TREM-1 attenuates RIPK3 mediated necroptosis in hyperoxia induced lung injury in neonates

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Abstract

Hyperoxia-induced injury to the developing lung, impaired alveolarization and dysregulated vascularization are critical factors in the pathogenesis of bronchopulmonary dysplasia (BPD); however, mechanisms for hyperoxia-induced development of BPD are not fully known. Here we show that the triggering receptor expressed on myeloid cells 1 (TREM-1) is upregulated in hyperoxia-exposed neonatal mice lungs as well as in TA and lungs of human neonates with respiratory distress syndrome (RDS) and BPD as an adaptive response to survival in hyperoxia. Inhibition of TREM-1 function using siRNA approach or deletion of TREM-1 gene in mice showed enhanced lung inflammation, alveolar damage and mortality of hyperoxia-exposed neonatal mice. The treatment of hyperoxia-exposed neonatal mice with agonistic TREM-1 antibody decreased lung inflammation, improved alveolarization and was associated with diminished necroptosis regulating protein receptor-interacting protein kinase 3 (RIPK3). Mechanistically, we show that TREM-1 activation alleviates lung inflammation and improves alveolarization through down-regulating RIPK3 mediated necroptosis and nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3)-inflammasome activation in hyperoxia-exposed neonatal mice. These data show that activating TREM-1, enhancing angiopoietin 1 signaling or blocking RIPK3-mediated necroptosis pathway may act as new therapeutic interventions to control adverse effects of hyperoxia on the development of BPD.

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Introduction

Hyperoxia-induced injury to the developing lung, impaired alveolarization and dysregulated vascularization are critical factors in the pathogenesis of BPD (1, 2); however, mechanisms for hyperoxia-induced development of BPD are not fully known. Following hyperoxia-induced acute lung injury (HALI) or mechanical ventilation during premature birth, damage-associated molecular patterns (DAMPs) are released from necrotic cells and recognized by pattern recognition receptors (PRRs) that include toll like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (3-5). The activation of these molecules has been recognized as a modulator of inflammation and decreased alveolarization in a mouse model of BPD (3) as well as in preterm infants (3). In recent years, a new family of the innate immune receptor has been identified -- known as triggering receptors expressed on myeloid cells (TREMs) -- that have been shown to modulate immune response due to their ability to amplify or decrease the signals induced by TLRs and NOD-like receptors (6, 7). However, the role of TREM-1 in neonatal HALI and contribution to the development of BPD is not known.

We tested the hypothesis whether TREM-1 expression is required for limiting lung inflammation, alveolar injury and survival in hyperoxia. We report that TREM-1 expression is increased in the lungs of hyperoxia-exposed neonatal mice as well as human neonates with respiratory distress syndrome (RDS) and BPD as an adaptive survival response. In hyperoxia-exposed neonatal mice, we observed that deletion of TREM-1 gene in mice leads to increased lung inflammation, alveolar damage and mortality. We further observed that increased lung inflammation is associated with enhanced necroptosis regulating protein receptor-interacting protein kinase 3 (RIPK3)-mediated necroptosis and nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3)-inflammasome activation in the lungs of hyperoxia-exposed neonatal mice and human

neonates with RDS and BPD. We next tested whether TREM-1 arbitrates protection to hyperoxia-exposed neonatal mice by blocking necroptosis regulating protein RIPK3-mediated necroptosis and NLRP3-inflammasome activation. The treatment of hyperoxia-exposed neonatal mice with agonistic TREM-1 antibody decreased NLRP3-inflammasome activation, improved alveolarization and was associated with diminished RIPK3-mediated necroptosis in the lungs of neonatal mice. Mechanistically, we show that TREM-1 alleviates pulmonary inflammation and alveolar injury by down regulating RIPK3 mediated necroptosis and NLRP3-inflammasome activation through induction of vascular endothelial growth factor-A (VEGF-A) and augmenting angiopoietin1 (Ang1) expression in lung of hyperoxia-exposed neonatal mice. Taken together, our data show that activating TREM-1, enhancing Ang1 signaling or blocking RIPK3 mediated necroptosis may represent novel therapeutic targets for HALI and BPD in neonates.

