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TLR Signaling Prevents Hyperoxia-Induced Lung Injury by Protecting the Alveolar Epithelium from Oxidant-Mediated Death

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Mechanical ventilation using high oxygen tensions is often necessary to treat patients with respiratory failure. Recently, TLRs were identified as regulators of noninfectious oxidative lung injury. IRAK-M is an inhibitor of MyD88-dependent TLR signaling. Exposure of mice deficient in IRAK-M (IRAK-M−/−) to 95% oxygen resulted in reduced mortality compared with wild-type mice and occurred in association with decreased alveolar permeability and cell death. Using a bone marrow chimera model, we determined that IRAK-M’s effects were mediated by structural cells rather than bone marrow-derived cells. We confirmed the expression of IRAK-M in alveolar epithelial cells (AECs) and showed that hyperoxia can induce the expression of this protein. In addition, IRAK-M−/− AECs exposed to hyperoxia experienced a decrease in cell death. IRAK-M may potentiate hyperoxic injury by suppression of key antioxidant pathways, because lungs and AECs isolated from IRAK-M−/− mice have increased expression/activity of heme oxygenase-1, a phase II antioxidant, and NF (erythroid-derived)-related factor-2, a transcription factor that initiates antioxidant generation. Treatment of IRAK-M−/− mice in vivo and IRAK-M−/− AECs in vitro with the heme oxygenase-1 inhibitor, tin protoporphyrin, substantially decreased survival and significantly reduced the number of live cells after hyperoxia exposure. Collectively, our data suggest that IRAK-M inhibits the induction of antioxidants essential for protecting the lungs against cell death, resulting in enhanced susceptibility to hyperoxic lung injury.

Previous work showed that TLRs play a role in mediating acute lung injury (26–28). More specifically, mice deficient in certain extracellular TLRs (e.g., TLR2/4) are more susceptible to hyperoxia lung injury (26, 28). The increased susceptibility of TLR4−/− mice was due to dysregulated epithelial cell death. Thus, both oxidative stress and infectious insults can activate common pathways to protect the host (29, 30).

IRAK-M (or IRAK-3) is a member of the IRAK family that functions as an important negative regulator of MyD88-dependent TLR signaling. IRAK-M disrupts the interactions of IRAK-1 and IRAK-4 from the TLR-signaling domain, resulting in inhibition of the signal-transduction pathways (31). Although originally described to be exclusively produced in myeloid cells (32, 33), IRAK-M was more recently found in epithelial cells (34, 35). Mice deficient in IRAK-M (IRAK-M−/−) display enhanced immune and inflammatory responses during infectious insults (36, 37), due in part to increased NF-κB and MAPK activation in response to TLR ligands (38). This more robust inflammatory response in IRAK-M−/− mice can be beneficial in the case of sepsis-induced immunosuppression (36) or bacterial infection (38, 39), but it potentiates lung injury in the setting of influenza viral infection (37).

The primary objective of this study was to determine the role of an important TLR-signaling inhibitor, IRAK-M, in regulating noninfectious lung injury. We hypothesized that IRAK-M plays an essential role in hyperoxic lung injury. Our findings indicate that IRAK-M enhanced susceptibility to hyperoxic lung injury, and we demonstrated a previously unrecognized role for IRAK-M in regulating the generation of antioxidants in the lungs.

Materials and Methods

Animals

A colony of IRAK-M−/− deficient mice bred on a B6 background for more than eight backcrosses was established at the University of Michigan (36). Age- and sex-matched specific pathogen-free 6- to 8-wk-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were housed in specific pathogen-free conditions within the University of Michigan Animal Care Facility. All animal experiments proceeded in accordance with National Institutes of Health policies on the human care and use of laboratory animals and were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Murine alveolar epithelial cell isolation and cell culture conditions

