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Inducible Activation of TLR4 Confers Resistance to Hyperoxia-Induced Pulmonary Apoptosis

Salman T. Qureshi,1,2* Xuchen Zhang,2† Erika Aberg,* Nicolas Bousette, † Adel Giaid, † Peiyong Shan,§ Ruslan M. Medzhitov,‡ and Patty J. Lee§

TLRs are essential mediators of host defense against infection via recognition of unique microbial structures. Recent observations indicate that TLR4, the principal receptor for bacterial LPS, may also be activated by noninfectious stimuli including host-derived molecules and environmental oxidant stress. In mice, susceptibility to ozone-induced lung permeability has been linked to the wild-type allele of TLR4, whereas deficiency of TLR4 predisposes to lethal lung injury in hyperoxia. To precisely characterize the role of lung epithelial TLR4 expression in the host response to oxidant stress, we have created an inducible transgenic mouse model that targets the human TLR4 signaling domain to the airways. Exposure of induced transgenic mice to hyperoxia revealed a significant reduction in pulmonary apoptosis compared with controls. This phenotype was associated with sustained up-regulation of antiapoptotic molecules such as heme oxygenase-1 and Bcl-2, yet only transient activation of the transcription factor NF-κB. Specific in vivo knockdown of pulmonary heme oxygenase-1 or Bcl-2 expression by intranasal administration of short interfering RNA blocked the effect of TLR4 signaling on hyperoxia-induced lung apoptosis. These results define a novel role for lung epithelial TLR4 as a modulator of cellular apoptosis in response to oxidant stress. The Journal of Immunology, 2006, 176: 4950–4958.
activation regulates the host response to hyperoxia by significantly reducing lung cell apoptosis through distinct effector mechanisms.

Materials and Methods

Luciferase assay

The human A549 lung alveolar epithelial cell line (American Type Culture Collection no. CCL-185) was grown in RPMI 1640 with 10% FCS, glutamine, and penicillin/streptomycin. Plasmid pSR-α (0.9 μg), containing human CD4TLR4, and pBIBIXLuc (0.1 μg), a NF-κB promoter-driven luciferase reporter plasmid, were transfected in duplicate wells either individually (as controls) or in combination using Lipofectamine 2000 reagent (Invitrogen); a total of 1 μg of DNA was used in each experiment by adding the empty pFLAG vector as required. Twenty-four hours later the cell supernatant was aspirated, and the cells were washed once with sterile PBS and lysed using 75 μl of buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton, and 1 mM EDTA). The lysate was centrifuged at 12,000 × g for 10 min at 20°C to pellet cell debris, and 5 μl of solution was used for a luciferase assay (Promega).

Production and identification of transgenic mice

The CD4hTLR4 plasmid construct (see Fig. 1) was linearized with XhoI; the DNA ends were filled in by treatment with Pfu Turbo polymerase (Stratagene) for 15 min at 72°C in the presence of 1 mM dNTPs. A 2-kb luciferase assay (Promega).

EMSA

For kinetic studies of NF-κB activation, binary transgenic mice and control littermates on regular food and water received a daily i.p. injection of 4 mg of doxycline in 100 μl of sterile water (equivalent to the daily dose from doxycline-supplemented chow) for 3 days. Mice were humanely euthanized, and nuclear protein extracts from lung tissues were prepared using NE-PER nuclear and cytoplasmic reagents (Pierce). Nuclear extracts were incubated with a 100× molar excess of biotinylated double-stranded oligonucleotide (5′-GGAGTTTGGGGCTGCTGGTTTCC-3′) (product size 214 bp), and for mouse β-actin the primers were sense (5′-GGTGCCGCTCTAGGCACAAA-3′) and antisense (5′-CTCTTTGATGTACCCAAGGTATTTCC-3′) (product size 540 bp). A reaction mixture (30 μl) was made according to the Access RT-PCR System (Promega), which consisted of 0.8 μg of total RNA, 10 μl of avian myeloblastosis virus/Thermus flavus 5′× reaction buffer, 1 μl of dNTP mix (10 mM each dNTP), 50 pmol of sense and antisense primers, 2 μl of 25 mM MgSO4, 1 μl of avian myeloblastosis virus reverse transcriptase (5 U/μl), and 1 μl of Thermus flavus DNA polymerase (5 U/μl). Conditions for RT-PCR were 1 cycle at 48°C for 45 min, 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 30 s, 50°C for 1 min, 68°C for 2 min, and 1 cycle at 68°C for 5 min.