Results

Hyperoxia induces TREM-1 expression localized to alveolar macrophages in neonatal mice lungs

To determine the contribution of TREM-1 to HALI, we first examined the expression of TREM-1 and its localization in the lung compartments of room air (RA; 21% O_2) or hyperoxia (60% O_2) exposed neonatal mice. We selected 60% O_2, as opposed to 80-100% O_2, to more closely mimic the supplemental O_2 exposure as currently used for human neonates, predisposing them to developing the more common moderate BPD versus the severe BPD phenotype (8). As shown in **Figs. 1A** and **1B**, the transcript and protein expression of TREM-1 were significantly higher in the lungs of hyperoxia-exposed neonatal mice compared to RA littermates at postnatal day (PN) 7. To evaluate the constitutive presence of TREM-1 within the pulmonary compartment, cells from bronchoalveolar lavage fluid (BALF) and lungs were collected from RA or hyperoxia-exposed

neonatal mice at PN7 for flow cytometric or confocal microscopic analysis, respectively. As shown in **Supplementary Figs. 1A** and **1B**, the expression of TREM-1 on alveolar macrophages (AM; CD11c positive cells) was higher in cells isolated from BALF as well as in lung tissue of hyperoxia-exposed neonatal mice compared to RA group.

To further confirm the induction and localization of TREM-1 by AM, we isolated AM from BALF of RA and hyperoxia-exposed neonatal mice at PN7 and measured transcript and protein expression of TREM-1 levels. As shown in **Figs. 1C** and **1D**, the transcript and protein expression of TREM-1 were significantly higher in AM isolated from neonatal mice exposed to hyperoxia compared to RA group. To corroborate these *in vivo* findings, we exposed murine macrophage-like RAW 264.7 cells to RA or hyperoxia for various time points and measured the transcript and protein levels of TREM-1. As shown in **Figs. 1E** and **F,** transcript levels of TREM-1 were elevated in a time-dependent manner in murine macrophage-like RAW 264.7 cells exposed to hyperoxia. Taken together, these results suggest that TREM-1 expression is enhanced in response to hyperoxia and is localized to AM in the developing lung.

TREM-1 expression is up-regulated in human neonatal lungs with RDS and BPD

To ascertain the relevant potential functional role of TREM-1 in the development of BPD, we assessed the relative expression of TREM-1 in the tracheal aspirates (TA) and lung tissues of human neonates with RDS and BPD. We found that the transcript level of TREM-1 was significantly higher in the TA of infants who subsequently developed BPD/died compared to those who did not develop BPD (**Fig. 1G**). The detailed characterizations of enrolled infants are shown in **Supplementary Table 1.** We next measured TREM-1 protein level in the lungs of human neonates at various stages of BPD development. We found that the protein level of TREM-1 was

significantly higher in lungs of neonates with RDS and BPD compared to term control babies (**Fig. 1H**). The detailed characterizations of this 2nd independent cohort of enrolled infants are shown in **Supplementary Table 2.** Finally, we confirmed the increased level of TREM-1 in lungs of neonates with RDS and BPD by immunohistochemical staining in a 3rd independent cohort of human neonatal lungs with RDS and BPD (**Fig. 1I**). The detailed characterization of enrolled infants is shown in **Supplementary Table 3.** Altogether, these data from experimental hyperoxia-exposed neonatal mice and three independent cohorts of human lung samples from RDS and BPD neonates confirmed that TREM-1 is induced during hyperoxia exposure in the developing lung.

