Activation of Toll-like receptor 2 impairs hypoxic pulmonary vasoconstriction in mice

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Petersen B, Bloch KD, Ichinose F, Shin H-S, Shigematsu M, Bagchi A, Zapol WM, Helman J. Activation of Toll-like receptor 2 impairs hypoxic pulmonary vasoconstriction in mice. Am J Physiol Lung Cell Mol Physiol 294: L300–L308, 2008. First published November 30, 2007; doi:10.1152/ajplung.00243.2007.—Toll-like receptors (TLRs) mediate inflammation in sepsis, but their role in sepsis-induced respiratory failure is unknown. Hypoxic pulmonary vasoconstriction (HPV) is a unique vasoconstrictor response that diverts blood flow away from poorly ventilated lung regions. HPV is impaired in sepsis and after challenge with the TLR4 agonist lipopolysaccharide (LPS). Unlike TLR4 agonists, which are present only in Gram-negative bacteria, TLR2 agonists are ubiquitously expressed in all of the major classes of microorganisms that cause sepsis, including both Gram-positive and Gram-negative bacteria and fungi. We tested the hypothesis that (S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys=OH, trihydrochloride (PamCys), a TLR2 agonist, impairs HPV and compared selected pulmonary and systemic effects of PamCys vs. LPS. HPV was assessed 22 h after challenge with saline, PamCys, or LPS by measuring the increase in the pulmonary vascular resistance of the left lung before and during left lung alveolar hypoxia produced by left mainstem bronchial occlusion (LMBO). Additional endpoints included arterial blood gases during LMBO, hemodynamic parameters, weight loss, temperature, physical appearance, and several markers of lung inflammation. Compared with saline, challenge with PamCys caused profound impairment of HPV, reduced systemic arterial oxygenation during LMBO, weight loss, leukopenia, and lung inflammation. In addition to these effects, LPS-challenged mice had lower rectal temperatures, metabolic acidosis, and were more ill appearing than PamCys-challenged mice. These data indicate that TLR2 activation impairs HPV and induces deleterious systemic effects in mice and suggest that TLR2 pathways may be important in sepsis-induced respiratory failure.

h ypoxic pulmonary vasoconstriction impairment; lung inflammation; sepsis

ACUTE LUNG INJURY AND THE ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) FREQUENTLY COMPLICATE SEPSIS. Sepsis-induced ARDS has the highest mortality rate of all causes of ARDS (30). Although hypoxic respiratory failure frequently occurs in sepsis, the precise mechanisms of sepsis-induced respiratory failure are incompletely understood. Hypoxic pulmonary vasoconstriction (HPV) is a unique vasoconstrictor response to alveolar hypoxia that diverts blood flow away from poorly ventilated lung regions, thereby reducing the right-to-left shunting of venous blood and optimizing systemic oxygenation when the lung is injured (37). HPV is impaired in ARDS and sepsis, which results in intrapulmonary shunting and at times systemic hypoxemia (11, 26). We previously reported that challenge with lipopolysaccharide (LPS) causes impaired HPV in mice (35). Although the detailed mechanisms that underlie HPV remain elusive, several factors seem to contribute. For example, increased pulmonary nitric oxide (NO) levels are required, but are not sufficient, to impair HPV in LPS-challenged mice (35). Also, reactive oxygen species (ROS) have been implicated in the pathogenesis of sepsis-induced HPV impairment on the basis of a study that indicated that HPV is preserved in septic mice that are treated with ROS scavengers (6).

In sepsis, components of microorganisms activate host cells to express inflammatory mediators such as cytokines and NO. Toll-like receptors (TLRs) are critically involved in sensing and generating inflammatory responses to infection (1, 2). TLRs are a family of pattern-recognition receptors that recognize conserved molecular motifs in microorganisms. Thus far, 13 TLRs have been described in mammals (9). Although TLRs recognize conserved patterns that are present in microorganisms, there are important differences between the TLRs. For instance, TLR4 mediates the inflammatory effects of LPS (endotoxin) from Gram-negative bacteria (27–29), whereas TLR2 mediates the inflammatory effects of lipoproteins from Gram-negative bacteria and also of components of Gram-positive bacteria and fungi (3, 10, 18, 25, 34). Furthermore, the patterns of inflammatory responses elicited by activation of TLR2 vs. TLR4 differ (9, 19, 36), and different TLR agonists have been found to profoundly influence the inflammatory effects of one another (7, 24). Thus far, two predominant intracellular TLR pathways have been identified (1, 41). The differences observed in inflammatory effects of TLR2 vs. TLR4 agonists, and the ability of TLR2 and TLR4 agonists to alter each other’s inflammatory effects, most likely derives, in part, from differential signaling through myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent intracellular pathways (7). Whereas TLR2 signals through only the MyD88-dependent pathway, TLR4 signals through both the MyD88-dependent and MyD88-independent pathways (1, 41). TLR2 and TLR4 are expressed in multiple cell types in the lung, including macrophages, endothelial cells, and epithelial cells (5, 12, 13). Although mouse aortic vascular smooth muscle cells have been reported to express TLR2, there is not yet conclusive evidence

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that pulmonary vascular smooth muscle cells express TLR2 (22, 42, 43). Challenge with TLR2 agonists has been shown to induce lung inflammation (15, 39). However, the effects of TLR2 activation on pulmonary function, including on the lung vasculature and on gas exchange, are not clear.

