Genetic Deletion of Toll-Like Receptor 4 on Platelets Attenuates Experimental Pulmonary Hypertension

Eileen M. Bauer, R. Savanh Chanthaphavong, Chhinder P. Sodhi, David J. Hackam, Timothy R. Billiar, Philip M. Bauer

Rationale: Recent studies demonstrate a role for toll-like receptor 4 (TLR4) in the pathogenesis of pulmonary hypertension (PH); however, the cell types involved in mediating the effects of TLR4 remain unknown.

Objectives: The objective of this study was to determine the contribution of TLR4 expressed on nonparenchymal cells to the pathogenesis of PH.

Methods and Results: TLR4 bone marrow chimeric mice revealed an equal contribution of TLR4 on nonparenchymal and parenchymal cells in the pathogenesis of PH as determined by measuring right ventricular (RV) systolic pressure and RV hypertrophy. However, the deletion of TLR4 from myeloid lineage cells had no effect on the development of PH because we found no difference in RV systolic pressure or RV hypertrophy in wild-type versus LysM-TLR4−/− mice. To explore the potential role of platelet TLR4 in the pathogenesis of PH, platelet-specific TLR4−/− mice were generated (PF4-TLR4−/− mice). TLR4−/− platelets from either global TLR4−/− or PF4-TLR4−/− mice were functional but failed to respond to lipopolysaccharide, demonstrating a lack of TLR4. PF4-TLR4−/− mice demonstrated significant protection from hypoxia-induced PH, including attenuated increases in RV systolic pressure and RV hypertrophy, decreased platelet activation, and less pulmonary vascular remodeling. The deletion of TLR4 from platelets attenuated serotonin release after chronic hypoxia, and lipopolysaccharide-stimulated platelets released serotonin and promoted pulmonary artery smooth muscle cell proliferation in a serotonin-dependent manner.

Conclusions: Our data demonstrate that TLR4 on platelets contributes to the pathogenesis of PH and further highlights the role of platelets in PH. (Circ Res. 2014;114:1596-1600.)

Key Words: blood platelets ■ hypertension, pulmonary ■ toll-like receptor 4

Pulmonary hypertension (PH) is a progressive and fatal disease with no cure. Historically, PH research has relied on concepts derived from hypoxic pulmonary vasoconstriction for the development of new therapies. Increasingly, however, thrombotic and inflammatory mechanisms are recognized as complicating and possibly initiating events in PH.

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Toll-like receptors (TLRs) are one family of receptors that the innate immune system uses to sense pathogens or tissue damage by recognizing molecular patterns in microbial products or endogenous molecules released by damaged tissues. TLR4, perhaps more than any other TLR, interfaces microbial and sterile inflammation by responding to bacterial lipopolysaccharides and endogenous ligands, including hyaluronic acid, heparin sulfate, high-mobility group box 1, and heat shock proteins. Recent studies demonstrate that the activation of TLR4 contributes to the pathogenesis of PH.

The lung is equipped to sense and respond to endogenous TLR4 ligands. The lung consists of parenchymal cells, including fibroblasts, endothelial cells (ECs), and smooth muscle cells, and nonparenchymal cells (NPCs), including alveolar macrophages, dendritic cells, granulocytes, and transiting leukocytes and platelets. Both parenchymal cells and NPCs express TLR4 and have intact TLR4 signaling pathways. The aim of this study was to determine the role of TLR4 on NPCs in the pathogenesis of PH.

