The expression of GAPDH was used as a loading control. Representative blots of three independent experiments are shown.

effect of hypoxia on PASM proliferation and the effect of an NADPH oxidase inhibitor, DPI. Hypoxia induced the proliferation of PASM, which was inhibited by DPI in a concentration-dependent manner, as demonstrated by BrdU incorporation assays (Figure 6). Furthermore, the effect of DPI on PASM proliferation was not due to induction of apoptosis, because DPI did not induce cleavage/activation of caspase-3, an indicator for apoptosis. These results confirmed that increased activation of NADPH oxidase mediated hypoxia-induced PASM proliferation.

## 5. DISCUSSION

In animal models of hypoxia-induced PAH, vascular remodeling and right ventricle hypertrophy are prominent characteristics (9;25). In the present study, we demonstrated that mice deficient in TLR4 spontaneously developed PAH, featuring increased RV pressure and hypertrophy of pulmonary arteries compared with wild type mice or mice with TLR2 deficiency. In contrast to hypoxia-induced PAH in the wild type and TLR2<sup>-/-</sup> mice, spontaneously developed PAH in TLR4<sup>-/-</sup> mice was



not increased by hypoxia, supporting a role of TLR4 in mediating hypoxia-induced PAH. Consistent with previous observations in endothelial cells that hypoxia decreases the expression of TLR4 via increased ROS (22), we found hypoxia decreased TLR4 expression in PASMC, supporting that hypoxia may contribute to PAH via down-regulation of TLR4.

Hypoxia-induced ROS production regulates vascular remodeling in PAH. Therefore, increased ROS production in PASMC from TLR4<sup>-/-</sup> mice may contribute to PAH in the TLR4<sup>-/-</sup> mice. As the major source of ROS in the vasculature, the NADPH oxidase family of enzymes is distributed throughout all three layers of the vascular wall (4,5). NADPH oxidase-dependent ROS generation has been described in pulmonary endothelial cells and smooth muscle cells (3,34-36). Nox4 and Nox1 are predominant NADPH oxidases in smooth muscle cells. Nox-derived ROS is thought to be a hypertensive signaling element in the vasculature (5,37-39). Hypoxia-induced activation of Nox1 and Nox4 leads to ROS production that plays an important role in pulmonary hypertension (4,7,9,10,36,40,41). Previous studies have demonstrated increased expression of NOX4 in patients with PAH; as well as in lungs and pulmonary artery of mice after chronic exposure to hypoxia (36). With the use of PASMC, we found that hypoxia induced the expression of Nox1 and Nox4. Furthermore, increased expression of Nox1 and Nox4 were determined in TLR4<sup>-/-</sup> PASMC. Accordingly, increased ROS and NADPH oxidases may contribute to hypoxia-induced PAH, as well as PAH in the TLR4<sup>-/-</sup> mice. However, the molecular mechanisms linking TLR4 and Nox1 and Nox4 activation are not known.

TLR4 was first characterized through studies of LPS hypo-responsive C3H/HeJ mice (42), which possess a point mutation that renders the receptor unable to transduce a signal (43). In addition to LPS, increasing evidence has demonstrated that host-derived endogenous ligands of TLRs such as heat shock protein 60, oxidized low-density lipoproteins and fibronectin are also able to activate immune responses through TLR4 and induce an inflammatory reaction that leads to atherosclerotic and injured arteries (20,44,45). In general, TLR4 deficiency protects mice from endotoxin, ischemia-reperfusion, and ozone-induced injury (24,46,47). As TLR4 has been well-studied as a microbial pattern receptor for human body's first line of defense against infectious agents, one would anticipate that TLR4 deficiency will result in hyposensitivity of host cells to bacteria or virus infection, a common risk for heart and lung disease. Therefore, we analyzed the serum concentrations of 22 cytokines and chemokines, including interleukins 1-17, interferon- $\gamma$ , MCP-1 and TNF $\alpha$ , using Bio-Plex Protein Array System and a Mouse Cytokine 22-plex Panel (Bio-Rad). However, we found that TLR4 deficiency did not affect serum levels of the tested cytokines and chemokines (data not shown), suggesting additional signals independent of the

role of TLR4 as a receptor for LPS might be involved in promoting PAH.

Upregulation and activation of Nox1 or Nox4 NADPH oxidases have been linked to activation of TLR4 signaling, in which TLR4 functions as a receptor for ligands such as LPS (48-52). Upregulation of Nox1 by *Helicobacter pylori*-derived LPS in guinea pig gastric pit cells is mediated through TLR4-dependent pathways (51,52). On the other hand, Nox4 has been shown to be a major downstream effector of TLR4 signaling in human aortic endothelial cells and SMC (48-50). Nox4 knockdown by siRNA inhibits TLR4 signaling-induced ROS production and NF $\kappa$ B signaling (48-50). Therefore these studies with LPS, a potent TLR4 activator, suggest a role of the Nox1 and Nox4 NADPH oxidase in mediating TLR4-activated signaling.

