Novel critical role of Toll-like receptor 4 in lung ischemia-reperfusion injury and edema

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1Division of Cardiothoracic Surgery, 2Division of Pulmonary and Critical Care Medicine, Cystic Fibrosis Pulmonary Research and Treatment Center, 3Carolina Cardiovascular Biology Center, 4Department of Pathology and Laboratory Medicine, and 5Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

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Zanotti G, Casiraghi M, Abano JB, Tatreau JR, Sevala M, Berlin II, Smyth S, Funkhouser WK, Burridge K, Randell SH, Egan TM. Novel critical role of Toll-like receptor 4 in lung ischemia-reperfusion injury and edema. Am J Physiol Lung Cell Mol Physiol 297: L52–L63, 2009. First published April 17, 2009; doi:10.1152/ajplung.90406.2008.—Toll-like receptors (TLRs) of the innate immune system contribute to noninfectious inflammatory processes. We employed a murine model of hilar clamping (1 h) with reperfusion times between 15 min and 3 h in TLR4-sufficient (C3H/H11002) and TLR4-deficient (C3H/HeJ) anesthetized mice with additional studies in chimeric and myeloid differentiation factor 88 (MyD88)- and TLR4-deficient mice to determine the role of TLR4 in lung ischemia-reperfusion injury. Human pulmonary microvascular endothelial monolayers were subjected to simulated warm ischemia and reperfusion with and without CRX-526, a competitive TLR4 inhibitor. Functional TLR4 solely on pulmonary parenchymal cells, not bone marrow-derived cells, mediates early lung edema following ischemia-reperfusion independent of MyD88. Activation of MAPKs and NF-κB was significantly blunted and/or delayed in lungs of TLR4-deficient mice as a consequence of ischemia-reperfusion injury, but edema development appeared to be independent of activation of these signaling pathways. Pretreatment with a competitive TLR4 inhibitor prevented edema in vivo and reduced actin cytoskeletal rearrangement and gap formation in pulmonary microvascular endothelial monolayers subjected to simulated warm ischemia and reperfusion. In addition to its well-accepted role to alter gene transcription, functioning TLR4 on pulmonary parenchymal cells plays a key role in very early and profound pulmonary edema in murine lung ischemia-reperfusion injury. This may be due to a novel mechanism: regulation of endothelial cell cytoskeleton affecting microvascular endothelial cell permeability.

microvascular permeability; endothelial cell; pulmonary edema

ACUTE LUNG INJURY IS A FEATURE of sepsis, systemic inflammatory response, and adult respiratory distress syndrome. Noncardiogenic pulmonary edema and impaired gas exchange are consequences of acute lung injury, irrespective of etiology. The mechanisms causing pulmonary edema due to acute lung injury are not well-understood. Ischemia-reperfusion injury (IRI), a form of acute lung injury occurring immediately following lung transplantation, is a frequent complication causing morbidity and mortality (26). A greater understanding of lung IRI is likely relevant to many types of acute lung injury and thus may benefit not only lung transplant recipients, but also substantial numbers of other patients with lung injury. Such knowledge would also facilitate retrieval of lungs from non-heart-beating cadaver donors for transplant and/or may assist in the salvage of lungs not considered suitable for transplant, thereby reducing the critical shortage of transplantable lungs (8, 11).

Reperfusion following an interval of ischemia results in an inflammatory response involving components of the innate immune system, including the complement and coagulation cascades. Both parenchymal and myeloid cells elaborate free radicals, nitric oxide, and pro- and anti-inflammatory cytokines (4, 5). Recently, there has been increasing awareness of the important contribution of the innate immune system, particularly the Toll-like receptors (TLRs), to a variety of noninfectious inflammatory processes (31).

TLR4 is the mammalian LPS receptor (3). On stimulation, TLR4 activates the innate immune system by phosphorylation of MAPKs and activation of NF-κB (34). In an earlier study, increased expression of ICAM-1 following lung transplantation was due to reperfusion, not the antecedent ischemia per se (9). Interestingly, ICAM-1 expression was increased and lung histology was similar 6 h after LPS administration and 6 h following lung transplantation. Thus we hypothesized that TLR4 contributed to acute lung injury due to IRI. We tested this hypothesis in a murine model of in situ left-lung IRI, comparing TLR4-deficient mice (C3H/HeJ) with TLR4-sufficient mice (C3H/OuJ). Functioning TLR4 not only contributes to the inflammatory response via MAPK and NF-κB signaling, but also TLR4 mediates rapid lung edema on reperfusion of ischemic lung. We confirmed this in TLR4−/− mice. In vitro, alteration of pulmonary microvascular endothelial cytoskeleton occurs during simulated ischemia-reperfusion; this was substantially reduced in our model by CRX-526, a competitive inhibitor of TLR4 (14), implying a possible mechanism for TLR4-mediated pulmonary edema due to IRI. Pretreatment of mice with CRX-526 also reduced edema formation.