Our prior studies (35) indicated that challenge with LPS impairs HPV in mice. LPS, a TLR4 agonist, is present only in Gram-negative bacteria. However, sepsis is also caused by Gram-positive bacteria (40–65%) and fungi (~10%) (31, 40). In fact, the incidence of Gram-positive bacteremia has increased over the past few decades, such that Gram-positive bacteremia now occurs more frequently than Gram-negative bacteremia (40). Thus it seems likely that non-TLR4 pathways also contribute to pulmonary inflammation and dysfunction in sepsis. We have found that several TLR2 agonists are shed by bacteria into human serum and into the blood of septic animals (14, 15, 17).

Because TLR2 agonists are ubiquitously expressed in all of the major classes of microorganisms and circulate in diverse sepsis models, we investigated the role of TLR2 activation in sepsis-induced respiratory dysfunction. We tested the hypothesis that challenge with (S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys3-OH, trihydrochloride (Pam3Cys), a synthetic bacterial lipopeptide TLR2 agonist, would impair HPV and induce deleterious pulmonary and systemic effects. We learned that activation of TLR2 causes profound impairment of HPV in mice.

**MATERIALS AND METHODS**

**Animals.** The Massachusetts General Hospital Subcommittee on Research Animal Care approved all of the animal studies. Male C57BL/6/J (wild-type) and TLR2-deficient (B6.129-Tlr2m1Ker/J, TLR2−/−) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6.129-Tlr2m1Ker/J heterozygotes were in crossed, then backcrossed to C57BL/6J for nine generations. Mice weighed between 18 and 30 g.

**Reagents.** Pam3Cys was purchased from EMC Micro Collections (Tubingen, Germany). Escherichia coli O55:5 LPS was purchased from List Biological Laboratories (Campbell, CA). The LPS was determined to be free of non-TLR4 bacterial components by using immunoblots for bacterial peptidoglycan-associated lipoprotein and murine lipoprotein, gold stains, and on the basis of the fact that the LPS did not activate macrophages prepared from LPS-hyporesponsive mice (7, 16). Angiotensin II was purchased from American Peptide (Sunnyvale, CA).

**Challenge of mice with saline, Pam3Cys, or LPS.** Mice received an intravenous injection of either saline, Pam3Cys (20 mg/kg), or LPS (20 mg/kg). These doses of Pam3Cys and LPS were established in pilot dose–response studies. Pam3Cys and LPS were dissolved in saline. The injectate volume for all conditions was 0.01 ml/g body wt. All studies were performed by using wild-type mice. Limited studies were also performed by using TLR2−/− mice, to verify that effects of Pam3Cys on HPV, arterial blood gas tensions, white blood cells (WBC), and physical appearance were mediated by TLR2 and not additional receptors.

**Measurement of HPV and arterial blood gas tension analysis in mice after treatment with saline, Pam3Cys, or LPS.** We used a model of HPV that was previously developed in our laboratory to assess pulmonary blood flow redistribution in response to alveolar hypoxia in mice (35). This model uses an open-chest approach, which provides access for placement of a flow probe around the left pulmonary artery and a catheter into the main pulmonary artery. In early experiments we found that, after a thoracotomy, mice that were ventilated with hypoxic gas mixtures died rapidly and did not develop a significant acute change in pulmonary vascular resistance (PVR). Thus we developed a model of unilateral alveolar hypoxia that is well tolerated after a thoracotomy and provides reliable and consistent changes in PVR in response to alveolar hypoxia (35). Left lung alveolar hypoxia is induced by reversibly occluding the left mainstem bronchus and allowing the lung to collapse. Left lung pulmonary vascular resistance (LPVR) and other hemodynamic parameters were obtained before and during left lung alveolar hypoxia induced by left mainstem bronchial occlusion (LMBO).