Methods

Animal procedures were performed in accordance with University of Pittsburgh Institutional Animal Care and Use Committee guidelines.
TLR4loxP/loxP mice were generated as described and interbred with C57BL/6 males (TLR4loxP/−;Ella-cre, TLR4loxP/loxP;PF4-cre, or TLR4loxP/−;PF4-cre) to generate the desired genotype. Wild-type (WT) mice were TLR4loxP/loxP mice without the introduction of Cre recombinase. Bone marrow (BM) was isolated from WT or TLR4−/− mice and transplanted into irradiated WT or TLR4−/− mice as described. PH was induced by exposure to chronic hypoxia (CH) for 21 days or the combination of SU5416 (a vascular endothelial growth factor receptor inhibitor) plus CH (SUH) as described. Right ventricular systolic pressure (RVSP) and RV hypertrophy (RVH) were measured by established methods. Pulmonary vascular muscularization and percent wall thickness were assessed as described. Bleeding time was measured as described. Platelet-rich plasma was prepared from whole blood anticoagulated with acid citrate dextrose. To isolate platelets, platelet-rich plasma was centrifuged at 800 g and platelets were resuspended in Tyrodes buffer. Serotonin and interleukin-6 were measured by ELISA. Platelet activation was determined using flow cytometry. The effect of platelets on human pulmonary artery smooth muscle cell (HPASMC) proliferation was assessed by coculturing lipopolysaccharide-activated platelets with HPASMC. Cell proliferation was assessed by 3H-thymidine incorporation. Statistical analyses were performed using GraphPad Prism software.

**Results**

**Equal Contribution of NPCs and Parenchymal Cell TLR4 to Hypoxia-Induced PH**

To deduce a role for TLR4 in NPCs versus parenchymal cells, we performed BM transplantation into irradiated mice to create WT and TLR4−/− BM chimeric mice. After transplantation and recovery, mice were exposed to CH to induce PH. WT mice that received WT BM developed PH as determined by measuring RVSP and RVH. PH was attenuated in TLR4−/− mice that received TLR4−/− BM (Figure 1A). Interestingly, WT mice that received TLR4−/− BM, or TLR4−/− mice that received WT BM, showed the same attenuation of PH as whole-body TLR4−/− chimeric mice.

**TLR4 on Myeloid-Derived Cells Does Not Contribute to Hypoxia-Induced PH**

TLR4 is highly expressed on cells of myeloid lineage, and these cells are often associated with response to TLR4 agonists. To investigate a role for TLR4 on myeloid-derived cells, we exposed WT or LysM-TLR4−/− mice to 3-week CH. The loss of TLR4 on myeloid-derived cells failed to attenuate the development of PH as assessed by measuring RVSP (Figure 1C) and RVH (Figure 1D).

**Functional Characterization of Platelet TLR4 Knockout Mice**

To investigate a role for platelet TLR4 in PH, we used platelet-specific TLR4−/− (PF4-TLR4−/−) mice. Platelets were isolated from WT, TLR4−/−, and PF4-TLR4−/− mice and stimulated with lipopolysaccharide. WT platelets upregulated surface P-selectin after lipopolysaccharide stimulation, whereas platelets from TLR4−/− or PF4-TLR4−/− mice did not (Figure 2A). Collagen induced P-selectin expression on platelets isolated from all 3 genotypes, demonstrating that TLR4−/− and PF4-TLR4−/− platelets were functional. Additionally, serotonin was increased in the supernatant of WT platelets, but not TLR4−/− or PF4-TLR4−/− platelets, after stimulation by lipopolysaccharide (Figure 2E).

To demonstrate the specificity of platelet knockout, we tested the effect of lipopolysaccharide on ECs isolated from the lungs of WT, TLR4−/−, or PF4-TLR4−/− mice. The ECs expressed CD31 but not smooth muscle α actin, demonstrating purity (Figure 2E). Lipopolysaccharide induced interleukin-6 release from ECs derived from WT or PF4-TLR4−/− mice, but not from ECs derived from TLR4−/− mice (Figure 2F).

**Genetic Deletion of Platelet TLR4 Attenuates Hypoxia-Induced PH**

We next exposed WT, TLR4−/−, and PF4-TLR4−/− mice to 3-week CH to induce PH. The deletion of TLR4 globally or on platelets significantly attenuated PH as assessed by measuring RVSP and RVH (Figure 3A and 3B).

**Genetic Deletion of Platelet TLR4 Attenuates Platelet Activation in Hypoxia-Induced PH**

Exposing mice to CH leads to decreased bleeding time and increased platelet surface P-selectin expression, indicative of platelet activation. In both the CH and SUH models, bleeding time decreased in WT mice but not in TLR4−/− or
PF4-TLR4−/− mice (Figure 3C). Similarly, platelet surface P-selectin expression was increased in CH and SUH WT but not TLR4−/− or PF4-TLR4−/− mice (Figure 3D).