MATERIALS AND METHODS

Male C3H/HeJ, C3H/OuJ, TLR4−/−, and C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and myeloid differentiation factor 88 null (MyD88−/−) mice were provided by Dr. Akira (Kyoto, Japan). Mice were maintained in a pathogen-free facility until they weighed 25–30 g and were 8–10 wk old. Reagents were from Sigma (St. Louis, MO) unless specified. Animal experimental protocols were approved by the University of North Carolina Institutional Animal Care and Use Committee.
Surgical model of murine lung IRI. Mice were anesthetized with ketamine (0.1 mg/g body wt) and xylazine (0.01 mg/g) intraperitoneally, followed by one-third of the initial dose hourly. Tracheotomy allowed mechanical ventilation with a tidal volume of 0.4 ml, respiratory rate of 120 breaths/min, inspiratory-to-expiratory ratio of 0.4, positive end-expiratory pressure (PEEP) of 3 cmH2O, and FiO2 of 1.0 (CIV-101; Columbus Instruments, Columbus, OH). The right jugular vein was cannulated for infusion of 2.5% albumin in 0.9% saline 450 μl/h by syringe pump (Medfusion 2010; Medex, Carlsbad, CA) to maintain hydration. Rectal temperature was monitored and maintained with a heating pad. The left pulmonary hilum was occluded for 1 h with a microvascular clamp through a left thoracotomy. Reperfusion began with removal of the clamp. Animals were euthanized at intervals ranging from 15 min to 3 h by cardiectomy, and both lungs were excised. The apical portion of each lung was excised and immediately weighed and then desiccated in a 60°C oven for 48 h and reweighed to determine wet-to-dry weight ratio (W/D). Remaining lung tissue was flash-frozen in liquid nitrogen and stored at −80°C. Lungs excised immediately after death served as controls.

Extravascular albumin extravasation with Evans blue dye. Extravascular albumin extravasation after 1-h IRI was assessed by the Evans blue dye (EBD) technique (39). After occlusion of the left hilum, 30 mg/kg EBD dissolved in 250 μl of 0.9% saline solution was injected into the right jugular vein. After 1 h of reperfusion, the chest was opened and a median sternotomy, the mice were euthanized by right ventriculotomy, the pulmonary trunk was cannulated with an 18-gauge Angiocath, and the left atrial appendage was amputated. Both lungs were flushed with normal saline to remove intravascular EBD, excised, and weighed. The lung tissue was suspended in formamide (100 mg lung tissue/1 ml formamide; Roche Diagnostics, Indianapolis, IN) and incubated for 24 h at 50°C. Specimens were then centrifuged (13,000 g 30 min), and 50 μl of supernatant were placed in 96-well plates for colorimetric assessment in a μQuant spectrophotometer (BioTek Instruments, Winooski, VT) at 620 nm. Relative optical density values were normalized by the weight of the samples.

Inflammation fixation for histology. After 60 or 180 min of IRI (n = 4 per strain per group), lung blocks were inflation-fixed through the trachea with 4% buffered paraformaldehyde at a constant pressure of 25 cmH2O for 24 h at room temperature and then embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin. Lungs from animals euthanized immediately after tracheotomy (n = 4/strain) served as controls.

Immunostaining for NF-κB translocation. Immunohistochemical staining of inflation-fixed lung tissue was performed using a rabbit polyclonal p65 antibody (ab31481; Abcam) at a 1:100 dilution. Immunohistochemical staining of virtually all nuclei.

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three times with PBS. Cells were incubated for 1 h with a 1:100 dilution of Alexa Fluor 568 phalloidin (Invitrogen) in PBS with 1% BSA and 0.05% Tween-20. Coverslips stained for F-actin were immediately examined with a Leica DMIRB inverted fluorescence differential interference contrast (DIC) microscope at ×20 and ×40 magnification to evaluate changes in cell shape and F-actin cytoskeleton. For each dish, three pictures were taken of contiguous fields near the center of the dish at ×40 with a Kodak digital camera at the same exposure time. A masked observer assessed actin stress fiber pattern of each cell as normal or abnormal. Quantitative analysis of gap area was performed using MetaMorph software. A second masked observer evaluated the pattern of actin stress fibers in photographs grouped at the same time points.

**Determination of viability for cell culture experiments.** In separate experiments performed in triplicate, HMVECs grown to confluence on P35 dishes underwent simulated IRI. At the same time points, cells and cell culture media or RL were assessed for lactate dehydrogenase (LDH) activity using the CytoTox 96 Non-Radioactive Cytotoxicity Assay following the manufacturer’s instructions (Promega, Madison, WI). Control samples were also taken at time 0 and 24 h to assess cell viability apart from the experimental model. Culture medium and RL were used as background controls to normalize the absorbance value from the other samples. Cytotoxicity was calculated as media LDH activity divided by total LDH activity (cell pellet and media). Viability was the inverse and expressed as percent viability at each time point.

**Bronchoalveolar lavage and alveolar macrophage cell culture.** Alveolar macrophages (AMs) from HeJ and OuJ mice were harvested by bronchoalveolar lavage (BAL) 120 days after BMT. The trachea was cannulated with a tailored 18-gauge catheter (Becton Dickinson, Sandy, UT). BAL was performed by slow tracheal delivery of 4 aliquots (35 μl/g body wt) of prewarmed, sterile, endotoxin-, calcium-, and magnesium-free, PBS with 0.2 mM EGTA. Lavage fluid was withdrawn by gentle suction, pooled for each mouse, and centrifuged at 250 g for 5 min. Cells were resuspended in RPMI 1640 (GIBCO BRL, Rockville, MD) containing 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA), penicillin G (100 U/ml), and streptomycin (100 μg/ml). Viability was consistently >95% by trypan blue exclusion. Cells were plated at 20,000/well in 96-well plates. After 2 h of incubation, plates were washed with PBS to remove nonadherent cells. Adherent AMs were cultured in RPMI 1640 at 37°C in a humidified incubator with 5% CO₂.