Murine type II alveolar epithelial cells (AECs) were isolated using the method developed by Corti et al. (40). Briefly, at designated time points following CO2 asphyxia, the pulmonary vasculature was perfused, and the lungs were first filled with 1 ml dispase (Worthington, Lakewood, NJ) and then 1 ml low melting point agarose and placed in ice-cold PBS to harden. The lungs were then subgrouped in disase for 45 min before being minced and incubated in DMEM with 0.01% DNase for 10 min. A single-cell suspension was obtained by passing the lung mince over a series of nylon filters. Myeloid cells were removed by first incubating cells with biotinylated Abs against CD32 and CD45 (BD Pharmingen, San Diego, CA) and then streptavidin-coated microbeads (Promega, Madison, WI), followed by negative selection using a magnetic tube separator. Mesenchymal cells were removed by overnight adherence in a Petri dish; the resulting nonadherent AECs were assayed immediately (ex vivo AECs), plated on fibronectin-coated dishes, or cultured in Teflon-coated vials (Cole-Palmer, Vernon Hills, IL) for designated time periods. Previous work showed that the day-3 time point has >90% pure AECs (41).

In a murine type H-like AEC line, MLE-12 (CRL-2110; American Type Culture Collection), was cultured under the following conditions: RPMI 1640, 2% FBS, 1× insulin transferrin solution, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 nM β-estradiol, and 10 nM hydrocortisone.

Hyperoxia exposure

Mice were exposed to hyperoxia by housing them in a 50 × 30 × 30-cm airtight chamber with a constant flow >95% oxygen. This system results in measured oxygen concentrations of 90–94% (41). Both mice exposed to hyperoxia and room air control mice were fed food and water ad libitum and kept on a 12-h dark–light cycle at room temperature. For in vitro studies, AECs were plated on fibronectin-coated plates or in Teflon-coated vials and then cultured either in a traditional incubator at 37°C supplemented with 5% CO2 or in a modular incubator chamber (Billups-Rothenburg, Del Mar, CA) that was filled with >95% O2 and then placed at 37°C for the designated time period.

Bronchoalveolar lavage

Mice were sacrificed, and bronchoalveolar lavage (BAL) was performed as previously described (41). Briefly, BAL was performed by instilling PBS containing 5 mM EDTA into the trachea in 1-m1 aliquots. Approximately 3 ml was retrieved per mouse, and the concentration of albumin was determined using a mouse albumin ELISA quantification kit (Bethyl Laboratories, Montgomery, TX). Lavaged cells from each group of animals were pooled and counted after RBC lysis with hypotonic solution. Cytospins (Thermo Electron, Waltham, MA) were prepared for determination of BAL differentials using a modified Wright stain.

Total lung leukocyte preparation by lung digestion

Lungs were removed from euthanized animals, and leukocytes were prepared as described (36). Briefly, lungs were minced with scissors to a fine slurry in 15 ml digestion buffer (RPMI 1640, 10% FCS, 1 mg/ml collagenase Roche Diagnostics, Indianapolis, IN), 30 µg/ml DNase [Sigma-Aldrich, St. Louis, MO] per lung and enzymatically digested for 30 min at 37°C. Any undigested fragments were further sheared by repeatedly withdrawing and ejecting the suspension through a 10-ml syringe. The total lung cell suspension was pelleted, resuspended, and separated by centrifugation through a 40% Percoll gradient to enrich leukocytes.

Assessment of cell death in whole lung and AECs

Cell death in whole lung and AECs was quantified by measuring histone-associated DNA fragments using an ELISA kit (Cell Death Detection ELISA apoptosis, Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions (42). In addition, AECs were isolated from wild-type (WT) and IRAK-M−/− mice from both room air control and in vivo hyperoxia-exposed mice; AECs from WT and IRAK-M−/− mice cultured in Teflon-coated vials and exposed to 95% O2 in vitro were stained with Annexin V/propidium iodide (PI) (BD Pharmaceuicals), following the manufacturer’s instructions. Immediately following the staining protocol, cells were analyzed on a BD FACS Calibur flow cytometer, and additional postanalysis was done using FlowJo software (Tree Star, Ashland, OR).