Determined methods of the surgical preparation and the measurement of LPVR have been described (35). Briefly, 22 h after challenge with saline, Pam3Cys, or LPS, wild-type and TLR2−/− mice were anesthetized, mechanically ventilated with an inspired oxygen fraction (FiO2) of 1.0 (respiratory rate 110–120 breaths per minute, peak inspiratory pressure 13 cmH2O, and positive end-expiratory pressure level 2–3 cmH2O), and then subjected to a thoracotomy. For hemodynamic measurements, an arterial line was inserted into the left carotid artery, a custom-made catheter was placed into the main pulmonary artery, and a flow probe was sited around the left pulmonary artery. Systemic arterial pressure (SAP), pulmonary arterial pressure (PAP), and left pulmonary arterial blood flow (QLPA) were continuously measured and recorded before and during LMBO. To estimate the LPVR, the inferior vena cava was partially occluded to transiently reduce cardiac output until QLPA was reduced by ~50%. LPVR was calculated from the slope of the QLPA/PAP relationship. The LMBO-induced increase in LPVR was expressed as the percentage increase from baseline LPVR after 5 min of LMBO vs. before LMBO.

After hemodynamic measurements were completed, arterial blood was sampled from the left carotid artery during LMBO at a FiO2 of 1.0. Blood gas tension analyses were measured by using a Rapid Lab 840 (Chiron Diagnostics, Medfield, MA). The alveolar-to-arterial difference in the partial pressure of O2 (A-aDO2) was calculated for wild-type mice. Partial pressure of O2 in the alveoli (PAIVO2) was calculated to be 550 mmHg based on the alveolar gas equation (PAIVO2 = [P atm – PaCO2] × FiO2 – PacO2/respiratory quotient), where P atm is the atmospheric pressure, PaCO2 is the partial pressure of water vapor in the alveoli, and PacO2 is arterial partial pressure of CO2. The A-aDO2 was calculated by using the following equation: A-aDO2 = PAIVO2 – arterial partial pressure of O2 (Pao2).

**Physical appearance of mice.** Wild-type and TLR2−/− mice were observed at 22 h after challenge. For each mouse, numeric “health” scores were assigned at intervals based on the several features with a numeric value (0 to 2): mobility (2 = very slow, 1 = slow, 0 = normal), appearance of the hair (2 = shiny, 1 = normal, 0 = none), presence of purulent drainage from the eyes (2 = copious, 1 = minimal, 0 = none), and diarrhea (2 = copious, 1 = minimal, 0 = none). In addition, changes of body weight were measured by weighing the mice both before and 22 h after challenge with saline, Pam3Cys, or LPS. Body temperature was measured at the beginning of the preparation for hemodynamic measurements by using a rectal thermometer probe (temperature control system; FHC, Bowdoinham, ME).

**Quantitative RT-PCR for inducible NO synthase and TNF-α, RNA was prepared from lungs of mice 2, 7, and 22 h after challenge by using RNA STAT-60 (Tel-Test, Friendswood, TX). Samples were prepared with RNase-free DNase (Qiagen, Valencia, CA), and RNA was further purified with RNaseasy/QIAamp Columns (Qiagen). cDNA was generated with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random primers (Promega) at 37°C for 1 h. Quantitative real-time PCR was performed with the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) by using TaqMan premade primers for TNF-α and inducible NO synthase (iNOS; Applied Biosystems). Postamplification dissociation curves were performed to verify the presence of a single amplification product. Ribosomal RNA (18S) was detected by using...
18S primers (18S forward: 5'-CGGCTACCACTCCAGGAA, 18S reverse: 5'-GCTGGAATTACCGCGGCT; DNA Oligonucleotide Synthesis Core, Massachusetts General Hospital). Changes in gene expression were normalized to 18S ribosomal RNA levels by using the relative cycle threshold method.

ROS. Luciferin-enhanced chemiluminescence was used to measure superoxide levels as described (21). Briefly, lungs were collected from wild-type mice 2 h after challenge with saline, Pam3Cys, or LPS. Lung tissue was preincubated with Krebs-HEPES buffer containing 10 μM NADPH and was transferred into wells containing Krebs-HEPES buffer supplemented with 10 μM NADPH and 5 μM Luciferin. Chemiluminescence was recorded over 60 s (Victor™; Perkin Elmer) and was reported as relative light units (RLU). Background chemiluminescence was subtracted from the RLU count. Superoxide production is expressed as RLU per milligram of dry lung tissue.

WBC concentration. To assess circulating WBC concentration, a 25-ml blood sample was obtained via the arterial line. WBC counts were performed by using a hemocytometer (Hauser Scientific, Horsham, PA) and a microscope.

Myeloperoxidase activity. Polymorphonuclear neutrophil (PMN) infiltration in the lungs was estimated by measuring myeloperoxidase (MPO) as described (15).