**NF-κB reporter assay.** Recombinant, first-generation E1/E3-deleted adenovirus serotype 5 vectors were prepared by the University of North Carolina at Chapel Hill (UNC) Gene Therapy Center Vector Core (38), and HMVECs and AMs were transfected as previously described for epithelial cells (54).

**Statistical analysis.** All data are reported as means ± SE. Groups were compared by ANOVA with Tukey post hoc test or by paired or unpaired t-tests using Statistica (Tulsa, OK).

**RESULTS**

**TLR4 is a key mediator of early pulmonary edema due to IRI.** Reperfusion of left lungs rendered ischemic by 1 h of hilar clamping induced early, pronounced fluid accumulation manifest by elevated W/D in the left lung of TLR4-sufficient (OuJ) mice within 15 min of reperfusion that persisted out to 3 h of reperfusion (Fig. 1A). In contrast, TLR4-deficient (HeJ) mice experienced significantly less edema following 15- and 30-min reperfusion and demonstrated earlier recovery; W/D after 1- and 3-h reperfusion was normal.

There was more perivascular and alveolar wall edema in inflation-fixed OuJ left lungs reperfused for 3 h compared with HeJ lungs (Fig. 1B). However, there was no histological difference in interstitial edema between strains after 1-h reperfusion (Fig. 1C). All 8 lung specimens after 1-h reperfusion and 4 HeJ specimens after 3-h reperfusion were judged to be normal and not different from 4 control specimens (2 HeJ, 2 OuJ) by a masked observer. We postulated that the increased interstitial edema in OuJ lungs after 3-h reperfusion was due to rapid alveolar flooding rendered undetectable by inflation fixation 60 min after reperfusion. Consistent with this hypothesis, left lungs from OuJ mice had increased EBD content [a measure of microvascular permeability to albumin (39)] compared with left lungs from HeJ mice and right lungs from both strains (Fig. 1D). Thus the difference in W/D was due to alveolar flooding occurring early in OuJ mice compared with HeJ mice, with later absorption of the fluid into the alveolar walls and interstitium.

TLR4 signaling downstream of receptor activation involves recruitment of adapter proteins including MyD88 and TRIF (34). Because TRIF is not present in murine endothelial cells (16), MyD88 signaling is the key adapter downstream of TLR4 in these cells. When we subjected MyD88−/− mice to 1 h of IRI, equivalent edema developed in MyD88−/− mice as in OuJ mice and C57BL/6J mice, the background strain for MyD88−/− mice (Fig. 1E). Thus TLR4-mediated lung edema due to IRI is independent of downstream signaling via the MyD88 adapter. To confirm that early edema was due to TLR4, we repeated experiments in TLR4−/− mice compared with C57BL/6J mice, the background strain. Figure 1F confirms that TLR4−/− mice had significantly less edema compared with C57BL/6J mice after 1-h hilar clamping and reperfusion for 15, 30, or 60 min. Response to lung injury differs among mouse strains (2, 6). Perhaps this is an explanation for slightly increased W/D in right lungs of C57BL/6J mice with increased reperfusion times and why there is still some persistent edema in left lungs of TLR4−/− mice after 60-min reperfusion, although the temporal pattern and extent of edema development is very similar to the TLR4-defective HeJ strain.

**TLR4 mediates early MAPK and NF-κB activation due to lung IRI.** HeJ mice demonstrate delayed (p38, ERK) or reduced (NF-κB, JNK) activation compared with early and sustained activation in OuJ mice (Fig. 2). Because some degree of MAPK and NF-κB activation was observed in HeJ mice, alternative pathways independent of functioning TLR4 are implicated. Immunostaining for the p65 component of NF-κB confirmed the time course of NF-κB activation depicted by IkBα degradation (Fig. 3). Surprisingly, NF-κB was activated in the right lung to the same extent at the same reperfusion times despite the lack of edema in the right lung. Thus NF-κB activation is not necessarily associated with edema development. p38 activation was apparent in left lungs from HeJ mice 3 h after reperfusion with normal W/D. Taken together with the rapidity of development, the acute phase pulmonary edema in this model does not appear to be due to MAPK or NF-κB activation.