Murine hyperoxia exposure

Both lines of binary transgenic mice and their transgenic negative littermates were placed on doxycline-supplemented chow (2.3 mg/kg) for 7 days before and during exposure to 100% O2 in a Plexiglas chamber. Uninduced binary transgenic mice on regular chow and their wild-type littermates on doxycline-supplemented chow were kept at room air. For assessment of lung injury, mice were removed from the chamber after 72 h of hyperoxia and humanely euthanized, and bronchoalveolar lavage was performed twice with 1 ml PBS (pH 7.4). Cell pellets were pooled and resuspended in PBS, and cell counts were done with a hemacytometer. The supernatant was used for protein determination by a standard Bradford assay (Pierce). Lung specimens were obtained for RNA and protein extraction, histology, apoptosis, and immunohistochemical studies.

Identification and quantification of pulmonary apoptosis

TUNEL assays were performed with the in situ cell death detection kit (Roche) as described previously (22).

Immunohistochemistry

Formalin-fixed, paraffin-embedded lung tissue sections were deparaffinized, rehydrated, washed with deionized water, and incubated with 3% hydrogen peroxide for 5 min followed by avidin D blocking solution (Vector Laboratories) and biotin blocking solution for 15 min each. Sections were then incubated with a 1/100 dilution of goat anti-HO-1 primary Ab (Santa Cruz Biotechnology) at 37°C for 2 h. After several PBS washes, sections were incubated with a biotinylated donkey anti-goat IgG secondary Ab at 37°C for 30 min and a peroxidase-conjugated streptavidin-biotin complex (Santa Cruz Biotechnology). Protein-DNA complexes were separated on Novex 6% retardation gels (Invitrogen). The gels were dried and exposed to BioMax MR film (Kodak).

Identification and quantification of pulmonary apoptosis

TUNEL assays were performed with the in situ cell death detection kit (Roche) as described previously (22).
Lung protein levels of I-κBα, HO-1, Bcl-2, Bcl-xL, and Bax were analyzed by standard immunoblotting techniques. Briefly, lung tissue protein from induced and uninduced binary transgenic mice was extracted in a buffer containing 62.5 mM Tris (pH 6.8) and 1× Roche protease inhibitor, quantified by a Bradford assay (Bio-Rad), electrophoresed in 12% acrylamide gels, and electrotransferred to a nitrocellulose membrane (Immobilon-P). Immunoblotting with primary Abs (I-κBα from Cell Signaling Technology, all others from Santa Cruz Biotechnology) was followed by HRP-conjugated IgG secondary Ig, and detection was performed with the Phototope-HRP detection system (Cell Signaling). To verify equivalent sample loading, membranes were stripped and reprobed with anti-β-tubulin Ab (Santa Cruz Biotechnology).