Genetic Deletion of Platelet TLR4 Attenuates Vascular Remodeling in Hypoxia-Induced PH
TLR4−/− or PF4-TLR4−/− CH or SUH mice exhibited less pulmonary vascular remodeling compared with WT mice (Figure 4A). Morphometric analysis of peripheral arterioles revealed less thickening of the vessel wall in TLR4−/− or PF4-TLR4−/− mice versus WT mice (Figure 4B). Likewise, there was less muscularization of peripheral arterioles in TLR4−/− or PF4-TLR4−/− mice versus WT mice (Figure 4C and 4D). Plasma serotonin was increased in CH WT mice but not TLR4−/− or PF4-TLR4−/− mice. Coculturing HPASMC with lipopolysaccharide-stimulated WT platelets, but not TLR4−/− platelets, promoted HPASMC proliferation (Figure 4E). This effect was blocked by GR127935, a 5HT1B receptor antagonist (Figure 4F). Figure 4G shows the proposed conceptual framework for how platelet TLR4 fits into TLR4 and serotonin signaling in PH.
Discussion

In addition to their role in hemostasis and thrombosis, platelets are mediators of inflammation and immune responses. They express several TLRs (TLR1, 2, 4, 6, 8, 9), pattern recognition receptors involved in the innate immune response by recognizing microbial structures and endogenous molecules released from damaged, stressed, or activated cells. Lipopolysaccharide, a TLR4 agonist, induces platelet P-selectin expression and interleukin-1β and ATP secretion and primes platelets to aggregate in response to low-dose thrombin. Thus, platelet TLRs bridge innate immunity and coagulation. Previous studies from our laboratory demonstrated a role for TLR4 in the pathogenesis of PH. The major finding of this study is that genetic deletion of TLR4 on platelets, but not myeloid cells, attenuates the pathogenesis of PH. Furthermore, platelets are activated to express surface P-selectin and release serotonin during hypoxia by TLR4-dependent mechanisms. Thus, the deletion of TLR4 globally or on platelets prevented CH- or SUH-induced PH, in vivo platelet activation, and pulmonary vascular remodeling. Importantly, this study is the first to show that genetic deletion of a platelet surface receptor can attenuate PH.

There is an emerging role of platelet-derived mediators, such as serotonin, thromboxane-A2, and growth factors in patients with severe PH. These vasoactive mediators promote vasoconstriction (thromboxane-A2, serotonin), thrombosis (thromboxane-A2), and proliferation of vascular smooth muscle cells, ECs, and fibroblasts (serotonin, platelet-derived growth factor). Furthermore, platelet aggregation is enhanced by the altered balance of proaggregatory molecules (thromboxane-A2) and antiaggregatory molecules (nitric oxide, prostacyclin).

The serotonin hypothesis of PH was postulated in the 1960s when it was discovered that women taking an indirect serotonergic agonist developed PH. More recently, it was recognized that patients with PH have markedly elevated plasma serotonin. Data demonstrate that serotonin released from ECs binds to serotonin receptors on PASMC or is taken up by PASMC via the serotonin transporter stimulating PASMC proliferation, migration, and contraction, thus contributing to vascular remodeling in PH. Furthermore, mice deficient in bone morphogenetic protein receptor 2 are more sensitive to serotonin-induced PH, which was associated with the inhibition of Smad1/5 phosphorylation. These data suggest that, in humans, increased serotonin could provide a second hit necessary for the development of PH caused by bone morphogenetic protein receptor 2 haploinsufficiency. In this study, we found that genetic deletion of TLR4 on platelets abrogated platelet activation and prevented the increase in plasma serotonin in 2 experimental models of PH. Furthermore, coculturing HPASMC with lipopolysaccharide-stimulated WT platelets, but not TLR4−/− platelets, promoted HPASMC proliferation via a mechanism that was dependent on the 5HT1B receptor. Together, our data suggest that TLR4 plays...
a role in platelet activation and serotonin release in PH and that platelets are an important source of serotonin in PH.