**TLR4 on lung parenchymal cells is necessary for edema formation due to lung IRI.** To determine the importance of functioning TLR4 on lung parenchymal vs. bone marrow-derived cells, particularly AMs, we created chimeric mice by lethally irradiating mice of each strain and reconstituting bone marrow by BMT. Replacement of AMs in chimeric animals was virtually complete 12 wk after BMT (Fig. 4A). In chimeric animals after 3 h of IRI, edema is apparent only when functional TLR4 is present on lung parenchymal cells (P), whether
Fig. 1. Toll-like receptor 4 (TLR4) is a key mediator of pulmonary edema due to ischemia-reperfusion injury (IRI). A: ischemia alone (0-h reperfusion) caused no increase in W/D. Following reperfusion, less edema develops in TLR4-deficient mice (HeJ), and is resolved by 1 h, but edema persists for 3 h in TLR4-sufficient mice (OuJ). LL, left lung; RL, right lung. *P＜0.05, ‡P＜0.001 compared with respective controls. P＜0.001 OuJ left lung compared with other lungs at 15-min, 30-min, 1-h, and 3-h reperfusion. n=6/group. ANOVA with Tukey post hoc. B: inflation-fixed (25 cmH2O) left lungs retrieved after 1-h hilar clamp and 3-h reperfusion show increased interstitial edema in peribronchial and perivascular spaces in TLR4-sufficient mice (OuJ) compared with TLR4-deficient mice (HeJ) (red arrows; top; ×40) and thicker alveolar walls (black arrows; bottom; ×200). Representative of 4 specimens. C: despite significant difference in W/D following 60-min reperfusion, there is no interstitial peribronchial/perivascular edema in TLR4-sufficient (OuJ) and TLR4-deficient (HeJ) mice (top; ×40) and no alveolar wall thickening (bottom; ×200). Representative of 4 specimens. These inflation-fixed sections look identical to control specimens (data not shown). D: Evans blue dye accumulation in left lungs retrieved after 1-h hilar clamping and 1 h of reperfusion supports the assumption that alveolar flooding is the reason for ↑ W/D in TLR4-sufficient mice (OuJ) compared with TLR4-deficient mice (HeJ). *P＜0.05, unpaired t-test; ‡P＜0.05, paired t-test. OD, optical density. E: left lungs retrieved after 1 h of hilar clamping and 1 h of reperfusion from myeloid differentiation factor 88-deficient (MyD88/−) mice (n=5) develop the same increase in W/D as TLR4-sufficient (OuJ) mice (n=6) and C57BL/6J mice (BL6; n=6), their background strain. F: TLR4/− mice develop significantly less edema than the background strain C57BL/6J mice after 1-h left hilar clamp and reperfusion for 5, 15, 30, 60, or 180 min (n=3 at 5, 15, 30, 180 min, n=6 otherwise). *P＜0.05, †P＜0.01, ‡P＜0.001, ANOVA with Tukey post hoc compared with respective controls; §P＜0.05 compared with other lungs at 5 min. For all other reperfusion times, P＜0.001, BL6 left lung compared with all others, ANOVA with Tukey post hoc. Although TLR4/− left lungs gain some weight with time, they are not different compared with right lungs of either strain at any time point except at 1-h reperfusion (P＜0.05). This small weight gain with time may be due to strain differences in susceptibility to lung injury (see text). Bars in B and C: top, 2.0 mm; bottom, 200 μm.
or not functioning TLR4 is present on myeloid cells (M) (Fig. 4B). The presence of TLR4-sufficient AMs or other myeloid cells does not result in lung edema if parenchymal cells do not possess functional TLR4 (P/H11002 M/H11001). Chimeric controls (OuJ into OuJ and HeJ into HeJ) showed no difference in pulmonary edema formation compared with nonirradiated strains (Fig. 4C), demonstrating that lethal irradiation had no impact on development of edema due to IRI. AMs from these “control chimerics” had the same response to LPS as AMs from the native strains (data not shown). Thus early edema formation due to IRI is attributable to functioning TLR4 on lung parenchymal cells.

A competitive inhibitor of TLR4 prevents pulmonary edema due to IRI. CRX-526 administered intravenously over 30 min (10 μg in 200 μL of saline) to OuJ mice 30 min before left hilar clamping prevented edema following 1 h of IRI (Fig. 5A). CRX-526 also prevented NF-κB activation in cultured HMVECs exposed to LPS (Fig. 5B).

Simulated WI causes actin cytoskeletal rearrangement and formation of gaps in the endothelial monolayer that are prevented by the TLR4 inhibitor CRX-526. Simulated ischemia resulted in disappearance or peripheral rearrangement of intracellular actin stress fibers and formation of gaps in the HMVEC monolayer (Fig. 6). Following simulated reperfusion, the gaps became smaller, and within 4 h, the cytoskeleton appeared normal, and monolayers resumed their original appearance.

In the presence of CRX-526, the area of monolayer gaps was significantly reduced during ischemia, and monolayers regained confluence more quickly after simulated reperfusion (Fig. 6B). The percentage of cells with altered actin cytoskel-

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Fig. 2. Activation of MAPKs and NF-κB due to IRI differs between strains. A: protein extracted from left lungs rendered ischemic × 1 h and then reperfused for 0, 15, 30, 60, or 180 min. Activation of JNK, ERK, p38, and NF-κB is apparent early in TLR4-sufficient mice (OuJ), whereas TLR4-deficient mice (HeJ) have minimal or no degradation of JNK, reduced activation of ERK until 180 min, and very delayed activation of p38. IκBα degradation occurs earlier in OuJ mice and later (180 min) in HeJ mice. n = 4 Each of HeJ and OuJ strains; controls, 2 HeJ, 2 OuJ mice euthanized and lungs retrieved expeditiously. Protein from controls were placed on each gel for each time point to allow comparison with normal lung for each time point. B: quantification of intensity by laser scanning. Phospho-total MAPKs and IκBα/β-actin were normalized by dividing each ratio by the mean ratio for controls. This makes each control = 1.0 with variability among the different control samples represented by error bars. Values are means ± SE. p46 and p54 JNK and p44 and p42 ERK have similar patterns and P values.

*P < 0.05, †P < 0.01, ‡P < 0.001 compared with controls by ANOVA with Tukey honestly significant difference (HSD) for multiple comparisons.
P, Phosphorylated.