Lung morphology. Lungs were collected 2, 7, and 22 h after challenge with saline, Pam3Cys, or LPS and were fixed by perfusing the airway and pulmonary artery with 3% paraformaldehyde and 0.1% glutaraldehyde, respectively. Tissue blocks were then embedded in resin (Technovit 7100; Heraeus Kulzer, Wehrheim, Germany), cut into 2-μm-thick sections, and stained with 0.05% toluidine blue. The distribution and number of WBC infiltrating the lungs was assessed by using light microscopy by an investigator who was blinded to the time and treatment. PMNs were identified on the basis of their morphology, and the quantity of PMNs was estimated by using a semiquantitative scoring analysis (0 = none, 1 = few, 2 = moderate, 3 = many).

Lung wet-to-dry ratios. Wet-to-dry ratios were quantified in the lungs of wild-type mice 22 h after challenge with saline, Pam3Cys, or LPS to quantify any lung edema as described (35). Mice were euthanized with pentobarbital, and both lungs were excised, blotted dry, and immediately weighed. Lungs were then dried to constant weight in a microwave oven for 60 min and were reweighed.

Pulmonary vascular response to infusion of angiotensin II. Twenty-two hours after challenge with saline or Pam3Cys, wild-type mice were prepared as described above for the HPV measurements. An additional vascular access catheter was placed in the right carotid artery for infusion of saline or angiotensin II (dissolved in saline) by using an infusion pump (Kont Scientific, Torrington, CT). Angiotensin II doses of 0.05, 0.5, and 5.0 μg·kg⁻¹·min⁻¹ were consecutively infused for 5 min. After a plateau was reached, the inferior vena cava was transiently occluded. LPVR was calculated from the slope of the QLPA/PAP relationship during the transient occlusions. The angiotensin II infusion was then discontinued, a final measurement of the QLPA/PAP relationship was performed, and the LPVR was calculated.

Statistical analysis. Data are expressed as means ± SE. P values < 0.05 were considered to be statistically significant. Statistical analyses were performed by using SigmaStat 3.0 (Systat Software). The effects of angiotensin II infusion on LPVR and expression of mRNA and MPO activity in the lung were compared by using a one-way ANOVA with post hoc Bonferroni tests for data that were normally distributed or by using a Kruskal-Wallis Test with post hoc Dunn’s test for data that were not normally distributed. For all other studies, t-tests were used for data that were normally distributed or Mann-Whitney rank sum tests were used for data that were not normally distributed.

RESULTS

Hemodynamic parameters before and during LMBO in mice challenged with Pam3Cys and LPS. To investigate the effects of Pam3Cys and LPS on HPV, the percent change in LPVR in response to LMBO was measured 22 h after challenge with saline, Pam3Cys, or LPS (Fig. 1). Hemodynamic parameters and left pulmonary blood flow before and during LMBO are included in Table 1. Saline-challenged mice exhibited a pro-

A

B

![Fig. 1. Hypoxic pulmonary vasoconstriction (HPV) is impaired by activation of toll-like receptor (TLR) 2. A: wild-type (WT) and TLR2 knockout (TLR2⁻/⁻) mice were challenged intravenously with saline, (S)-[2,3-bis(palmitoxyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys(4-OH), trihydrochlo-rolylamine (Pam3Cys), or lipopolysaccharide (LPS) (n = 7/group). After 22 h, pulmonary arterial pressure (PAP) and left pulmonary artery blood flow (QLPA) were recorded during transient reductions of cardiac output. Left lung alveolar hypoxia was induced by left mainstem bronchus occlusion (LMBO). LPVR was calculated before and during LMBO from slope of QLPA/PAP relationship during reduction of cardiac output. Data shown are percent change of cardiac output. Left lung alveolar hypoxia was induced by left mainstem bronchus occlusion (LMBO). LPVR was calculated before and during LMBO from slope of QLPA/PAP relationship during transient reductions of cardiac output. Data shown are percent change of cardiac output. After 22 h, both groups were infused with saline followed by increasing doses of angiotensin II (0.05, 0.5, and 5.0 μg·kg⁻¹·min⁻¹, 5 min at each dose), followed by saline. PAP and QLPA were recorded during transient reductions of cardiac output during infusion of saline and angiotensin II, and LPVR was calculated from slope of QLPA/PAP relationship. *P < 0.01 for angiotensin II at 0.0 vs. 5.0 μg·kg⁻¹·min⁻¹ in both saline and Pam3Cys-challenged groups. #Final measurements were performed during saline infusion after discontinuation of angiotensin II infusion.](http://ajplung.physiology.org/Download)
found increase of LPVR in response to LMBO (112 ± 4%, Fig. 1). In pilot experiments, we found that HPV is not impaired in mice challenged with Pam3Cys at a dose of 4 mg/kg (112 ± 9%, n = 7, data not shown), but HPV is significantly impaired at a dose of 20 mg/kg (38 ± 6%, P < 0.001 vs. saline, Fig. 1). Challenge with LPS (20 mg/kg) markedly and similarly impaired the increase of LPVR in response to LMBO (37 ± 7%, P < 0.001 vs. saline, Fig. 1). There were no significant differences in heart rate or systemic arterial pressure before and during LMBO in any of the groups (Table 1), suggesting that systemic hemodynamics were stable and did not account for differences in left lung blood flow that were observed between the groups. Despite significant differences in the percent increase in LPVR, there were not significant differences in baseline PAP or the rise in PAP following LMBO in wild-type mice that were challenged with saline, Pam3Cys, and LPS (Table 1). Although left pulmonary blood flow was reduced during LMBO vs. baseline in all groups, there was more of a reduction in left pulmonary blood flow during LMBO in saline-treated vs. Pam3Cys or LPS-treated wild-type mice (Table 1).