Suppression or deletion of TREM-1 gene is associated with increased pulmonary alveolar injury and mortality

To evaluate the functional role of TREM-1 on the outcome of pulmonary alveolar injury and mortality in hyperoxia-exposed neonatal mice, we first studied loss-of-function of TREM-1 using a siRNA approach to inhibit TREM-1 function or completely abolish TREM-1 function using TREM-1/3- deficient mice. We selected TREM-1/3 deficient mice because previous studies have shown that TREM-1 lies adjacent to TREM-3 gene and that these 2 genes are likely to have complementary functions (9). Additionally, TREM-3 is a pseudogene in humans; hence, TREM-1/3 deficient mice would reflect the lack of functionality of TREM-1 in humans (9). As shown in **Fig. 2A**, TREM-1 transcript levels were significantly decreased (75%) in the lungs of neonates treated with TREM-1-siRNA compared with the scrambled siRNA (scr-siRNA) treated group. We next determined the effect of TREM-1 silencing on hyperoxia-exposed neonatal mice. Surprisingly, we found that inhibition of TREM-1 led to enhanced lung inflammation as manifested by increased neutrophil recruitment (**Fig. 2B)** and inflammatory cytokine interleukin (IL*)*-1β (**Fig. 2C**) in the lungs of neonatal mice exposed to hyperoxia. This increased lung inflammation was also allied with impaired alveolarization as demonstrated by increased chord

length (**Figs. 2D** and **2E**) and decreased radial alveolar count (**Fig. 2F**) in the lungs of TREM-1-siRNA mice exposed to hyperoxia. These detrimental effects of TREM-1 inhibition were concomitant with decreased vascularization (**Fig. 2G**), increased apoptosis (**Fig. 2H**) and higher mortality of hyperoxia-exposed TREM-1-siRNA neonatal mice, as compared to the scr-siRNA mice group (**Supplementary Fig. 2**).

To finally confirm the loss-of-function of TREM-1 in pulmonary inflammation and alveolar damage and the survival response to hyperoxia, we challenged newborn TREM-1/3–deficient and control WT mice to hyperoxia or RA till PN7. Similar to the results of TREM-1-siRNA treated mice exposed to hyperoxia, we found that TREM-1/3-deficient neonatal mice also showed enhanced neutrophil recruitment (**Fig. 2I**) and myeloperoxidase levels (**Fig. 2J**) in the lungs. In addition, we noted robust lung inflammation as manifested by increased inflammatory cytokines IL-6 and IL-1β (**Figs. 2K** and **L**), increased alveolar damage as demonstrated by enhanced chord length, decreased radial alveolar count (**Figs. 2M-O**) and increased mortality in hyperoxia-exposed TREM-1/3-deficient neonatal mice (**Fig. 2P**). Importantly, we did not detect any changes in lung morphometry, inflammation and mortality in TREM-1/3-deficient neonatal mice exposed to RA as compared to wild type (WT) littermates. These results suggest that enhanced TREM-1 expression may be essential for protecting developing lung against hyperoxia-induced lung inflammation, alveolar injury and survival during hyperoxia.

TREM-1 activation decreases lung inflammation and alveolar injury in hyperoxia-exposed neonatal mice

Because we found the inhibition of TREM-1 expression in lungs led to increased lung inflammation, alveolar injury and mortality in hyperoxia-exposed neonatal mice, we reasoned that augmenting pulmonary TREM-1 expression would attenuate pulmonary alveolar injury and

improve survival. To test this hypothesis, we treated hyperoxia or RA exposed neonatal mice with the TREM-1 agonist antibody on alternate days starting at postnatal (PN) day 2 through PN6. The control group was treated similarly with an isotype antibody. Consistent with the known agonistic activity of TREM-1 antibody (10, 11), we found that augmentation of TREM-1 in hyperoxia-exposed neonatal mice led to decreased neutrophil recruitment (**Fig. 3A)** and myeloperoxidase levels (**Fig. 3B**) and reduced lung inflammation as demonstrated by decreased levels of pro-inflammatory cytokines IL-6 and IL-1β in the lungs. (**Figs. 3C** and **D**), and improved alveolarization as manifested by decreased chord length and increased radial alveolar count (**Figs. 3E**-**G**). Furthermore, these improvements in lung alveolarization was also accompanied with decreased staining of 8-Oxo-2'-deoxyguanosine in the lung (8-oxo-dG) (**Supplementary Fig. 3**). Notably, we did not detect any changes in lung morphometry and inflammation in RA groups of neonatal mice treated with TREM-1 agonist or isotype antibodies. Collectively, these results suggest that stimulating TREM-1 activation using the TREM-1 agonist antibody protects against HALI through reduction of lung inflammation.