Fig. 3. Immunostaining for p65 component of NF-κB. Minimal nuclear localization in control (freshly euthanized) with marked nuclear staining (3+/+) in 60-min reperfused samples in TLR4-sufficient (OuJ) mice compared with TLR4-deficient (HeJ) strain where staining was graded 1–2+. IκBα staining intensity complemented IκBα degradation (Fig. 2) except that IκBα levels appear equivalent in HeJ and OuJ strains at 180-min reperfusion despite more p65 staining in OuJ animals at 180-min reperfusion. We interpret this to mean some recovery of IκBα protein in OuJ mice 180 min postreperfusion. Curiously, p65 staining was the same for right (R) and left (L) lungs in both strains, implying equivalent NF-κB activation in both lungs following reperfusion of the left ischemic lung. Bars = 100 μm.
etaton was decreased in monolayers by CRX-526 (Fig. 6).

Fig. 4. Functioning TLR4 on lung parenchymal cells is necessary for development of edema due to IRI. Panel A: parenchymal cells; M, marrow-derived cells; +, intact TLR4 (OuJ); −, nonfunctional TLR4 (HeJ). A: alveolar macrophages (AMs) retrieved by bronchoalveolar lavage (BAL) were infected with Ad.NF-BLUC and Ad.CMV-LacZ and then incubated with 1μg/ml PBS or LPS. Firefly luciferase/β-galactosidase (fluc/β-gal) activity shows complete replacement of recipient marrow from either HeJ strain (P−M−) or OuJ strain (P−M+). AMs retrieved from nonirradiated HeJ and OuJ mice served as controls. LPS stimulation resulted in a 60-fold increase in luciferase activity in both native OuJ AMs and in AMs retrieved from chimeric strain P−M+, n = 4 Experiments/group; P < 0.0001. AMs retrieved from irradiated mice reconstituted with same strain marrow behaved in the same manner (data not shown). B: functioning TLR4 on parenchymal cells (P) is necessary for development of edema after 3-h IRI. P−M− chimeras developed significant increase in W/D. However, even if AMs had functioning TLR4 (P−M+), W/D was not elevated. Thus functioning TLR4 on AMs is not sufficient for development of edema but may amplify edema in mice with functioning TLR4 on lung parenchymal cells; W/D was slightly higher in P+M+ animals compared with P+M−, but the difference was not significant (NS). n = 5/Group; *P < 0.05, †P < 0.01 compared with W/D of P−M− lungs (ANOVA with Tukey HSD). C: chimeras with restored bone marrow (P−M−, P+M+) had the same W/D as the intact strains (OuJ, HeJ) demonstrating that lethal radiation and bone marrow transplant (BMT) had no effect on development of edema due to IRI.

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DISCUSSION

Our studies demonstrate a novel pivotal role for TLR4 as a major upstream effector of early edema consequent to reperfusion of ischemic lung. OuJ mice with functioning TLR4 quickly develop substantial edema on reperfusion after 60 min of hilar clamping due to rapid alveolar flooding. Increased W/D persists at least for 3 h, when increased alveolar wall thickness and interstitial edema are apparent in inflation-fixed specimens. In contrast, HeJ mice with defective TLR4 develop significantly less edema that resolves quickly, so that within 1 h of reperfusion, W/D is normal, and inflation-fixed histology is also normal after 3 h of reperfusion. We confirmed the role of TLR4 in pulmonary edema formation due to IRI by performing experiments in TLR4−/− mice. Increased edema due to functioning TLR4 is apparent within 5 min of reperfusion. MyD88-deficient animals developed edema when reperfused similar to TLR4-sufficient OuJ and C57BL/6J mice, the background strain for MyD88−/− and TLR4−/− mice. Thus MyD88, the downstream adapter protein implicated in activation of signaling cascades by many TLRs including TLR4, does not appear to be involved in development of edema due to IRI.

In addition to a role in edema formation very early after reperfusion of ischemic lung, functioning TLR4 mediates early activation of signaling pathways associated with inflammation. Functioning TLR4 resulted in early phosphorylation of p38 (observed during ischemia), early phosphorylation of ERK and JNK, and early activation of NF-κB following reperfusion. This time course is very similar to MAPK and NF-κB activation following lung transplantation in rats (unpublished observations). TLR4-deficient HeJ mice showed delayed (p38, ERK) or reduced (NF-κB, JNK) activation, implying involvement of alternative activation pathways other than TLR4. Delayed activation of p38 and ERK in TLR4-deficient mice was not associated with increased W/D nor was NF-κB activation in the right (nonischemic) lung of TLR4-sufficient mice (Fig. 3). In a rat model of in situ left lung IRI, W/D of the right lung is also not increased, although MAPKs are activated in the right lung after reperfusion of the left lung (unpublished observations). Thus, although functioning TLR4 is implicated in development of early edema due to IRI, our data suggest that early edema formation due to reperfusion of ischemic lung is not due to paracrine stimulation by an endogenous TLR4 ligand.
not dependent on activation of MAPKs or NF-κB and is not dependent on MyD88.

Our chimeric animal studies show that functioning TLR4 on lung parenchymal cells, not myeloid cells, is critical for early lung edema formation due to IRI. AMs clearly contribute to lung IRI (32), and functioning TLR4 on bone marrow-derived Kupffer cells contributes to liver IRI assessed biochemically 3 and 6 h postreperfusion (47). However, this study had a different time course, and the two models and outcomes are not necessarily comparable; liver edema was not assessed. Functioning TLR4 on AMs contributed substantially to airway inflammation 4 or 8 h after LPS inhalation (18). Depletion of AMs reduced permeability edema in a rat model of lung IRI (31). However, our data strongly suggest that IRI-induced early pulmonary edema is due to increased capillary leak very early after reperfusion. Our experiments in chimeric mice clearly show this requires functioning TLR4 only on parenchymal lung cells, not myeloid-derived AMs.