Gene knockdown of HO-1 and Bcl-2 in vivo by short interfering RNA (siRNA)

HO-1 siRNA and a nonspecific siRNA scrambled duplex were both synthesized by Dharmacon Research as described previously (23). Bcl-2 siRNA and Bcl-2 mismatched control siRNAs were gifts from Athersys Europe. We have previously shown that intranasal administration of siRNA has a maximal effect if given 16 h before lung injury and achieves lung specificity (23). Therefore, each mouse was anesthetized with methoxyflurane for 10 min. Sections were then incubated with a 1/100 dilution of mouse CD4 epitope (Fig. 1), was previously shown to induce NF-κB-controlled immune response genes in the THP-1 monocytic cell line (24). To determine whether this construct was also functional in the lung epithelium, transient transfections of CD4hTLR4 were performed in the human A549 adenocarcinoma cell line with or without the pBiXLuc reporter plasmid (Fig. 2). Compared with transfection of either plasmid alone, activation of the NF-κB transcription factor, reflected by a 30-fold up-regulation of luciferase activity, was observed following cotransfection of both plasmids, demonstrating the signaling activity of the human CD4hTLR4 chimera in lung epithelial cells.

Inducible lung-specific transgenic targeting of CD4hTLR4 to the murine lung

To determine the spatial expression pattern of CD4hTLR4 using a binary tetracycline-regulated transgenic system with selective targeting to the airways (Fig. 3), mice with stable integration and expression of active TLR4 in the airways (Fig. 3), mice with stable integration and expression of active TLR4 in the airways. The rat CC10 promoter targets expression of a rtTA to the airway epithelium. Binding of the transactivator to upstream DNA sequences and induction of CD4hTLR4 transcription occurs only in the presence of doxycycline (Dox), hGHpa, human growth hormone intronic and polyadenylation sequences.
and CD4hTLR4 expression was evaluated by RT-PCR using specific primers for the human transgene sequence. CD4hTLR4 expression was confined to the lung and was clearly induced in both transgenic lines after doxycycline administration (Fig. 4A). Pulmonary transgene expression was not detectable by RT-PCR during the first three days of doxycycline administration and was maintained for at least 35 days in the presence of continuous doxycycline treatment (data not shown). Anti-CD4 immunoblotting of whole lung protein extract from binary transgenic mice treated with either regular or doxycycline-supplemented food demonstrated inducible expression of the CD4hTLR4 protein (Fig. 4B) that was localized to the airways by immunohistochemical staining (Fig. 4C). CD4hTLR4 expression appeared slightly higher in the lungs of transgenic line 2 compared with transgenic line 1 (Figs. 4, A and B).

**Transient activation of NF-κB and up-regulation of A20 by inducible CD4hTLR4**

To characterize the in vivo activation of NF-κB in response to the induction of CD4hTLR4, mouse lung protein was subjected to immunoblotting with an Ab to IκBα, a protein that retains NF-κB in the cytoplasm (Fig. 5A). Degradation of IκBα was observed within 4 h after the administration of doxycycline and was sustained for >24 h. Interestingly, the lung IκBα level returned to baseline within 48 h, suggesting that NF-κB in response to ong-

**FIGURE 5.** Activation and regulation of NF-κB by CD4hTLR4. A, Immunoblotting of lung protein lysates from uninduced binary transgenic mice (No dox) and at various time points after doxycycline administration, demonstrating that CD4hTLR4-mediated degradation of IκBα (IκB). Ab to β-tubulin was used as loading control. B, Electromobility shift assay of nuclear protein extracts from uninduced binary transgenic mice (0) and at various time points after doxycycline administration demonstrates transient activation of NF-κB. An unbound probe (Unb) was used as a control. C, RT-PCR analysis of A20 expression in lungs of an uninduced binary transgenic mouse (No dox) and two independent lines of binary transgenic mice (dox 1 and dox 2) treated with doxycycline for 7 days. Amplification of β-actin was performed as a control. Each lane represents a single mouse. Results shown are representative of three independent experiments.