Previously, TREM-1 has been shown to be crucial for modulating macrophage polarization (12, 13). We reasoned that TREM-1 activation may also polarize macrophages to the M2 phenotype to result in a protective response in the lung during hyperoxia. Consistent with this hypothesis, we found that TREM-1 activation polarizes macrophages to the M2 phenotype as manifested by increased levels of M2 markers (Ym1, KLF4 and Arginine 1) and decreased expression of M1 markers (inducible nitric oxide synthase or iNOS, IL-6 and Ccl2) in whole lungs and AM (**Figs. 3H-K**). These data suggest that TREM-1 activation is required to resolve lung inflammation and injury during hyperoxia exposure to the developing lung.

Increased NLRP3-inflammasome activation is associated with increased lung necroptosis in human neonates with RDS and BPD

Recently, it has been reported that NLRP3-inflammasome, a key mediator of inflammation, is activated in the lungs of neonatal mice (3) and rat (14) exposed to hyperoxia and in the TA of preterm infants with respiratory failure (3). Consistent with their findings, we now detected an increased expression of NLRP3-inflammasome markers -- NLRP3, Caspase-1 and IL-1β -- in human neonatal lungs at various stages of BPD development (**Fig. 4A**) and in the lungs of neonatal mice exposed to hyperoxia (**Supplementary Fig. 4**). These results further strengthen the involvement of NLRP3-inflammasomal activation in the development of BPD; however, the mechanism(s) for NLRP3-inflammasome activation are not known.

We have previously shown that the high-mobility group box-1 (HMGB1) protein, which is released from necrotic cells, is higher in TA from premature infants who develop BPD (15) and now we have confirmed our earlier result with the increased HMGB1 levels in the lungs of human infants developing BPD (**Fig. 4B**). These finding suggest that lung necroptosis may have a crucial role in the development of BPD. Necroptosis, a form of programmed necrotic cell death, amplifies NLRP3-inflammasome activation (16, 17) and has been shown to play a critical role in lung diseases such as adult RDS (ARDS) (4, 18-20), chronic obstructive pulmonary disease (COPD) (21) and sepsis (22, 23). We reasoned that increased NLRP3-inflammasome activation might also be functionally related to increased necroptosis in lungs of human infants developing BPD and neonatal mice exposed to hyperoxia. To test this possibility, we first verified the expression and activation of RIPK3, a regulator protein in the necroptosis pathway in human lungs of neonates with RDS and BPD and in the lungs of neonatal mice exposed to hyperoxia. As shown in **Fig. 4B**, phosphorylation of RIPK3 was significantly increased in human neonatal lungs at various stages of BPD development. Consistent with increased RIPK3 activation in human neonatal lungs at

various stages of BPD, neonatal mice exposed to hyperoxia also showed increased activation of RIPK3 in the lungs (**Supplementary Fig. 5**).

To further explore the involvement of RIPK3 mediated necroptosis, we measured mixed lineage kinase like (MLKL), a downstream substrate of RIPK3 which is necessary for RIPK3 kinase activity to execute necroptosis (3, 16). We found increased phosphorylation of MLKL in the lungs of human infants with RDS and BPD (**Fig. 4B**), and in the lungs of neonatal mice exposed to hyperoxia (**Supplementary Fig. 5**). Collectively, these results from human infants with RDS/BPD and hyperoxia-exposed neonatal mice suggests that lung necroptosis during hyperoxia may be involved in the activation of NLRP3-inflammasome in the neonatal lung.