We previously showed (35) that challenge with LPS does not impair the murine pulmonary vasoconstrictor response to infused angiotensin II, indicating that LPS does not impair HPV via nonspecific effects on pulmonary vascular contractile function. To assess the possibility that the effects of Pam3Cys on HPV could be due to nonspecific effects of Pam3Cys on pulmonary vascular contractile function, we compared the effects of infusing increasing doses of angiotensin II on LPVR in wild-type mice 22 h after challenge with saline (n = 5) or with Pam3Cys (n = 6) (Fig. 1B). In saline-treated mice, the LPVR increased from 74 ± 14 at baseline to 165 ± 7 mmHg·ml⁻¹·min⁻¹·g⁻¹ during infusion of angiotensin II at 5.0 μg·kg⁻¹·min⁻¹ (P < 0.01). In Pam3Cys-challenged mice, the LPVR increased from 89 ± 8 at baseline to 173 ± 24 mmHg·ml⁻¹·min⁻¹·g⁻¹ during infusion of angiotensin II at 5.0 μg·kg⁻¹·min⁻¹ (P < 0.01). LPVR returned to baseline for both saline and Pam3Cys-challenged mice following discontinuation of the angiotensin II infusion (89 ± 9 and 96 ± 11 mmHg·ml⁻¹·min⁻¹·g⁻¹, respectively). There were no significant differences in the LPVR between saline and Pam3Cys-challenged mice at any dose of angiotensin II. These data suggest that the effects of Pam3Cys on LPVR in response to LMBO are not due to a generalized impairment of the pulmonary vascular contractile function.

Pam3Cys or LPS causes reduced systemic arterial oxygenation during LMBO. To estimate the impact of HPV on systemic arterial oxygenation, we measured arterial blood gas tensions in wild-type mice during LMBO while the right lung was ventilated with 100% oxygen. These data are shown in Table 2. The values of PaO₂ were significantly decreased in mice that were challenged with Pam3Cys (P < 0.001) or with LPS (P < 0.05) compared with mice that were challenged with saline (Fig. 2). The PaO₂ values were not significantly different from the control value of 189 ± 14 mmHg in saline-treated mice (Fig. 2).

Table 2. Arterial blood gas tension analysis in mice after intravenous challenge with saline, Pam3Cys, or LPS

<table>
<thead>
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<th>Challenge</th>
<th>Wild-Type</th>
<th>TLR2−/−</th>
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<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Pam3Cys</td>
</tr>
<tr>
<td>PaO₂, mmHg</td>
<td>258 ± 21</td>
<td>149 ± 11*</td>
</tr>
<tr>
<td>PaCO₂, mmHg</td>
<td>30 ± 2</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>pH</td>
<td>7.37 ± 0.02</td>
<td>7.35 ± 0.01</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td>19.2 ± 1.0</td>
<td>18.4 ± 0.4</td>
</tr>
<tr>
<td>BE, mmol/l</td>
<td>−6.9 ± 1.3</td>
<td>−7.6 ± 0.6</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>13.0 ± 0.4</td>
<td>12.4 ± 0.4</td>
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</table>

Values are means ± SE. Arterial blood gas tension analysis was performed at the end of the hemodynamic studies during LMBO in wild-type and TLR2−/− mice while breathing 100% oxygen. PaO₂, arterial partial pressure of O₂; PaCO₂, arterial partial pressure of carbon dioxide; BE, base excess. *P < 0.05 vs. saline; †P < 0.05 vs. Pam3Cys.
LPS caused a significant metabolic acidosis as indicated by a decrease in systemic arterial oxygenation; $\Delta$PaO2 values after challenge with either saline, Pam3Cys, or LPS. Compared with saline, treatment with either Pam3Cys or LPS induced lung expression of $\text{Pam3Cys}$ mRNA (Fig. 3A). There were differences between Pam3Cys and LPS in terms of the time course and magnitude of iNOS mRNA induction. Pam3Cys caused increased levels of iNOS mRNA that were statistically significant at 2 h ($P < 0.05$ vs. saline) but not at 7 or 22 h. In contrast, LPS caused a more sustained and marked iNOS induction than Pam3Cys ($P < 0.001$). After a challenge with LPS, levels of iNOS mRNA were increased at 2 h, peaked at 7 h, and remained elevated at 22 h ($P < 0.001$).