To determine whether TLR4 activation within the airway epithelium modulates the response to hyperoxia, binary transgenic mice induced with doxycycline for 7 days were exposed to 95% oxygen for 72 h. Transgenic negative littermates as well as uninduced binary transgenic mice were used as controls. No significant differences in lung protein levels, cell infiltration (as determined by bronchoalveolar lavage cell counts), or survival were observed between transgenic mice and controls (data not shown). Compared with controls, a significant reduction in the degree of apoptotic lung cell death in response to hyperoxia was observed by TUNEL.
staining in both lines of induced binary transgenic mice (Fig. 6, A–D). Double immunostaining of lung sections from hyperoxia-exposed TLR4 transgenic mice using TUNEL to label apoptotic cells and SP-C to label type II alveolar epithelial cells showed that type II cells were apoptotic. In addition, TUNEL labeling was detected in cells that appeared to be macrophages and type I alveolar epithelial cells (Fig. 6E).

In vivo activation of HO-1 via CD4hTLR4

To identify the mechanisms whereby TLR4 expression modulates pulmonary apoptosis in response to hyperoxia, we investigated expression of HO-1, a stress-response gene that is known to have antioxidant and antiapoptotic properties (25). To determine whether induction of HO-1 through constitutively active human CD4hTLR4 occurs in the murine lung, RT-PCR analysis of RNA from binary transgenic mice that were induced with doxycycline for 0–10 days (for sustained induction) was performed using a specific primer pair (Fig. 7A). HO-1 mRNA expression was strongly induced in response to CD4hTLR4 activation. Specific RT-PCR of the same samples demonstrated equivalent expression of β-actin at all time points, confirming that an equal quantity of mRNA was assayed from individual mice. Up-regulation of HO-1 protein expression in response to CD4hTLR4 was also demonstrated by immunoblot analysis of lung tissue with a specific HO-1 Ab (Fig. 7B).

Up-regulation of Bcl-2 family proteins through CD4hTLR4

To further evaluate the regulation of hyperoxia-induced pulmonary apoptosis in binary transgenic mice, immunoblot analysis of whole lung protein was performed using Abs specific for members of the Bcl-2 family (Fig. 8). Induction of CD4hTLR4 up-regulated the pulmonary expression of Bcl-2 and Bcl-xL, two proteins that regulate the intrinsic pathway of apoptosis. In contrast, the expression

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** A–C, Photomicrographs of lungs exposed to hyperoxia. TUNEL staining of lung tissue sections from wild-type mice in room air (A), wild-type mice exposed to 72 h of hyperoxia (B), and doxycycline-treated binary transgenic mice exposed to 72 h of O2 (C). Magnification is ×200. D, Quantification of lung cell apoptosis among the groups. Data represent mean ± SE (n = 3); *, p < 0.05 compared with corresponding room air; #, p < 0.05 compared with wild-type (WT) exposed to 72 h of O2. E, TUNEL/SP-C costaining. Sections were stained for TUNEL (green) and SP-C (red); double-stained cells appear yellow. Arrows represent type I epithelial cells, single arrowheads indicate type II epithelial cells, and double arrowhead indicates an alveolar macrophage. Magnification is ×400. Results shown are representative of three independent experiments.

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Induction of HO-1 by CD4hTLR4. A, RT-PCR analysis of HO-1 and β-actin expression using whole lung RNA from individual binary transgenic mice fed regular chow (0) or treated with doxycycline (Dox)-containing chow for 3–10 days. MW, molecular weight marker. B, Immunoblot of whole lung protein lysate from binary transgenic mice treated for increasing durations with doxycycline (Dox)-containing food. Each lane represents a single mouse. Results shown are representative of three independent experiments.
level of the proapoptotic protein Bax was not significantly changed in binary transgenic mice. This finding suggests that another mechanism by which CD4hTLR4 inhibits hyperoxia-induced apoptosis is alteration of the relative expression of proapoptotic and antiapoptotic members of the Bcl-2 family.

siRNAs targeting lung HO-1 or Bcl-2 reverse the antiapoptotic effect of CD4hTLR4 during hyperoxic lung injury