We used an in vitro model to explore possible mechanisms of rapid edema formation due to IRI, focusing on pulmonary microvascular endothelial cells. Earlier studies demonstrated significantly increased filtration coefficient and W/D of rat lungs after 1 h of ischemia, attributed to endothelial dysfunction (23), although altered lung epithelial fluid clearance can also contribute to pulmonary edema (30). Other investigators have employed hypoxia reoxygenation in cell culture models of IRI (37, 59), but we (28) previously demonstrated lung acidosis to pH 6.8 and no significant hypoxia in rat lungs left in situ at 37°C 1 h after cardiac arrest. Thus hypoxia is not a feature of lung ischemia, particularly in lungs inflated with 100% oxygen, so our cell culture model of IRI accurately reflects in vivo events (although nutrient depletion and development of acidosis would be more gradual in vivo). During simulated ischemia, HMVECs undergo structural alterations with reorganization of actin stress fibers from the center to the periphery of endothelial cells, associated with the development of gaps in the monolayer, not due to increased cell death.

Following simulated reperfusion, HMVEC cytoskeletal morphology normalizes, and a confluent monolayer is restored. Although it is difficult to assess the endothelial cell cytoskeleton in vivo, we speculate that the in vitro phenotype is similar to the changes that increase permeability on reperfusion and cause rapid alveolar flooding in vivo. It takes time for the lung to clear this excess fluid. W/D is still elevated, and there is histological evidence of increased interstitial fluid in TLR4-sufficient mice 3 h after reperfusion, whereas lungs from TLR4-deficient mice have a normal histological appearance (Fig. 1B).

Our studies in MyD88−/− mice imply that traditional TLR4-mediated signaling for gene transcription is not related to TLR4-mediated development of edema. Although the TRIF activation pathway may be operational in HMVECs, the time course of actin cytoskeletal alteration in vitro and the time course of activation of MAPK and NF-κB signaling cascades in vivo strongly suggest that TLR4-mediated edema development and alteration of actin cytoskeleton are not related to traditional TLR4-mediated signaling for gene transcription. Additional studies simulating cold IRI in cultured endothelial cells show a similar pattern of NF-κB and MAPK activation due to simulated IRI, i.e., activation of p38 during simulated ischemia, and prominent activation of MAPKs and NF-κB with simulated reperfusion (unpublished observations). Thus TLR4-mediated actin cytoskeletal alterations during simulated ischemia and TLR4-mediated activation of signaling cascades appear to be separate events that occur at different times.

CRX-526 prevented NF-κB activation in HMVECs exposed to LPS, confirming it is a TLR4 inhibitor. When administered to Ouj mice 1 h before hilar clamping, CRX-526 prevented edema formation in vivo after 60-min reperfusion. Treatment of HMVECs exposed to simulated IRI in vitro with micromolar doses of CRX-526 resulted in significantly reduced alteration of actin cytoskeleton, significantly decreased gap formation, and quicker recovery. Thus, through mediation of endothelial actin cytoskeleton, TLR4 may be

Fig. 5. A competitive inhibitor (Inhib) of TLR4 (CRX-526) prevents lung edema due to IRI and TLR4-mediated activation of NF-κB. A: W/D of left lungs of Ouj mice pretreated with vehicle or 10 μg CRX-526 in 200 μl of normal saline administered over 30 min, 30 min before left hilar clamping for 1 h and 1-h reperfusion. Mice pretreated with CRX-526 had the same W/D as HeJ mice 1 h postreperfusion in Fig. 1A. n = 5/Group; *P = 0.0014 compared with right lung of same animal by paired t-test; P = 0.0023 compared with left lung of mice pretreated with CRX-526 by unpaired t-test. B: concentrations of CRX-526 from 100 μg/ml to 0.1 μg/ml successfully inhibited NF-κB activation (based on luciferase/β-gal activity) following stimulation of human pulmonary microvascular endothelial cells (HMVECs) in 96-well plates with LPS (10 or 5 ng/ml), but CRX-526 had no impact on TNF stimulation of NF-κB activation (black bars). n = 4/Group; *P < 0.05 compared with other values at same time point by ANOVA.
an important effector of IRI-induced edema in humans, and the TLR4 receptor may be a promising target for pharmacological intervention to mitigate IRI and other forms of acute lung injury. Pretreatment of mice with eritoran, another TLR4 inhibitor, reduced myocardial infarction size in a murine model of cardiac IRI (41). It is possible that CRX-526 may have effects on other receptors or pathways besides TLR4, but our in vivo data in HeJ and TLR4−/−mice clearly show that functional TLR4 substantially contributes to IRI-induced pulmonary edema.
mediates edema due to IRI? Our in vitro experiments failed to find that acid (29), or hyaluronan fragments (22, 46) explain how TLR4 is involved in IRI.

We investigated TLR4 expression and function in human thoracic microvascular endothelial cells (HMVECs) in vitro. TLR4 is present in HMVECs, and HMVECs stimulated with a TLR4 ligand (e.g., lipopolysaccharide) release cytokines that mediate inflammation.