Inhibition or deletion of RIPK3 reduces necroptosis and NLRP3-inflammasome activation
Next, we examined the functional relationship between RIPK3 mediated necroptosis and NLRP3-inflammasome activation using pharmacological or molecular approaches. We repressed RIPK3 activity using the RIPK3 inhibitor GW440139B (0.5µM) (also known as GW'39B) which disrupts RIPK3-MKLKL complex and inhibits necroptosis (24) and also utilized RIPK3 specific null mutant mice to confirm the role of RIPK3 mediated necroptosis in NLRP3-inflammasome activation. We found that RIPK3 inhibitor GW'39B significantly decreased phosphorylation of MLKL which is required for executing necroptosis (**Fig. 5A**). This decreased lung necroptosis was also associated with diminished NLRP3-inflammasome activation as manifested by decreased NLRP3, activated caspase-1 and IL-1β expression in lungs of hyperoxia-exposed neonatal mice (**Fig. 5B**) suggesting that necroptotic cell death during hyperoxia mediates NLRP3-inflammasome activation in the lung. We next examined whether inhibition of RIPK3 also ameliorated pulmonary inflammation and alveolar injury in hyperoxia-exposed neonatal mice. As shown in **Figs. 5C-F**, RIPK3 inhibition attenuated lung inflammation, as measured by decreased neutrophil recruitment,

myeloperoxidase and levels of pro-inflammatory cytokines tumor necrosis factor (TNF)-α, IL-6 and IL-1β in the lungs. This reduction in lung inflammation was associated with a marked reduction in pulmonary alveolar injury as manifested by improved alveolarization (decreased chord length and increased radial alveolar count), compared to hyperoxia group not receiving any treatment (**Figs. 5G** and **H** and **Supplementary Fig. 6**).

Similar to the results of RIPK3 inhibitor GW'39B, the RIPK3 null mutant mice also showed robust protection against hyperoxia-induced acute lung inflammation and alveolar injury by reducing lung necroptosis and NLRP3-inflammasome activation (**Figs. 5I-R**). Altogether, these findings indicate that suppression of RIPK3 expression by a RIPK3 inhibitor or genetic deletion of RIPK3 reduces lung inflammation and attenuates pulmonary alveolar damage in hyperoxia-exposed neonatal mice through inhibition of NLRP3-inflammasome activation.

TREM-1 deletion is associated with increased lung necroptosis and NLRP3-inflammasome activation

Because we found that TREM-1 inhibition induced lung inflammation in hyperoxia-exposed neonatal mice, we reasoned that TREM-1 may be involved in lung necroptosis and NLRP3-inflammasome activation. To test this hypothesis, we examined the markers for RIPK3-mediated necroptosis and NLRP3-inflammasome activation in TREM-1/3- deficient mice exposed to hyperoxia and compared to WT littermates. As shown in **Fig. 6A,** activated forms of RIPK3 and its downstream substrate phosphorylation of MLKL is increased in the lungs of TREM-1/3- deficient neonatal mice exposed to hyperoxia as compared to WT neonatal mice. This increased RIPK3-mediated necroptosis is also associated with increased NLRP3-inflammasome activation in TREM-1/3- deficient neonatal mice (**Fig. 6B**) suggesting that TREM-1 may be involved in the regulation of RIPK3 mediated lung necroptosis during hyperoxia exposure to neonates.

TREM-1 activation suppresses RIPK3 mediated necroptosis through induction of angiopoietin 1 expression

Finally, to address whether diminution of lung inflammation and alveolar injury by TREM-1 activation is arbitrated through the suppression of RIPK3 mediated necroptosis, we treated hyperoxia-exposed neonatal mice with the TREM-1 agonist antibody and measured HMGB1, RIPK3 and its downstream protein MLKL. We found that TREM-1 agonist antibody reduced HMGB1 expression and RIPK3 mediated necroptosis as manifested by decreased expression of RIPK3 and its downstream protein MLKL phosphorylation in the whole lung as well as macrophages isolated at PN7 and exposed to hyperoxia in culture (**Figs. 7A-D**). This decrease in lung necroptosis is also associated with significant decrease NLRP3-inflammasome activation as demonstrated by diminished expression of inflammasome markers; NLRP3, active caspase 1 and IL-1β in the lungs of neonatal mice treated with TREM-1 agonist antibody (**Fig. 7B**).