It was surprising to find that the loss of TLR4 on myeloid cells did not influence the disease course because myeloid cells are important responders to TLR4 ligands. It is possible that TLR4 on myeloid cells works toward counter purposes in PH, thus masking the role of TLR4 on individual cell types. It may also be a limitation of the CH mouse model that the role of these cells in sensing endogenous TLR4 ligands is diminished or absent because of the mild inflammatory phenotype. Future studies will be necessary to sort out the role of TLR4 on myeloid cells in the pathogenesis of PH.

In summary, this study demonstrates the importance of platelet TLR4 in PH because its deletion from platelets improved disease outcome. These data suggest that platelet TLR4 is a proximate promoter of platelet activation and serotonin release in PH. We proffer that drugs interrupting TLR4 interaction with its endogenous ligands may limit platelet activation and inflammation and lead to better therapies for PH.

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Disclosures
None.

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5. Thorpe AM, Hunter KD, Mowat AM, Birmark CA, Dryden DW, Maclean GJ, Scott D, Forsyth RC, Sleigh BJ, Higginson C, Idris AA. Platelet TLR4 is a proximate promoter of platelet activation and serotonin release in PH. Platelet TLR4 contributes to PH. Indeed, the deletion of TLR4 from these cells had no effect on PH. Platelets, in addition to their role in thrombosis, are mediators of inflammation and play a role in platelet activation and serotonin release in PH.

Novelty and Significance

What Is Known?

• Toll-like receptor 4 (TLR4) contributes to the pathogenesis of pulmonary hypertension (PH) via largely unknown mechanisms.
• Platelets participate actively in thrombus formation and produce (e.g., thromboxane-A2), store (e.g., serotonin), and release mediators that may contribute to the initiation or aggravation of PH.
• Platelets express functional TLR4, and TLR4 agonists stimulate release of soluble mediators from platelets.

What New Information Does This Article Contribute?

• TLR4 on both parenchymal and nonparenchymal cells (NPCs) contribute to the pathogenesis of PH.
• Platelet TLR4 is a proximate promoter of platelet activation and serotonin release in PH.
• Serotonin released from platelets may contribute to increased pulmonary vascular smooth muscle cell proliferation and pulmonary vascular remodeling.

Despite evidence for thrombotic and inflammatory mechanisms in PH, no drugs have been developed to target these mechanisms. Recent data from our laboratory demonstrate a role for the innate immune receptor TLR4 in PH. We sought to determine the role of TLR4 on bone marrow (BM)–derived cells in PH. BM chimera experiments revealed an equal contribution of TLR4 on NPCs and parenchymal cells. Although myeloid lineage cells are most often associated with TLR4 responses, the deletion of TLR4 from these cells had no effect on PH. Platelets, in addition to their role in thrombosis, are mediators of inflammation and express functional TLR4. We, therefore, hypothesized that platelet TLR4 contributes to PH. Indeed, the deletion of TLR4 on platelets attenuated platelet activation, serotonin release, pulmonary vascular remodeling, and PH in 2 experimental models of the disease. These data demonstrate that platelet TLR4 is a proximate promoter of platelet activation and serotonin release in PH and suggests that drugs interrupting TLR4 interaction with its endogenous ligands may limit platelet activation and inflammation and lead to better therapies for PH.
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SUPPLEMENTARY MATERIALS

Genetic deletion of TLR4 on platelets attenuates experimental pulmonary hypertension

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Supplemental Methods

Animals. Male wild-type (WT) (TLR4loxP/loxP) mice, cell-specific, and global TLR4−/− mice were bred at our facility and used at the age of 8–12 weeks. All mice developed were on a C57BL/6 genetic background. Animal protocols were approved by the animal care and use committee of the University of Pittsburgh (Pittsburgh, PA), and experiments were performed in strict adherence to the National Institutes of Health Guidelines for the Use of Laboratory Animals.