Pam3Cys and LPS induce a similar pattern and magnitude of TNF-α mRNA expression. After challenge with Pam3Cys and LPS, the lung’s TNF-α mRNA expression was highest at 2 h ($P < 0.001$ vs. saline) and remained elevated at 7 and 22 h ($P < 0.05$ vs. saline, Fig. 3B). There were no significant differences in the levels of TNF-α mRNA induction between Pam3Cys- and LPS-treated mice (Fig. 3B).

Pam3Cys and LPS augment pulmonary ROS levels. In a pilot study, we found that treatment with LPS causes increased lung superoxide production, which peaks at ~2 h and declines to baseline at 4 to 7 h (data not shown). Thus we compared superoxide production in the lungs 2 h after challenge with saline, Pam3Cys, or LPS. Compared with treatment with saline, treatment with either Pam3Cys or LPS caused a statistically significant increase of ROS production ($P < 0.05$ vs. saline, Fig. 4). There was no significant difference in ROS levels in the lungs of mice that were treated with Pam3Cys or LPS.

Pam3Cys and LPS decrease the circulating WBC concentration. Circulating WBC concentration was measured 22 h after challenge with saline, Pam3Cys, or LPS. Challenge with either Pam3Cys or LPS decreased the WBC concentration compared with saline ($P < 0.001$, Fig. 5A). The reduction of WBC concentration induced by LPS at 22 h was significantly greater than the decrease in WBC levels induced by Pam3Cys ($P < 0.05$ for LPS vs. Pam3Cys).

Pam3Cys and LPS increase lung MPO activity. Pam3Cys and LPS both caused a sustained increase in the MPO activity levels at 2, 7, and 22 h ($P < 0.001$ vs. saline, Fig. 5B). LPS induced significantly higher MPO levels in the lung at 2 h than Pam3Cys ($P < 0.001$ for LPS vs. Pam3Cys).

Table 3. Physical appearance, change in body weight, and rectal temperature of wild-type vs. TL2R−/− mice 22 h after intravenous challenge with saline, Pam3Cys, or LPS

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Wild-Type</th>
<th>TL2R−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Pam3Cys</td>
</tr>
<tr>
<td>Physical appearance</td>
<td>0.1±0.1</td>
<td>1.3±0.2*</td>
</tr>
<tr>
<td>Change in body wt, g</td>
<td>0.6±0.2</td>
<td>-1.2±0.3*</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>37.1±0.4</td>
<td>36.8±0.4</td>
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Values are means ± SE. *$P < 0.05$ vs. saline; †$P < 0.05$ vs. Pam3Cys.
recruitment without histological evidence of infiltration. We verify that Pam3Cys impairs HPV via TLR2, rather than through additional receptors, the LMBO-induced increase in physical appearance, weight loss, and circulating WBC. To obtain from Pam3Cys- vs. LPS-challenged mice at any time (data not shown).

abundance and localization noted between the lung sections of LPS-challenged mice. There were no differences in the lungs of LPS-challenged mice at these three times and were also primarily located in the intravascular compartment of the lungs of LPS-challenged mice. There were no differences in abundance and localization noted between the lung sections obtained from Pam3Cys- vs. LPS-challenged mice at any time (data not shown).

TLR2 mediates the effects of Pam3Cys on HPV, blood gases, physical appearance, weight loss, and circulating WBC. To verify that Pam3Cys impairs HPV via TLR2, rather than through additional receptors, the LMBO-induced increase in LPVR was also measured in TLR2−/− mice. Compared with results in wild-type mice, treatment with saline or Pam3Cys did not compromise HPV in TLR2−/− mice (Table 1 and Fig. 1). As expected, HPV was impaired in both wild-type and in TLR2−/− mice that were treated with LPS. After completing the measurement of HPV, the systemic arterial blood gas tension was measured in TLR2−/− mice challenged with either saline, Pam3Cys, or LPS (Table 2 and Fig. 2). There were no significant differences in PaO2 values of TLR2−/− mice that were treated with saline vs. Pam3Cys (P > 0.05). In contrast, and as expected on the basis of use of TLR4 and not TLR2, challenge with LPS caused a significant decrease in PaO2 in TLR2−/− mice (P < 0.05 vs. saline).