To specifically demonstrate the individual contribution of HO-1 or Bcl-2 induction to TLR4-mediated attenuation of lung TUNEL staining during hyperoxia, we administered targeted and nonspecific intranasal siRNAs to TLR4 transgenic mice, followed by exposure to hyperoxia. HO-1 siRNA significantly increased the number of TUNEL-positive lung cells in TLR4 transgenic mice compared with mice treated with nonspecific siRNA or mice exposed to hyperoxia alone (Fig. 9). Similarly, Bcl-2 siRNA also significantly increased the number of TUNEL-positive lung cells in TLR4 transgenic mice compared with mice treated with mismatched Bcl-2 siRNA or mice exposed to hyperoxia alone (Fig. 10). Together, these data indicate that epithelial CD4hTLR4-mediated induction of either HO-1 or Bcl-2 confers profound antiapoptotic properties in the lung during hyperoxia.

Discussion

The lung epithelium is a direct interface between the environment and the internal milieu of the host and must defend against infectious challenge as well as oxidant stress associated with airborne pollutants, particulate matter, and inhaled gases. The TLR family provides a critical first line of defense against pulmonary infection through recognition of unique microbial structures and initiation of an inflammatory and adaptive immune response (26, 27); however, its role in other aspects of pulmonary host defense is less well understood. During respiratory failure, hyperoxia may be a necessary and lifesaving intervention, yet it has the potential to cause severe and potentially fatal lung damage. The deleterious effects of hyperoxia are mediated in large part by the formation of ROS that provoke epithelial and endothelial cell death, increased pulmonary capillary permeability, and, ultimately, severe tissue destruction (28, 29). Several recent observations suggest that TLRs participate in the response to oxidant stress. First, TLR4 was identified as a strong candidate gene for ozone-induced lung permeability based...
on linkage analysis of recombinant inbred mouse strains (30). Second, ROS have been linked to TLR4 inflammatory signaling in neutrophils (31). Finally, mice deficient for the TLR4 gene are predisposed to lung injury and death following prolonged hyperoxia (20). Based on these observations, we hypothesized that activation of TLR4 in the airway epithelium, the surface that is directly exposed to hyperoxia, is a critical mechanism for protection against oxidant lung injury and death. To test this possibility, we developed a transgenic mouse model in which the cytoplasmic domain of human TLR4 that is constitutively active in monocytes (24) could be inducibly expressed by a doxycycline-dependent transactivator under the control of a CC10 promoter. The activity of this construct in the lung epithelium was confirmed by transient transfection of the human A549 type II alveolar cell line along with a NF-κB-responsive luciferase reporter plasmid. Strong induction of luciferase activity was observed in the presence of both constructs, suggesting that activation of TLR4-dependent signaling pathways in the lung could also be achieved in vivo through a transgenic expression approach.

Using a binary doxycycline-dependent expression system, two independent lines of transgenic mice exhibiting inducible lung-specific expression of CD4hTLR4 were generated and studied following exposure to 72 h of hyperoxia (32). A significant reduction in the level of pulmonary apoptosis was observed among doxycycline-treated transgenic mice compared with control animals. Further analysis revealed that the HO-1 and Bcl-2 proteins were induced in lung tissue following transgenic TLR4 expression. HO-1 is the inducible isoform of the rate-limiting enzyme that catabolizes heme into biliverdin, free iron, and carbon monoxide (33). HO-1 is subject to transcriptional regulation by diverse stimuli and is known to have strong anti-inflammatory and antiapoptotic effects (33). In the current study, TLR4 activation before hyperoxia resulted in transient NF-κB activation as well as strong and sustained up-regulation of HO-1 transcription. Despite the presence of a NF-κB-like site in its regulatory regions, HO-1 has not been found to regulated by NF-κB in response to any of its inducers (25). We have previously shown that the mitogen-activated protein kinases, AP-1, Nrf2, and the STAT proteins are critical regulators of HO-1 in response to oxidant stress and, therefore, are potential regulators of Tlr4-mediated HO-1 induction (25, 34, 35). The specific contribution of HO-1 in this setting was confirmed by abrogation of the antiapoptotic phenotype through intranasal administration of unmodified siRNA, an approach that has been previously shown to result in potent lung-specific gene knockdown (23). The Bcl-2 protooncogene is the founding member of an intracellular protein family that regulates apoptosis (36). Bcl-2 family members may either promote or inhibit cell death, depending on the relative...
abundance of individual gene products at a particular time. Doxy-cycline-dependent TLR4 activation in the mouse airway epithelium resulted in strong and sustained up-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL, but did not influence the expression of the proapoptotic protein Bax, invoking another mechanism to explain the pulmonary phenotype of TLR4 transgenic mice in hyperoxia. The role of Bcl-2 as a modulator of cell death in hyperoxia was also confirmed by blocking the antiapoptotic phenotype of TLR4 transgenic mice through in vivo gene knockdown with specific intranasal siRNA.