Generation of TLR4loxP/loxP and Cellular-Specific TLR4−/− Mice. In brief, the TLR4loxP allele was created by inserting loxP sites within introns 1 and 2 and flanking exon 2 of TLR4. Mice homozygous for TLR4loxP were generated by Ozgene (Bentley, WA). TLR4loxP/loxP mice were interbred with stud males (TLR4loxP/−; EIIα-cre, TLR4loxP/−; LyzM-cre, or TLR4loxP/−; PF4-cre) to generate the desired genotype. Mice homozygous for Cre recombinase linked to the lysozyme (LysM), platelet factor 4 (PF4), and EIIa promoter are commercially available from The Jackson Laboratory (Bar Harbor, ME). Tg male mice used for experiments were confirmed to be a desired genotype by standard genotyping techniques. WT mice used in this study were TLR4loxP/loxP mice without the introduction of Cre recombinase. Global TLR4−/− mice were globally lacking the loxP flanked exon 2 (i.e., they were global homozygotes for the same mutation contained within the conditional KO mice). Sodhi et al. have recently provided a detailed description of the novel TLR4−/− mice used in this study 1.

Chronic Hypoxia and Su5416-Hypoxia Mouse Model. Eight to ten week old male mice were placed into a partially ventilated Plexiglass chamber (Biospherix,) and exposed to chronic hypoxia (FIO2=0.10, 90% nitrogen) for 21 or 42 days under normobaric conditions. Mice maintained in room air served as normoxic controls. For the Su5416-Hypoxia model the mice were injected weekly with Su5416 (20mg/kg) beginning on day 0 and maintained for 3 weeks in hypoxia as described by Ciuclan et al. 2

Right Ventricular Systolic Pressure. Right ventricular systolic pressure (RVSP) was measured essentially as described 3. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and ventilated via tracheotomy with room air. Body temperature was monitored and regulated with a rectal temperature probe and heating pad. RVSP was determined by placing a 1 F solid-state pressure transducing catheter (Millar Instruments, Houston, TX, USA) directly into the right ventricle (RV). Data were acquired using a PowerLab data acquisition system and LabChart Pro software (AD Instruments).

Right Ventricular Hypertrophy. Following hemodynamic measurements the vasculature was flushed with PBS, the heart was excised and right heart hypertrophy was determined by the ratio of the weight of the RV to the left ventricle (LV) plus septum (Fulton index) or the ration of weight of the RV to body weight. The right lung was tied off, dissected and flash frozen, and the left lung was perfused with paraformaldehyde (4%) for embedding in paraffin.

Assessment of Pulmonary Vascular Remodeling. Pulmonary vascular remodeling was assessed by counting the number of partially and fully muscularized peripheral arterioles (35–100 mm) per high-power field (200x total magnification). For each mouse, at least 20 highpower fields were analyzed in multiple lung sections. Wall thickness % was determined by measuring the thickness at four points on pulmonary arterioles using the Java-based image processing program ImageJ (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemistry. Paraffin-embedded lung sections (5 μm) were baked 60 min at 55°C, deparaffinized in xylenes and rehydrated through decreasing alcohol concentrations (three xylenes, 2x100%, 1x95%, 1x90%, 1x70% ethanol, 1xPBS, for 3 min each) followed by antigen retrieval in citrate buffer by using a microwave. Smooth muscle α-actin staining was performed
as described 4.

**Bleeding Time.** Mice were anesthetized with isoflurane and cut was made 3 mm from the tip of the tail. After transection, the tail was placed in a beaker filled with 37°C phosphate buffered saline, and bleeding time was recorded. After bleeding succession another 30 sec was waited for possible rebleeding. Bleeding was stopped at 7 min and all tails were cauterized.

**Preparation of Platelet Rich Plasma:** Mouse blood was obtained via cardiac puncture at the time of sacrifice in 1/7 volume acid-citrate dextrose (ACD; 85mM trisodium citrate, 110mM dextrose, and 78mM citric acid) as an anti-coagulant. The blood is spun at room temp for 15 min at 1000 rpm to obtain platelet rich plasma.