Flow cytometry

A total of 1 × 106 cells was incubated with anti-CD16/CD32 (FcBlock; BD Biosciences), stained with an Ab against CD45, permeabilized (BD Biosciences, San Jose, CA), and stained with primary Abs against TLRs (Abcam, Cambridge, U.K.) or its normal rabbit serum isotype control (Jackson ImmunoResearch, West Grove, PA) and prosurfactant protein C (Millipore, Billerica, MA) or its normal rabbit serum isotype control (Jackson ImmunoResearch). After sufficient washing, directly conjugated secondary Abs (Jackson ImmunoResearch) were added, and samples were fixed and run in the University of Michigan flow cytometry core. Data were analyzed using flow cytometry analysis software (FlowJo, version 7.5), and representative flow plots are shown in Supplemental Fig. 2.

Bone marrow transplantation

WT and IRAK-M−/− mice received 13 Gy of total body irradiation ([137]Cs source) delivered in two fractions separated by 3 h. Bone marrow (BM) was harvested from the femur of donor mice, and a cell mixture of 5 × 107 cells was resuspended in media. BM was transplanted into the lethally irradiated recipients via tail vein infusion (0.2 ml total volume), resulting in a syngeneic transplantation with no major or minor MHC mismatch. All experiments with BM transplantation (BMT) mice were performed 6–8 wk post-BMT, as previously cited (43).

Western blotting

AECs were plated at 3 × 105 cells/well and lysed in buffer containing RIPA (Sigma) supplemented with protease inhibitors (Roche Diagnostics). For immunoblot analysis, 20 µg protein was loaded onto 10% SDS-PAGE gels, subjected to electrophoresis, and transferred to membranes (Millipore). Membranes were incubated with Abs against IRAK-M (1:1,000; Abcam) or β-actin (1:10,000; Abcam). Signals were developed with an ECL Plus Western blot detection kit (Amersham, Arlington Heights, IL).
Semiquantitative real-time PCR

Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA). Gene-specific primers and probes were purchased from Integrated DNA Technologies (Coralville, IA). Whole lung and AECs were harvested from each group, and the mRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). The average cycle threshold (44) was determined for each sample from a given experiment. Relative gene expression (using the equation 2−ΔΔCT) was calculated using the comparative Ct method, which assesses the difference in gene expression between the gene of interest and an internal standard gene (β-actin) for each sample to generate the ΔΔCt. The average of the control sample was set to 1 for each experiment, and the relative gene expression for each experimental sample was compared with that.

Nrf-2 activity assay

The nuclear-binding activity of Nrf-2 was measured using a DNA-binding ELISA (TransAM Nrf-2; Active Motif, Carlsbad, CA) on both whole lung tissue and AECs. First, a single-cell suspension was made from whole lungs by cutting tissue into small pieces, followed by homogenizing the tissue using a Dounce homogenizer. Nuclear and cytoplasmic fractions were isolated from the resulting single-cell suspension or from isolated AECs using an extraction kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents; Thermo Scientific). The resulting nuclear fraction was added to the DNA-binding ELISA, and the binding of the activated transcription factor to the consensus sequence was quantified by colorimetric assay.

HO-1 inhibition

Mice were injected with 100 μl tin protoporphyrin (TP; Sigma; 20 μmol/kg resuspended in 0.1 N NaOH) or vehicle control (0.1 N NaOH) i.p. daily for the duration of the experiment. For in vitro inhibition of HO-1, AECs were treated with TP (10 μM) or vehicle control (0.001% DMSO) for the designated time period.

Statistical analysis

All data are expressed as mean ± SEM and were analyzed using Prism 5.0 statistical program (GraphPad Software). Comparisons between two experimental groups were performed with the Student t test. Comparisons among three or more experimental groups were performed using ANOVA with a post hoc Bonferroni test to determine significance. A p value < 0.05 was considered significant.

