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*J Immunol* published online 1 June 2012
http://www.jimmunol.org/content/early/2012/06/01/jimmunol.1103124

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/06/01/jimmunol.1103124.4.DC1

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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. The Journal of Immunology, 2012, 189: 000–000.

A dministration of high concentrations of oxygen during mechanical ventilation is a common life-sustaining intervention for patients with respiratory failure (1). Although a therapeutically necessary treatment, hyperoxia can result in mortality as high as 40% (2). Despite many clinical trials and promising targets, no pharmacologic treatment for hyperoxic lung injury has been identified (2). To study hyperoxia, several animal systems have been developed to model oxidative toxicity in humans (3). Specifically, exposing mice to 95% pure oxygen leads to pulmonary edema and respiratory failure, progressing to death by 96 h (4).

Under physiological conditions, reactive oxygen species generated as a consequence of cell metabolism are reabsorbed and converted into harmless byproducts (5). However, during hyperoxia, free oxygen radicals generated in the lung overwhelm the antioxidant systems (6). To cope with oxidative stress, the lung has developed a highly conserved and adaptive system to recognize and respond to stress, which includes water and lipid-soluble compounds (such as glutathione, ascorbate, vitamin E, and uric acid), detoxifying enzymes (such as superoxide dismutase, peroxodases, and catalase), and proteins (such as heat shock proteins and heme oxygenase-1 (HO-1)) (7, 8). HO-1, a member of the phase II antioxidant enzymes, catalyzes the degradation of heme into biliverdin, iron, and carbon monoxide (8). Induction or overexpression of HO-1 prior to oxidative stress protects cells against further injury (9–12). In addition, exogenous transfer of HO-1 protects animals against hyperoxic lung injury (13). The transcription factor NF (erythroid-derived)-like 2 (Nrf-2) is the master regulator of phase II antioxidant enzymes, including HO-1 (14, 15). Under homeostatic conditions, Nrf-2 is sequestered in the cytoplasm by the cytoskeletal-associated protein, Keap1 (14). However, under conditions of oxidative stress, Nrf-2 is phosphorylated (16–19), escapes Keap1-mediated degradation, and translocates into the nucleus (20) to initiate transcription of antioxidant genes (17). Previous work showed that the upregulation of Nrf-2 and the subsequent expression of antioxidant genes have a critical protective role against pulmonary hyperoxic injury (16, 21). In addition, the conditional deletion of Nrf-2 in Clara cells is sufficient to exacerbate hyperoxic lung injury (22).

TLRs are membrane-spanning receptors that signal through conserved adaptor proteins (such as MyD88 and TRIF) and recognize evolutionarily conserved motifs important in inflammation and immunity (23, 24). Although originally defined as pathogen-recognition receptors, TLRs also recognize endogenously produced molecules, including heat shock protein 60, high mobility group box I, β-defensins, surfactant protein A, and hyaluronan...
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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-ml 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. Cyto-fluorimetry (Thermo Electron, Waltham, MA) were prepared for determination of BAL differentials using a modified Wright stain.

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 ELISAplus, 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 Pharmacaems), following the manufacturer’s instructions. Immediately following the staining protocol, cells were analyzed on a BD FACSCalibur 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 (Abcam, Cambridge, U.K.) or its hamster IgG 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 (137Cs 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 × 106 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 nitrocellulose (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 set was 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)
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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 like AECs (MLE-12). We observed a significant 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 hyperoxia 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 incubated 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. Hyperoxia 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 exposure. Data were combined to verify that there was no difference in the frequency of any of the populations in the room air groups, and only after exposure to hyperoxia were there significant 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− population (~90% of the cells), we found that 82% of the remaining cells were positive for either pro-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 functional in AECs. In addition, the expression of IRAK-M in myeloid cells, we showed that IRAK-M is also expressed and 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.