**Preparation of Washed Platelets:** For the preparation of mouse platelets, 6- to 8-wk-old mice of either sex were anesthetized with an i.p. injection of pentobarbital and blood was drawn by cardiac puncture in 1/7 volume ACD. Blood from five to six mice was pooled. Platelets were isolated by centrifugation (2000 RPM 10 min). The pelleted platelets were washed twice with CGS buffer (0.12 M sodium chloride, 0.0129 M trisodium citrate, and 0.03 D-glucose (pH 6.5)), resuspended in freshly made Tyrode’s buffer (140 mM NaCl, 0.36 mM Na2HPO4, 3 mM KCl, 12 mM NaHCO3, 5 mM Heps, and 10 mM glucose, pH 7.3), and allowed to rest for at least 1 h at 37°C before use.

**Measurement of IL6 and Serotonin.** IL6 and serotonin were measured using commercially available ELISA kits per the manufacturer’s instructions.

**P-selectin expression:** PRP was prepared and labeled with PE-anti-mouse-CD41 and FITC-anti-mouse-CD62P (BD bioscience) antibodies following the manufacturer’s protocol for “staining platelets for activation”. PE-IgG1-k or FITC IgG1-λ were used as isotype control. Cells were analyzed by FACS (Guava-easy-cyte *HT*) using Guava Express Pro 8.1 software, gating for CD41 positive cells (platelet marker).

**Cultured murine lung endothelial cells:** Murine lung endothelial cells were isolated by modifications of an immunobead protocol 5. Briefly, mouse lungs were rinsed in PBS, finely minced and digested in collagenase (Type I, 100u/ml,) for 60min at 37°C with occasional agitation. The mixture was filtered through 100 µm cell strainer, centrifuged and washed twice in medium. The cell suspension was incubated with rat anti-mouse PECAM monoclonal antibody (Pharmingen) 30 min at 4°C. The cells were washed twice with buffer to remove unbound antibody, and resuspended in binding buffer containing the appropriate number of washed magnetic beads coated with sheep anti-rat IgG (Dynal; Oslo, Norway). Cells attached to the beads were washed 4-5 times in cell culture medium, and then digested with trypsin/EDTA to detach beads from the cells. Bead-free cells are centrifuged and resuspended for culture. Cells were cultured in EGM2-MV media from Lonza at 37°C with 5% CO2. Cells were used at passage 1 or 2.

**Cultured Cells.** Human pulmonary artery smooth muscle cells (HPASMC) were from Lonza. HPASMC were grown in SBM cell culture media supplemented with the SBM2 bullet kit (Lonza). Cells were maintained at 37°C in a humidified cell culture incubator with 5% CO2 and used between passage 4-9.

**Coculture of Platelets with HPASMC.** HPASMC were subcultured into 24-well culture plates in at an initial density of 12,500 cells per well/cm². An equal number of HPASMC was plated in each well to exclude variation due to differences in cell number. Twenty-four hours later, HPASMC were made quiescent by incubation in fresh growth factor and serum free media for 24h. After 24h washed platelets (300,000) were placed into transwells (0.2 µm diameter, Transwell™, Becton Dickinson, Bedford, MA) and activated with LPS (100ng/ml) or left
undisturbed for 30 min at 37°C. After 30 min the transwells were placed into the wells with the HPASMC for an 60 min and then removed. All HPASMC were pretreated with the antibiotic polymyxin A to prevent activation of TLR4 by LPS used to treat the platelets. Some HPASMC were additionally treated with the 5HT1B receptor inhibitor GR127935 (1 μM).

**HPASMC Proliferation.** Proliferation of HPASMC was determined by measuring [3H]-incorporation as previously described[18]. Briefly, cells were cultured as described above in “Platelet, HPASMC Co-culture”. Once the transwell with platelets was removed 0.2μCi [3H]thymidine was added. After 24h the cells were washed twice with ice-cold PBS, and 1ml of ice-cold 10% trichloroacetic acid (Sigma T0699) was added to each well for a 30-min incubation at 4°C, after which each well was washed with 1 ml of ice-cold 10% trichloroacetic acid. To each well 0.5ml of 0.4 N NaOH, 0.1% (wt/vol) SDS was added, and the plates were incubated for 1h at room temperature. The contents of each well were then transferred to 7ml scintillation vials containing 4.5ml of Pico-Fluor-15 scintillation mixture (ICN) and counted in a liquid scintillation spectrometer.

References