Results

Deletion of IRAK-M reduces susceptibility to hyperoxic lung injury

Previous work showed that the presence of TLRs is essential in mediating noninfectious lung injury, including hyperoxic lung injury (26–28); however the role of IRAK-M, an important negative regulator of MyD88-dependent TLRs, has not been examined. Therefore, WT and IRAK-M−/− mice were exposed to 95% O2 either continuously for up to 144 h (Fig. 1A) or transiently (Fig. 1B) for 96 h and then returned to room air. Compared with WT mice, IRAK-M−/− mice displayed reduced susceptibility to hyperoxic-induced mortality (no surviving WT mice after 5 d in hyperoxia compared with 60% survival in IRAK-M−/− mice) at this time point (Fig. 1A). IRAK-M−/− mice showed a significant survival advantage in the transient exposure model as well (Fig. 1B). To determine the mechanisms accounting for reduced susceptibility to mortality in the IRAK-M−/− mice, lung permeability was measured by quantifying the amount of albumin present in the BAL fluid. After exposure to O2 for 72 h, WT mice have significantly more protein influx into the alveolar spaces compared with IRAK-M−/− mice (Fig. 1C). In addition, significantly more cell death (as determined by DNA–histone complexes) was found in the lungs of WT mice exposed to hyperoxia for 72 h compared with IRAK-M−/− mice (Fig. 1D).

To determine effects on cell recruitment, we measured the influx of leukocytes in both WT and IRAK-M−/− oxygen-exposed animals compared with room air controls. Hyperoxia exposure results in minimal lung inflammation, and there was little to no cellular recruitment to the airways or the lung interstitium in the WT mice after 24 or 72 h of oxygen exposure (Supplemental Fig. 1A). Importantly, there was no difference in the recruitment of leukocytes in IRAK-M−/− animals compared with WT mice after hyperoxia. In addition, there was no difference in the total number of leukocytes found in the BAL fluid or isolated from a whole-lung digest (Supplemental Fig. 1A), and there was no difference in the composition of leukocytes isolated from the BAL fluid or from the whole-lung digestion from IRAK-M−/− mice compared with WT mice (Supplemental Fig. 1B). Finally, there was no difference in the production of cytokines (IL-1β, TNF-α, IFN-γ, or GM-CSF) or chemokines (MCP-1, MIP-2, or KC) from whole lungs of WT and IRAK-M−/− mice exposed to hyperoxia for 24, 48, or 72 h compared with room air controls (data not shown). Thus, improved survival in IRAK-M−/− mice during hyperoxia was associated with decreased lung permeability and cell death but not changes in influx of recruited leukocytes or production of inflammatory cellular mediators.

Expression of IRAK-M in structural cells, rather than BM-derived cells, is required for regulating hyperoxic lung injury

BM chimeras were generated to determine which IRAK-M-expressing cells of the lung were responsible for regulating susceptibility to hyperoxia. BM was harvested from donor WT or IRAK-M−/− mice and transplanted into lethally irradiated WT or IRAK-M−/− recipient mice. The mice were rested for 6 wk, because previous work by our group showed a complete reconstitution of the leukocyte populations in the lung at this time point (43). The transplanted mice were then exposed to continuous or transient hyperoxia, and survival was assessed. As shown in Fig. 2, mice in which hematopoietic cells were deficient in IRAK-M expression (IRAK−/→WT BMT) succumbed to hyperoxia with the same lethality as did WT→WT transplanted mice. However, if mice lacked IRAK-M in structural cells (e.g., WT→IRAK BMT)
were exposed to 95% O2 for 72 h and then removed and monitored at room

exposed to 95% O2 continuously (assessed when WT

We next assessed the amount of cell death occurring in WT and

FIGURE 2. Expression of IRAK-M on structural cells, but not BM-
derived cells, reduces susceptibility to hyperoxic lung injury. BM was
harvested from WT or IRAK-M−/− animals and transplanted into lethally irradiated WT or IRAK-M−/− donor mice, as described in Materials and

Materials and Methods. (A) Survival was assessed when the WT→WT BMT mice, IRAK-M−/−→WT BMT mice, and WT→IRAK-M−/− BMT mice were exposed to 95% O2 continuously (n = 10 mice/group). (B) Survival was assessed when WT→WT BMT mice and WT→IRAK-M−/− BMT mice were exposed to 95% O2 for 72 h and then removed and monitored at room
 airflow for an additional 4 d (n = 10 mice/group).

but it was expressed by hematopoietic cells, then survival was increased upon exposure to either continuous (Fig. 2A) or tran-
sient O2 (Fig. 2B). Therefore, expression of IRAK-M in structural
cells was responsible for mediating susceptibility to hyperoxic lung injury.