The TLR4 antagonist CRX-S526 was used to block TLR4 signaling.

<table>
<thead>
<tr>
<th>Time</th>
<th>Vehicle</th>
<th>CRX-S526</th>
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<tr>
<td>15-min WI</td>
<td>Stress fibers across the middle of the cells look more prominent, and the edges are well-defined by peripheral actin.</td>
<td>Actin a bit more prominent at the periphery, but there are still stress fibers crossing the cell centers.</td>
</tr>
<tr>
<td>1-h WI</td>
<td>A few gaps are present. Actin stress fibers are mainly peripheral.</td>
<td>Actin is less pronounced and is more peripheral. Possibly some ruffling going on at the cell edges? There may be some gaps.</td>
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<td>15-min rep</td>
<td>Actin staining looks more peripheral around the cell-to-cell borders. The overall cell appearance is more &quot;cobblestone.&quot;</td>
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<td>4-h rep</td>
<td>Actin appears a little disorganized and more peripheral; some variability between the panels.</td>
<td>Very prominent stress fibers crossing the cell centers. Less at the periphery. Fairly normal appearance.</td>
</tr>
<tr>
<td>24-h rep</td>
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Six photographs of human pulmonary microvascular endothelial cells (HMVECs) stained with phalloidin (×400) at each time point were randomly chosen and grouped in PowerPoint slides. No times were identified. The photos were studied and assessed for quality and location of actin stress fibers in HMVECs at designated time points in the experiment are displayed. Control, normal HMVECs in cell culture media; IRI, ischemia-reperfusion injury; WI, warm ischemia (media replaced with Ringer lactate); rep, simulated reperfusion (Ringer lactate replaced with cell culture media).

Does a released ligand, such as HSP-70, a saturated fatty acid (29), or hyaluronan fragments (22, 46) explain how TLR4 mediates edema due to IRI? Our in vitro experiments failed to demonstrate a soluble ligand capable of altering HMVEC shape in cell culture or activating NF-kB in highly sensitive AMs. Our experiments do not rule out the possibility of release of a ligand that binds quickly and avidly to TLR4 or other receptors and is not present in sufficient quantity in conditioned media (RL removed from cells that experienced cytoskeletal alteration) to activate TLR4 on AMs or alter HMVEC actin cytoskeleton. A TLR4 ligand released into the circulation following reperfusion of the ischemic left lung may explain why NF-kB activation was apparent in the contralateral lungs (Fig. 3), but NF-kB activation was not associated with development of edema in the right lungs. It is difficult to understand which TLR4 ligand would be involved in our cell culture experiment of simulated warm IRI. Why should replacing cell culture media with pyrogen-free commercial RL release a TLR4 ligand? If a TLR4 ligand were released in vivo during ischemia, then one would expect to see at least activation of NF-kB at 0-min reperfusion in Fig. 2. Thus our in vitro results suggest that TLR4-mediated alteration in endothelial morphology during ischemia may be independent of soluble TLR4 ligand released by endothelial cells.

TLR4 has been implicated as a contributor to several other forms of acute lung injury, including ozone-induced increased permeability (27) and inhaled LPS (17). Paradoxically, TLR4−/− mice are more susceptible to hyperoxia than wild-type mice (60). In IRI models, other investigators, until now, have studied events occurring hours or days after reperfusion, based on the assumption that any effects of TLR4 were mainly related to transcriptional regulation. TLR4-deficient mice have reduced myocardial infarction size 24 h after 1-h ligation of the left anterior descending coronary artery (35) and reduced levels of aspartate aminotransferase 1 and 3 h after reperfusion of liver lobes rendered ischemic for 45 min (53). TLRs were implicated in a rat model of renal IRI, but the earliest studies were performed 24 h after reperfusion (25). TLR4 protein and mRNA were elevated for days after renal IRI, and TLR4−/− mice as well as MyD88−/− animals had less renal dysfunction and less histological evidence of IRI 24 h after reperfusion (52). TLR4 contributes to markers of inflammation in a murine cardiac transplant model (24). During the course of our studies, Shimamoto et al. (42) reported that TLR4−/− mice experienced less increased permeability index following 3 h of reperfusion using a similar model of lung IRI. TLRs have multiple roles including both pattern formation during embryogenesis and recognition of pathogen-associated molecular patterns (PAMP; Refs. 21, 44). TLR4 has been implicated in other types of noninfectious inflammatory conditions (31, 33).

Other TLRs are involved in lung injury. Jiang et al. (22) showed reduced bleomycin-induced lung injury in double knockout TLR4/TLR2−/− mice. We have begun to study IRI in TLR2−/− mice; these animals develop less edema than C57BL/6J mice (unpublished observations). Our study is the first to show effects of TLR4 so early after injury and the first to implicate TLR4 in early development of edema due to IRI.

Our results are most consistent with TLR4 regulation of pulmonary microvascular endothelial barrier function in lung IRI by TLR4-mediated microvascular endothelial cell cytoskeletal alteration contributing to rapid development of permeability.