Body weight, temperature, and physical appearance were assessed at 22 h after challenge of TLR2−/− mice with saline, Pam3Cys, or LPS (Table 3). There were no differences observed in either saline- or Pam3Cys-challenged TLR2−/− mice. However, LPS-challenged TLR2−/− mice appeared acutely ill and had both a decreased body weight and reduced rectal temperature.

In the TLR2−/− mice, the circulating WBC concentrations were not reduced after either treatment with saline or with Pam3Cys (Fig. 5A, P > 0.05). As expected on the basis of intact TLR4 signaling, the WBC concentration was reduced in TLR2−/− mice that were challenged with LPS.

DISCUSSION

Unlike the systemic artery smooth muscle cell, the pulmonary artery smooth muscle cell constricts when exposed to hypoxemia. HPV acts to preserve systemic arterial oxygen tension and is impaired in severe acute lung injury, and especially in ARDS due to sepsis. The most important finding of our study is that TLR2 activation profoundly impairs HPV. We report that activation of TLR2 induces systemic arterial hypoxemia during LMBO and causes pulmonary inflammation and deleterious systemic effects, including weight loss and a reduced circulating WBC concentration. Activation of TLR4 pathways likewise impairs HPV, causes pulmonary inflammation, and reduces the circulating WBC concentration. How-

![Fig. 3. Inducible nitric oxide synthase (iNOS) mRNA and TNF-α mRNA levels in lungs from mice challenged with Pam3Cys and LPS. Lung RNA was prepared 2, 7, or 22 h after challenge with saline, Pam3Cys, or LPS. iNOS (A) and TNF-α (B) mRNA levels were determined by quantitative RT-PCR, and results are expressed as fold change vs. lungs from saline-treated mice; n = 8 mice/group; *P < 0.05 vs. saline; #P < 0.05 vs. Pam3Cys.

![Fig. 4. Reactive oxygen species (ROS) generation in lungs from mice that were challenged with either saline (Sal), Pam3Cys (P3C), or LPS. Superoxide production in freshly isolated lung tissue was detected by using Lucigenin-enhanced chemiluminescence 2 h after challenge with saline (Sal), Pam3Cys (P3C), or LPS. Superoxide production is expressed as relative light units (RLU) per milligram of dry tissue; n = 8 mice/group; *P < 0.05 vs. saline; #P < 0.05 vs. Pam3Cys.](http://ajplung.physiology.org/10.1152/ajplung.00365.2007)
ever, despite the fact that TLR2 and TLR4 activation both caused a similar degree of impairment of HPV, activation of TLR4 caused the mice to appear more profoundly ill and to develop a metabolic acidosis and reduced temperature, whereas activation of TLR2 did not cause these latter effects. Because the pulmonary vasoconstrictive response to angiotensin II was intact and unchanged following challenge with Pam3Cys, we do not believe that nonspecific dysfunction of the vasoconstrictor contractile apparatus contributes to the effects of TLR2 activation on LPVR observed in these studies.

TLRs are critical host sensors of infection that initiate inflammatory responses to all classes of microorganisms. Because LPS is uniquely produced by Gram-negative bacteria, TLR4 is believed to be primarily involved in sensing and initiating host responses to infection with Gram-negative bacteria. However, acute respiratory failure and ARDS also occur in sepsis due to other classes of microorganisms, including Gram-positive bacteria, fungi, viruses, and parasites. TLR2 agonists are broadly expressed in different classes of microbes, including Gram-negative and Gram-positive bacteria, and in fungi. Thus our finding that TLR2 activation impairs HPV suggests that TLR2 activation may be an important inducer of the respiratory complications of sepsis due to a broad range of microorganisms.

In these studies, we observed that compared with saline, challenge with Pam3Cys and LPS caused an impaired LPVR response to LMBO but that the PAP rise induced by LMBO was equal in wild-type mice that were challenged with saline, Pam3Cys, and LPS. Although we cannot provide a definitive explanation for the discrepancy between LPVR responses and PAP responses, we believe that the LPVR is a dynamic measurement of pulmonary vasomotor tone, whereas the PAP is a static measurement. The PAP reflects the sum total of different aspects of the cardiopulmonary system. Several factors may contribute to the PAP but do not seem to be crucial for HPV, including the cardiac output and cardiac processes that result in increased left atrial pressure, increased intrathoracic pressure, and perivascular or interstitial pulmonary edema. In contrast to PAP, the LPVR is a dynamic measurement reflecting the dynamic tone changes in the PAP/pulmonary arterial blood flow relationship. This test is far more sensitive for detecting physiological differences between groups than the simple evaluation of PAP.