IRAK-M is expressed in AECs and is upregulated in response to TLR ligands or hyperoxia

Initial studies characterizing IRAK-M suggested that its expression
was restricted to monocytes and macrophages (38); however, more
recent work showed that IRAK-M is expressed in many cells (34, 35). To verify that IRAK-M is expressed in AECs, we examined the
mRNA and protein expression of IRAK-M in response to TLR
ligands and hyperoxia in both primary AECs and murine type II-
like AECs (MLE-12). We observed a significant increase in the protein
expression of IRAK-M in primary WT AECs stimulated with TLR ligands LPS and IL-1β (data not shown). In response to hyperoxia, there was an increase in the mRNA expression levels of IRAK-M in whole lung tissue (data not shown) and AECs (Fig. 3A), most notably after 24 h. In addition, we observed a nearly 4-
fold increase in the protein expression of IRAK-M in WT AECs in
response to hyperoxia exposure after 24 h, which remained elevated at 72 h (2-fold increase; Fig. 3B, 3C). To verify that the expression
of IRAK-M in response to the different stimuli was not due to macrophage and/or monocyte contamination, we exposed MLE-12
cells to hyperoxia in vitro and measured the expression of IRAK-M. As shown in Fig. 3D, an increase in the expression of IRAK-M was
observed in MLE-12 cells exposed to hyperoxia for 72 h.

Decreased cell death in AECs isolated from IRAK-M−/− mice
after hyperoxic exposure

We next assessed the amount of cell death occurring in WT and
IRAK-M−/− AECs during hyperoxia exposure in vitro. AECs
were harvested from WT and IRAK-M−/− mice and then incu-
bated in room air conditions or 95% oxygen for 72 h. The amount
of cell death was quantified by assessing the amount of DNA-
histone complexing in these cells. Hyperoxic exposure resulted in a
time-dependent increase in cell death in AECs from WT mice (Fig. 4A). In comparison, the amount of cell death measured in
IRAK-M−/− AECs was significantly reduced after 72 h of O2
exposure. To assess cell death in AECs from in vivo-exposed
animals, we examined the expression of two markers of cell
death: Annexin V and PI. Fig. 4B shows a representative flow plot
in which WT AECs have more Annexin V+ (early cell death) and
Annexin V/PI double-positive cells (late cell death or necrosis) than do IRAK-M−/− AECs after 72 h of in vivo hyperoxia ex-
posure. Data were combined to verify that there was no differ-
ce in the percentage of any of the populations in the room air
groups, and only after exposure to hyperoxia were there signif-
icant differences in the percentage of live cells (double-negative)
and Annexin V/PI-stained cells (Fig. 4C). To confirm that cells examined from the in vivo-treated mice were indeed AECs,
cells were stained with Abs against CD45 (common leukocyte
marker), surfactant protein C (type II AECs), and T1-α (type I
AECs) or their isotype controls. Gating on the CD45−
MHC-II− population (>90% of the cells), we found that 82% of the remaining
cells were positive for either surfactant protein C or T1-α,
confirming that the majority of the cells isolated ex vivo are
AECs (Supplemental Fig. 2).