TLR4 signals by recruiting the intracellular adaptor molecule MyD88 or by engaging an alternative adaptor called the Toll/IL-1R domain-containing adaptor inducing IFN-β or TRIF. MyD88-dependent gene expression is mediated primarily by early phase activation of the transcription factor NF-κB and includes proinflammatory cytokines such as TNF-α, whereas TRIF uses IFN regulatory factor 3 to induce IFN-β and IFN-dependent gene products and contributes to the late phase of NF-κB activation (37). Interestingly, sustained transgenic expression of the active signaling domain of TLR4 in the mouse lung was associated with transient activation of NF-κB, most likely due to a well-documented autoregulatory feedback loop (38). Induction of CD4+ TLR4 also increased expression of A2O, a gene encoding a zinc finger protein that suppresses TNF- and TLR-mediated NF-κB activation by deubiquitylation of TNFR-associated factor 6. These two mechanisms are likely to account for the lack of observable pulmonary inflammation in this model and highlight the tight regulation of TLR activity in vivo to prevent the consequences of overwhelming immune activation (39).

Lung damage secondary to hyperoxia has a complex pathophysiology that is characterized by inflammation, cell necrosis, and apoptosis secondary to the toxic effects of excess reactive oxygen intermediates (40). The diversity of cell types in the lung and the associated differences in susceptibility or responsiveness complicates the analysis of hyperoxic lung injury (40). Numerous host defense mechanisms have been implicated in the response to hyperoxia, and each may influence various aspects of cell death, survival/stress responses, inflammation, and cell growth (41). Our studies have been focused on the role of TLR4 in lung epithelial integrity and demonstrate that activation of innate immunity through TLR4 in a subset of airway epithelial cells is sufficient to modulate lung cell apoptosis in a model of severe hyperoxia. This cell type-specific transgenic approach may account for the fact that no difference in survival or other parameters of acute lung injury was observed, and it does not address the potential contribution of other TLRs or TLR signaling by other lung cell types (such as vascular endothelium) that might influence organ damage or the overall outcome following hyperoxia. Previous reports have also shown that a selective antiapoptotic strategy is not sufficient to attenuate hyperoxia-induced injury and death (42). Recent reports indicate that innate immune activation exerts a complex influence on cell survival; induction or inhibition of apoptosis depends on both the specific TLR that is activated as well as the cell type on which it is expressed and its functional state (43–48). Only one other study has examined the role of TLRs in defense against oxidative stress; in this case, in vitro resistance of cardiac myocytes to the cytotoxic effect of hydrogen peroxide was shown to be mediated by TLR2-dependent activation of the transcription factors NF-κB and AP-1 (49).

Our observations contribute to an emerging paradigm linking innate immune recognition to host defense against oxidant stress. By modulating host cell signaling pathways, TLRs in the lung appear to serve a dual role by protecting the host from environmental oxidants as well as invading microbes. Therapeutic manip-ulation of pulmonary innate immunity may lead to improved strategies for the prevention and management of oxidant-induced acute lung injury.

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Disclosures

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