Table 1. Pattern of actin stress fibers in HMVECs due to simulated warm IRI

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Fig. 7. TLR4-mediated alteration of HMVEC cytoskeleton due to IRI is ligand-independent. A: quantification of gap area by MetaMorph software. WI-RL, media replaced by RL for 60 min; WI-RL/M, RL from this ischemia experiment diluted with equal volume of fresh media; RL/M, fresh RL mixed with equal volume of fresh media (glucose concentration is halved). HMVECs exposed for 60 min to WI-RL or RL/M do not develop significant gaps compared with HMVECs when media is replaced with RL (WI-RL). *P<0.01. B: RL removed from HMVECs following 1 h of simulated ischemia (RL-WI) and placed on transfected AMs retrieved from HeJ and OuJ mice (n = 4 each). There is no activation of NF-κB by this RL-WI or fresh RL.
edema when ischemic lung is reperfused. This is a novel and potentially very important role for the TLR4 receptor. Gong et al. (15) showed knockdown of TLR4 in HMVECs prevented increased permeability after 6-h exposure to LPS, and TLR4-associated activation of Src PTKs. Our cell culture studies did not address whether MyD88 or the TRIF pathway downstream of TLR4 was involved in endothelial cytoskeletal alteration in response to ischemia. However, our observations that edema due to IRI occurs in MyD88−/− mice coupled with the absence of the TRIF pathway in murine endothelial cells (16) and our data that edema due to IRI occurs irrespective of MAPK and NF-κB activation suggest that edema mediated by TLR4 occurs independent of TLR4-mediated transcriptional events. The absence of the TRIF pathway has not been documented in human endothelial cells to our knowledge. In addition to modulating the Src PTKs, we postulate that TLR4 may be interacting through RhoA and Rac to modify actin cytoskeleton. Several studies have observed changes in the actin cytoskeleton in response to TLR4 ligands such as LPS (48, 49), a role for RhoA downstream of LPS signaling (13, 43) and Rac activation has been observed (51).

Two phases (early and late) have been described in many forms of acute lung injury, dating back to early studies of the effect of endotoxin infusion (36) or activated complement (12). Our study clearly implicates TLR4 on pulmonary microvascular endothelial cells for early development of lung edema due to IRI, and through downstream signaling, TLR4 likely contributes to the later phase as well. Thus TLR4 may be an important link between the two phases of acute lung injury and may have two distinct roles: mediating alteration of endothelial actin cytoskeleton and mediating gene transcription through MyD88 and TRIF/TRIF-related adapter molecule (TRAM) adapter proteins that have downstream effects on signal transduction pathways including MAPKs, NF-κB, and possibly other signaling pathways. The point missense mutation of TLR4 in HeJ mice affects an intracellular portion of the TLR4 receptor at the interface with the MyD88 adapter, disrupting its recruitment, accounting for inability of the HeJ strain to respond to LPS (55). Perhaps this mutation also reduces endothelial cytoskeletal alteration in response to ischemia, prompting us to speculate that this may also be the site of interaction of TLR4 with the endothelial cytoskeleton. The endothelial cytoskeleton, particularly actin stress fibers, plays a critical role in regulation of pulmonary vascular permeability (7). The cytoskeleton may also function as an intracellular communication system or signaling scaffold (19).

An alternative explanation for our findings is that the absence of functional TLR4 alters the phenotype of endothelial cells in some way that renders them less susceptible to ischemia-induced cytoskeletal alteration. Zhang et al. (58) showed the TLR4−/− mice tend to develop emphysema as they age and noted that TLR4−/− mice have a different phenotype compared with wild-type mice in that they appear to have decreased antioxidant capacity and increased oxidant burden. This was attributed to increased Nox3 expression in lung tissue and endothelial cells from TLR4−/− mice. Thus, although a direct link between TLR4 and the cytoskeleton seems likely, it is also possible that indirect effects of nonfunctioning TLR4 may alter the phenotype of the cells contributing to cytoskeletal disruption due to IRI.

Irrespective of the mechanism, our findings have important clinical implications, not only for the opportunity to modify IRI for recipients of lung or other organ transplants, but also because many types of acute lung injury have strikingly similar phenotypes. Longer follow-up studies are necessary to understand whether TLR4 inhibition is beneficial or impairs lung integrity. TLR4/TLR2−/− mice had reduced markers of inflammation in BAL fluid early after bleomycin lung injury but suffered increased later mortality due to apoptosis in the lung (22). If transient TLR4 inhibition could safely reduce IRI, then this might benefit all organ transplant recipients and facilitate transplantation of lungs from non-heart-beating donors (NHBDs). This could provide lung transplants for thousands of patients with end-stage lung disease. Administration of a TLR4 inhibitor, via the airway to a NHBD (45), the pulmonary artery in an ex vivo perfusion circuit (10, 20, 50), or both, might mitigate IRI when lungs retrieved from NHBDs are subsequently transplanted.

ACKNOWLEDGMENTS

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Portions of this work have been presented at national meetings and appear in print as published abstracts (1, 56, 57).

G. Zanotti was a visiting research scholar from the Division of Cardiac Surgery, San Matteo Hospital, University of Pavia, Pavia, Italy, and is now a resident physician in the Department of Surgery, Duke University Medical Center, Durham, NC. M. Casiraghi was a visiting research scholar from the Department of Thoracic Surgery, Vita-Salute San Raffaele University, Milan, Italy, and is now with the Division of Cardiovascular Medicine, Gill Heart Institute, University of Kentucky, Lexington, KY.

GRANTS

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DISCLOSURES

UNC has filed a provisional patent on the discovery that CRX-526 prevents IRI-induced pulmonary edema and reduces actin cytoskeletal arrangement and gap formation in cultured HMVECs subjected to simulated IRI.

REFERENCES