IRAK-M suppresses antioxidant production during hyperoxic lung injury

Previous data showed that AECs and mice lacking IRAK-M were
protected against cell death during hyperoxic exposure (Figs. 1, 4).
To determine whether the absence of IRAK-M resulted in the
differences in the generation of antioxidants, we examined the
activity of the redox-sensitive transcription factor, Nrf-2, and the
expression of the downstream phase II antioxidant, HO-1. Whole
lungs were collected from room air control and hyperoxia-exposed
WT and IRAK-M−/− mice. Nuclear and cytoplasmic fractions
were made from a single-cell suspension of whole lung tissue, and
the amount of DNA binding to the Nrf-2 consensus site from the
nuclear fraction was measured. Compared with WT mice, Nrf-2
translocation was greater in lungs from IRAK-M−/− mice at both
24 and 48 h posthyperoxic exposure (Fig. 5A). In addition, Nrf-2 activity was upregulated earlier (24 h) in AECs isolated from
IRAK-M−/− mice exposed in vivo to hyperoxia (data not shown).
HO-1 is an important antioxidant induced by Nrf-2 during oxidant
stress, and HO-1 mRNA expression was elevated in response to
hyperoxia in both whole lung (Fig. 5B) and AECs (Fig. 5C) from
IRAK-M−/− mice compared with WT controls. We also examined
the production of glutathione peroxidase-2, because previous work
showed that expression of this antioxidant enzyme is also Nrf-2
dependent (45). There was no difference in mRNA expression of
GPx-2 in whole lung and AECs from WT and IRAK-M−/− mice
exposed to hyperoxia (data not shown). In addition, we measured
the activity of the enzyme superoxide dismutase in whole lung and
AECs from WT and IRAK-M−/− mice exposed to hyperoxia and
found no difference in the activity of this enzyme in response to
hyperoxia (data not shown). Taken together, these data suggest
that IRAK-M selectively inhibits the early activation of HO-1 in
response to hyperoxia.

Inhibition of HO-1 enhances susceptibility of IRAK-M−/− mice
to hyperoxic lung injury through increased AEC death

We used the HO-1 inhibitor TP to determine the role of HO-1 in
regulating oxidant injury. WT and IRAK-M−/− mice were treated
i.p. with either TP or vehicle control and then exposed to hyperoxia for 84 h before removing to room air and monitoring survival. Treatment with TP did not appreciably alter susceptibility of WT mice to hyperoxia, because we observed 100% mortality by 96 h in both the TP- and the vehicle control-treated groups (Fig. 6A). By comparison, treatment of IRAK-M−/− mice with TP during hyperoxia exposure resulted in 100% mortality compared with only 20% mortality of vehicle-treated IRAK-M−/− mice (Fig. 6A). When the duration of transient hyperoxia was shortened to 72 h, treatment of IRAK-M−/− mice with TP resulted in 60% mortality compared with only 10% mortality observed in vehicle control-treated IRAK-M−/− mice (data not shown).

To assess the role of HO-1 in AEC responses in vitro, AECs were harvested from WT or IRAK-M−/− mice and cultured in vitro in the presence of TP or vehicle control in room air or under hyperoxic conditions for 72 h, and the amount of cell death was assessed via Annexin V/PI flow cytometer staining. As shown previously, there was no difference in the number of live AECs isolated from WT and IRAK-M−/− mice and incubated in room air conditions; however, there was a greater number of viable IRAK-M−/− AECs after in vitro hyperoxia exposure compared with cells isolated from WT mice (Fig. 4). Inhibition of HO-1 in WT AECs exposed to O2 did not significantly alter the number of live cells; however, treatment of IRAK-M−/− AECs with TP in the presence of O2 significantly decreased the number of live cells (Fig. 6B). These data indicate that inhibition of HO-1 in IRAK-M−/− mice increases the susceptibility of these mice to hyperoxia in vivo and to increased AEC death in vitro.

**Discussion**

Collectively, our data indicate that IRAK-M plays a novel role in regulating responses to hyperoxic lung injury. The deletion of IRAK-M results in reduced susceptibility to both continuous and transient hyperoxia exposure as the result of decreased alveolar permeability and cell death. Using BM chimeras, we showed that expression of IRAK-M on structural cells, rather than leukocytes, is important in mediating responses to hyperoxia. Despite initial studies suggesting that IRAK-M was exclusively expressed in myeloid cells, we showed that IRAK-M is also expressed and functional in AECs. In addition, the expression of IRAK-M in AECs increases their susceptibility to cell death and decreases the expression of key antioxidant pathways. Treatment of IRAK-M−/− mice with an HO-1 inhibitor increased their susceptibility to hyperoxic lung injury. Taken together, these data show that IRAK-M inhibits the early generation of antioxidants from AECs, which are essential for protecting the host against oxidative lung injury.