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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 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.
The amount of Nrf-2 activity was measured from the nuclear fraction of a single-cell suspension of whole-lung homogenates from WT or IRAK-M−/− mice, as described in Materials and Methods. Data represent the fold change in absorbance, which was calculated as the difference in Nrf-2 expression at the given time point of hyperoxia exposure compared with the Nrf-2 expression from room air control animals (n = 5 mice/group). (B) Expression of HO-1 was determined by isolating mRNA from whole lungs of WT and IRAK-M−/− mice from room air- and O2-exposed animals and performing a semiquantitative real-time RT-PCR analysis. Data shown are mean ± SEM (n = 4 samples/group from three separate experiments). (C) Expression of HO-1 was determined by isolating mRNA from AECs of in vivo O2-exposed WT and IRAK-M−/− mice and performing a semiquantitative real-time RT-PCR analysis. Data shown are mean ± SEM (n = 3 samples/group from two separate experiments).

We did not observe consistent differences in Bcl-xL, Bcl-2, or caspase-3 activation in AECs or whole lungs from IRAK-M−/− mice in response to hyperoxia compared with WT cells or lungs (data not shown). Additional work needs to be done to dissect the mechanism of cell death occurring in our model as a result of hyperoxic lung injury.

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-κB 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-κB–signaling pathways (65). Recent studies suggested that NF-κB signaling inhibits the Nrf-2 pathway through the interaction of p65 and Keap1 (66). Conversely, other investigators showed that activation of NF-κB 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-κB and MAPKs was demonstrated in IRAK-M−/− macrophages stimulated with LPS (38). Consistent with these findings, we observed enhanced NF-κB 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, as described in Materials and Methods, Data shown are the mean number of live cells (Annexin V−/PI−) ± SEM (n = 3 animals/group from three separate experiments).

FIGURE 5. Expression of IRAK-M suppresses early antioxidant generation in response to hyperoxia. WT and IRAK-M−/− mice and AECs were exposed to hyperoxia for the designated time periods. (A) The amount of Nrf-2 activity was measured from the nuclear fraction of a single-cell suspension of whole-lung homogenates from WT or IRAK-M−/− mice, as described in Materials and Methods. Data represent the fold change in absorbance, which was calculated as the difference in Nrf-2 expression between O2-exposed and room air control animals (n = 3 samples/group from two separate experiments). *p < 0.05.

FIGURE 6. Inhibition of HO-1 decreases survival of IRAK-M−/− mice and AECs to elevated O2 exposure. (A) WT and IRAK-M−/− mice were treated i.p. with TP (20 μmol/kg resuspended in 0.1 N NaOH [pH 7.4]) or vehicle control (0.1 N NaOH [pH 7.4]) daily and exposed to 95% O2 for 84 h before removing to room air to continue to assess survival (n = 5 animals/group). (B) AECs were isolated from WT and IRAK-M−/− mice and cultured in vitro in Teflon-coated plates in the presence of TP (10 μM) or vehicle control (0.001% DMSO) and then were incubated under room air or hyperoxic conditions for 72 h. Cells were then washed, stained with Annexin V and PI, according to the manufacturer’s protocol, and assayed immediately by flow cytometry. Data shown are the mean number of live cells (Annexin V−/PI−) ± SEM (n = 3 animals/group from three separate experiments).
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 enhanced either by forced expression or in the absence of IRAK-M, alter the viability of AECs isolated from WT mice after hyperoxia.

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.

Acknowledgments

We thank Carol Wilke for expertise in the BMT model.

Disclosures

The authors have no financial conflicts of interest.

References

Ballinger et al. Sup Fig 1
Isotype control                           SPC-APC
Isotype control                             T1α-APC
CD45-PE

Ballinger et al. Sup Fig 2
Supplemental figure legend:

Sup Figure 1: No difference in recruited cells to the lung after hyperoxia exposure

WT and IRAK-M−/− mice were either housed in room air or exposed to O2 for 24 or 72 h. BAL or lung digest cells were collected and quantified as described in the material and methods section. Quantification of total cells (A) and differential cell counts (B) from WT and IRAK-M−/− mice isolated from BAL fluid and whole lung digest n=4 mice/group/time point and treatment.

Sup Figure 2: Verification of AECs from in vivo treated animals

AECs were collected from WT mice and stained according to the material and methods section. Blots show the percent of CD45 negative cells that are positive for either the isotype control or SPC (type II cell marker) or T1α (type I cell marker) n=4 animals/group.