Our finding that TLR4<sup>-/-</sup> mice developed PAH is consistent with previous observations that TLR4 is essential for maintaining normal homeostasis of gut, lung and vasculature independent of its well established role as the LPS receptor that mediates the inflammatory signals (23;53;54). TLR4 deficiency has been shown to confer susceptibility to lethal oxidant lung injury (53). Lack of TLR4 or MyD88 causes emphysema in mice (23). Furthermore, TLR4 and MyD88 are necessary for intestinal epithelial homeostasis and protection from injury (54). We found that spontaneously developed PAH in the TLR4<sup>-/-</sup> mice was not enhanced by hypoxia, which is consistent with the observations by Young *et al* that TLR4 deficiency conferred resistance to hypoxia-induced pulmonary hypertension (55). Of note, different strains of mice were used in that study; and the basal level of right ventricular pressure and RV weight seem higher in the TLR4<sup>-/-</sup> mice (55). Recent studies from Bauer *et al* demonstrated that platelet but not myeloid-derived cells contributed to the effects of TLR4 in hypoxia-induced PAH (56). They also found that high mobility group box 1 mediated the function of TLR4 in PAH (57). However, the underlying mechanisms by which TLR4 maintains basal vascular homeostasis have not been fully elucidated. TLR4 deficiency in lungs confers susceptibility to lethal oxidant lung injury (53), which is associated with increased expression of anti-apoptotic signals, such as Bcl-2 and phosphorylated-AKT protein (53). TLR4 deficiency was associated with decreased inflammatory response induced by hypoxia (55). Increased oxidative stress, as determined by ROS production, as well as Nox expression, has been found in lung endothelial cells from TLR4 deficient mice (23). Similar to this later observation, we found increased ROS production in the PASMC from TLR4<sup>-/-</sup> mice, which contributed to increased proliferation of these cells.

NOX-derived ROS have also been shown to have a pro-survival effect (11-13). In addition, ROS serves as second messengers to induce cell proliferation



in a variety of cells (11;58-60). Increased production of ROS is associated with greater proliferation in tumor cells (61). In SMC, increased Nox4-derived ROS promotes proliferation; whereas inhibition of Nox4 reduces ROS production and SMC proliferation (50;60). The increased ROS production in TLR4<sup>-/-</sup> PASMC was associated with increased expression of Nox1 and Nox4, the two predominant NADPH oxidase isoforms in the SMC. The proliferative role of Nox1 and Nox4 in SMC is supported by many studies. NOX1 has been referred as a "mitogenic oxidase 1", which is related to its mitogenic effect on SMC proliferation (62). Nox1 induces the generation of hydrogen peroxide that promotes cell growth and transformation (40). Overexpression of NOX1 enhances vascular hypertrophy (38). In Nox1-deficient mice, angiotensin II-induced SMC proliferation was inhibited (56). Several recent studies have demonstrated an important role of Nox4 in regulating SMC proliferation (36,50,60,63,64). In resting SMC, NADPH oxidase activity largely depends on NOX4 expression (63). The expression of NOX4 is upregulated in patients with PAH (36). Hypoxia upregulates NOX4 in homogenized lung tissue, pulmonary arterial vessels and medial PASMC (36), supporting an important role of NOX4 in pulmonary vascular remodeling in chronic hypoxia-induced PAH. Treatment of PASMC with Nox4 siRNA reduced ROS production and cell proliferation induced by transforming growth factor-beta1 (60). Consistently, increased Nox4 promotes proliferation and inhibition of Nox4 reduces proliferation of PASMC (50,60). Using a NADPH oxidase inhibitor, we confirmed that hypoxia-induced PASMC proliferation was mediated by NADPH oxidase. Therefore, upregulation of Nox1 and Nox4 expression in PASMC from TLR4<sup>-/-</sup> mice and in PASMC exposed to hypoxia may mediate increased PASMC proliferation and thus vascular remodeling in PAH.

Taken together, the present studies demonstrating that TLR4-deficient mice spontaneously develop PAH that is not enhanced by hypoxia, and that hypoxia reduces TLR4 expression, suggest that TLR4 plays an important role in maintaining normal pulmonary vasculature and hypoxia induces PAH via TLR4. Inhibition of TLR4 in PASMC, by genetic ablation or hypoxia was associated with increased expression of the Nox1 and Nox4 NADPH oxidase, which contributed to increased PASMC proliferation and vascular remodeling. These results demonstrate a novel function of TLR4 in regulating the development of PAH and identify a novel regulatory axis that is responsible for TLR4 deficiency-induced vascular hypertrophy and remodeling. Our studies provide novel insights into the crosstalk between TLR4 and oxidative stress signaling in regulating cell proliferation, which will likely be useful for our understanding of other heart and lung diseases that are characterized by increased vascular hypertrophy and remodeling.

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