Observations made over the past decade have illuminated the concept that TLRs evolved not just to protect the host against infectious insults but also to protect against noninfectious injuries. Previous studies showed that mice deficient in extracellular TLRs are more susceptible to acute lung injury (26–28). The enhanced susceptibility of TLR4-deficient mice to hyperoxia was associated with an inability to upregulate Bcl-2 and phospho-Akt (28). In addition, both TLR2/4 double-knockout mice and MyD88-deficient mice have decreased survival and increased AEC death in response to hyperoxic lung injury (26). Furthermore, cell death was attenuated after in vitro hyperoxia exposure when TLR4 was exclusively expressed on AECs (46). Interestingly, mice deficient in the intracellular MyD88-independent TLR3 were protected from hyperoxic lung injury that occurred in association with decreased alveolar permeability leak and reduced activation of apoptotic cascades compared with WT mice (47). These data suggest that MyD88-signaling pathways are critical for protecting the lung against oxidative injury. Consistent with this notion, MyD88−/− mice...
mice were protected against intestinal ischemia and reperfusion injury, as manifested by decreased microvascular leak and bacteremia (48). Our findings are in line with the previously published work regarding the role of TLRs in mediating noninfectious lung injury (26).

Epithelial cells in the lung express intracellular and extracellular TLRs and TLR-signaling molecules, suggesting that their presence is important for mediating lung immunity and inflammation (25). Specifically, expression of extracellular TLRs was shown to be regulated by a variety of noninfectious insults to the lung, including hemorrhagic shock (49), mechanical ventilation with high tidal volumes (50), and bleomycin administration (51). Previously, IRAK-M was found to be expressed in primary human gingival epithelial cells (35) and human biliary epithelial cells (34) and was upregulated by TLR4- and TLR1/2-specific agonists. Previously, our laboratory showed that AECs from IRAK-M−/− mice produced increased amounts of inflammatory cytokines and chemokines in response to infectious challenge (37). IRAK-M expression by AECs in response to noninfectious stimuli has not previously been described (Fig. 3).

A cardinal feature of hyperoxic lung injury is AEC death resulting in the loss of alveolar integrity and increased alveolar permeability (5, 7); however, the mechanism of cell death during hyperoxia exposure is uncertain (52–56). We observed that the absence of IRAK-M reduces the injury and cell death in AECs after hyperoxia (Figs. 1, 3). The preponderance of evidence suggests that AEC death during hyperoxia occurs as the result of a combination of programmed cell death through apoptosis and unscheduled, premature cell death through necrosis (57, 58). More recently, however, it was suggested that autophagy, a homeostatic stress-response pathway, may also play a role in protecting the epithelial cells against oxidant injury (59, 60). There is conflicting evidence regarding the role of Bcl-2 family members in hyperoxic lung injury, because the overexpression of one antiapoptotic molecule, Bcl-xL, protects from oxygen-induced cell death (61), whereas the overexpression of another antiapoptotic molecule, Bcl-2, in type II AECs did not prevent hyperoxic lung injury in mice (62). A recent study showed that oxidants generated in response to hyperoxia activate the intrinsic cell death pathway through the proapoptotic molecules BAX or BAK, leading to the death of epithelial cells.