Despite inducing a similar degree of impairment of HPV and weight loss, there were marked differences in the physical appearance, the rectal temperature, and the acid-base status of mice challenged with either Pam3Cys or LPS. At 22 h, LPS-challenged mice had a metabolic acidosis and a reduced rectal temperature and were observed to have piloerection, lethargy, diarrhea, and purulent drainage from their eyes. In contrast, Pam3Cys-treated mice did not have a metabolic acidosis, had a normal rectal temperature, and only exhibited mild piloerection. There were also some differences in the time course and magnitude of iNOS expression between Pam3Cys and LPS-treated mice. Both Pam3Cys and LPS caused an early peak in iNOS expression. However, the iNOS peak level was higher and was more sustained following challenge with LPS, remaining substantially elevated at the 7-h time point. It is probable that high levels of NO irreversibly damage the HPV, leading to enduring impairment of HPV. This concept is supported by our prior studies indicating that early, but not late, treatment with NO synthase inhibitors protects against LPS-induced impairment of HPV (35).

We found that activation of either TLR2 or TLR4 increased ROS production in the lung. ROS have been implicated in the physiology and pathophysiology of HPV (4, 38), and antioxidants have been shown to have beneficial effects in sepsis (8, 32, 33, 44). In addition, only the early administration of ROS scavengers at 3 h after LPS challenge protects against late impairment of murine HPV measured at 22 h, whereas there was no effect when ROS scavengers were administered later and shortly before the measurement of HPV at 22 h (6). As described above for the role of early iNOS upregulation in the pathogenesis of later impairment of HPV, it is likely that the early production of ROS causes early and enduring damage to HPV.

In a clinical context, it is likely that during sepsis multiple TLR agonists circulate in the bloodstream simultaneously and that the amount and diversity of circulating TLR agonists will determine the physical, physiological, and chemical manifestations of infection. The discrepancy in physical appearance, temperature, acid-base status, and patterns of expression of inflammatory markers between mice that were treated with...
Pam3Cys vs. LPS raises the possibility that divergent pathways mediate aspects of TLR2 and TLR4 activation on the lung and on systemic parameters. There are reported differences of the TLR2 and TLR4 intracellular pathways. Whereas TLR2 activates through the intracellular adapter molecule MyD88, TLR4 activates both MyD88-dependent and MyD88-independent pathways (1, 20, 23). We recently reported (7) that the differences between the MyD88-dependent and MyD88-independent intracellular TLR pathways are important in the synergy, priming, and tolerance of inflammatory responses induced by exposure to different TLR agonists. The current study raises the possibility that MyD88-dependent pathways mediate the impairment of HPV and weight loss, whereas MyD88-independent pathways mediate other manifestations such as the reduction in core temperature, diarrhea, and lethargy. If this is the case, then the presence of both LPS and TLR2 agonists during sepsis may cause more profound physiological responses. However, it is also possible that the differences observed between mice that were treated with LPS vs. Pam3Cys were caused by differences in dose responses of different TLRs and that these differences would not have been observed had we used higher doses of Pam3Cys. In the present studies, we chose to treat mice with the same dose (by weight) of LPS and Pam3Cys. The dose was chosen based on our prior work indicating that HPV is impaired by LPS at 20 mg/kg (unpublished data) and because in pilot experiments we established that Pam3Cys at a dose of 4 mg/kg did not impair HPV, whereas Pam3Cys at a dose of 20 mg/kg did impair HPV to a similar degree as LPS at the same dose.

Activation of TLR2 and TLR4 caused an early and sustained increase of pulmonary MPO levels. Lung histology revealed that there was an early and sustained accumulation of PMNs in the intravascular space of Pam3Cys- and LPS-challenged mice, without evidence of PMN transmigration into the interstitium or the alveolar space. The mechanisms involved in PMN TLR2 and TLR4-induced accumulation in the lung’s vasculature without diapedesis into the interstitial or alveolar spaces remain uncertain. Additional studies will be required to determine whether or not accumulation of PMNs in the pulmonary vasculature contributes to sepsis-induced impairment of HPV.

Our studies show that activation of TLR2 profoundly impairs HPV and induces pulmonary inflammation and systemic manifestations that are consistent with those observed during clinical sepsis. Although the degree of HPV impairment was similar, there were marked differences in effects of Pam3Cys vs. LPS, suggesting that divergent pathways may mediate some of the inflammatory effects due to TLR2 or TLR4 activation. Our laboratory studies provide insights into the role of TLRs in the pathogenesis of sepsis-induced pulmonary dysfunction. Because TLR2 agonists are ubiquitously expressed in the various classes of microorganisms that cause clinical sepsis, we believe these studies are important and shed light on the role of TLR2 and TLR2-mediated pathways in the pathogenesis of sepsis-induced acute lung injury and arterial hypoxemia.

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REFERENCES


25. L308 TOLL-LIKE RECEPTOR 2 ACTIVATION IMPAIRS HPV IN MICE