FIGURE 4. The absence of IRAK-M in AECs caused less cell death during hyperoxia exposure. AECs were isolated from WT or IRAK-M−/− mice. (A) AECs were plated on fibronectin-coated plates and cultured in hyperoxia (95% O2) or room air (supplemented with 5% CO2) for 72 h. The amount of cell death was assessed by measuring the DNA–histone complexing that occurred after hyperoxia exposure. Data shown are mean ± SEM, with four samples/group from two separate experiments. (B and C) Because viable AECs cannot be removed from fibronectin-coated plates, myeloid cells and mesenchymal cells were adherence purified from the AEC preparation by an overnight incubation, as described in Materials and Methods. The next day, the remaining nonadherent cells, AECs, were stained with Annexin V and PI and immediately analyzed by flow cytometry, as described in Materials and Methods. (B) Data shown are a representative flow plot image from each of the groups. (C) Bar graph represents mean ± SEM with four samples/group from three separate experiments.
The generation of antioxidants is required for the resolution of oxidant injury. The transcription factor Nrf-2 is essential for protecting against oxidative injury, because Nrf-2 knockout mice are more susceptible to hyperoxia and are less capable of generating antioxidants (16). In addition, upregulation of Nrf-2 was shown to be protective in oxidative lung injury models (16, 21, 22). We found that, in the absence of IRAK-M, AECs upregulate Nrf-2 more quickly, because there was greater Nrf-2 translocation into the nuclear fraction in IRAK-M−/− mice at 24 h post-hyperoxia compared with WT mice (Fig. 5). Interestingly, at 72 h of O2 exposure, there was no difference in the amount of Nrf-2 activation in IRAK-M−/− and WT mice; therefore, we believe that this early activation of Nrf-2 induces the expression of antioxidant molecules, allowing for protection against hyperoxic lung injury. Although our work demonstrated an important role for IRAK-M in regulating Nrf-2 activation, previous studies indicated that NF-kB has an important role in the activation of antioxidants (38), which also contributes to protection against oxidant injury (27, 64). Other investigators have begun to examine the potential cross-talk of Nrf-2- and NF-kB–signaling pathways (65). Recent studies suggested that NF-kB signaling inhibits the Nrf-2 pathway through the interaction of p65 and Keap1 (66). Conversely, other investigators showed that activation of NF-kB and the MAPK pathway can promote Nrf-2 activation and the downstream generation of protective antioxidants in hyperoxia (67, 68). Enhanced activation of both NF-kB and MAPKs was demonstrated in IRAK-M−/− macrophages stimulated with LPS (38). Consistent with these findings, we observed enhanced NF-kB transcriptional activity in the lungs of IRAK-M–deficient mice during hyperoxia compared with similarly treated WT mice (M. Ballinger, unpublished observations). Additional work needs to be performed to fully dissect the role that each of these important transcription factors plays in regulating responses to oxidant lung injury.

The antioxidant HO-1 was shown to be important in conferring protection against hyperoxic lung and vascular injury. For example, the overexpression of HO-1 in pulmonary epithelial cells protected cells against hyperoxia-induced cell death (9), and treatment of animals with an HO-1 overexpression vector protected against hyperoxic lung injury (13). Although HO-1–deficient animals are more susceptible to pulmonary ischemia and reperfusion injury, they are not more susceptible to hyperoxic lung injury (69). Based on the conflicting data in HO-1 knockout mice, we chose to inhibit HO-1 via a pharmacologic approach. Treatment of IRAK-M−/−
mice with the HO-1 inhibitor TP resulted in a significant decrease in survival during hyperoxia compared with vehicle control-treated mice. We did not observe any difference in survival in WT mice treated with TP compared with vehicle control (Fig. 6), which is consistent with other reports showing no effect on survival in hyperoxia-exposed mice in which HO-1 is genetically deleted or inhibited (70, 71). Likewise, HO-1 inhibition did not alter the viability of AECs isolated from WT mice after hyperoxia exposure. Conversely, HO-1 is protective when expression is enhanced either by forced expression or in the absence of IRAK-M. The observation that both survival in vivo and AEC viability in vitro were diminished considerably in IRAK-M−/− mice, but not WT mice or cells treated with TP, is consistent with this notion. It appears that, in the absence of IRAK-M, AECs rely heavily on the production of HO-1 for cell survival at basal conditions, as well as during oxidant stress. A recent publication provides some support for this concept, because, in vivo, the basophil expression of HO-1 is critical for liver homeostasis (72). Thus, additional work is needed to be able to determine the role of IRAK-M in mediating both the basophil and inducible expression of HO-1 in AECs.

In conclusion, we identified a novel role for IRAK-M in regulating the production of antioxidant molecules in AECs during hyperoxic lung injury. It appears that IRAK-M functions as a “break” in regulating inflammatory responses, which paradoxically include activating key antioxidant pathways in AECs during hyperoxia exposure. In the absence of IRAK-M, there is more rapid induction of Nrf-2, increased HO-1 production, and decreased cell death and alveolar permeability changes. Although additional work needs to be done to understand the signal-transduction pathway causing the Nrf-2 generation of antioxidants in epithelial cells, this study provides the framework for future studies. More importantly, these data may help to identify new molecular targets that could be translated to protect the lungs of mechanically ventilated patients.

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Disclosures

The authors have no financial conflicts of interest.

References

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