Given the fact that TREM-1 is also found in endothelial cells, we decided to investigate if there was a role for angiogenic agents in this pathway. We found that decreased lung necroptosis was also associated with increased expression of angiopoietin 1 (Ang1) and decreased expression of Ang2 proteins (**Figs. 7C** and **D**) suggesting that TREM-1 may show attenuation of necroptosis through activation of Ang1.

To investigate whether Ang1 expression is directly involved in the repression of RIPK3 activity, we treated TREM-1/3-deficient mice with recombinant Ang1 during hyperoxia and measured RIPK3-mediated necroptosis and Ang protein expression in the lung. As expected, we found that administration of recombinant Ang1 caused significantly increased Ang1 protein levels, enhanced expressions of vascular endothelial growth factor (VEGF)-A, Tie-2 (receptor for Ang1 and 2) and decreased level of Ang2 in the lungs of TREM-1/3-deficient mice (**Fig. 8A**). These changes in

angiopoietin protein expression were associated with decreased expression of RIPK3 and its downstream substrates MLKL activation in the lungs of TREM-1/3-deficient mice (**Fig. 8B**). We, and others, have previously shown that increased Ang2, an antagonist of Ang1, caused necrosis upon hyperoxia exposure in developing and adult mice lungs (25, 26). Taken together, our results suggest that TREM-1 may, in part, regulate RIPK3 mediated necroptosis through the VEGF-A mediated Ang1/2 signaling pathway in HALI (**Fig. 8C**).

Discussion

TREM-1 has been reported as an amplifier of the inflammatory response to bacterial infection and sepsis (7, 27, 28). Several previous studies have shown that treatment with TREM-1 inhibitors can act as a therapeutic strategy to prevent excessive systemic inflammation resulting in decreased severity of sepsis (27, 28) and mortality (12, 29). In contrast, other studies have recognized TREM-1 as a survival factor for the host against bacterial infection and sepsis (9). For example, it has been demonstrated that deletion of TREM-1/3- gene in mice leads to increased local as well as systemic cytokine production and enhanced mortality during polymicrobial induced sepsis (29, 30). These results suggest that TREM-1 has a sundry role in inflammation and infection and further investigation is required to explore the functional role of TREM-1 in various diseases. Here, we explored a new role of TREM-1 in hyperoxia-induced pulmonary alveolar injury and its consequence in the development of BPD. We demonstrated, for the first time, that TREM-1 expression is increased in the lungs of neonatal mice and human neonates with RDS and BPD in hyperoxia as an adaptive response to protect the lung from oxygen and/or ventilation-induced injury. This is evident from our finding that silencing TREM-1 expression in lungs or genetic

deletion of TREM-1 in mice showed increased pulmonary alveolar injury and mortality when exposed to hyperoxia. Further, we observed that augmentation of TREM-1 expression in neonatal lungs reduces inflammation and pulmonary alveolar injury and is associated with decreased NLRP3-inflammasome activation and RIPK3-mediated necroptosis.

Previously it has been reported that TREM-1/3-deficient adult mice infected with *Pseudomonas aeruginosa* showed a markedly increased mortality; however, they found a decreased neutrophil recruitment to the lungs following *Pseudomonas aeruginosa* challenge (9). We report here that TREM-1/3-deficient neonatal mice have increased neutrophil recruitment during hyperoxia. This discrepancy in results may be due to differential stage of lung development / age of mice (neonate vs adult) and/or mode of injury (hyperoxia vs bacterial infection) that we used in our study.

RIPK3-mediated necroptosis has been identified in several lung diseases such as ARDS (4, 18-20), COPD (21), and sepsis (22, 23). We now add neonatal HALI and BPD to this list and identify TREM-1, Ang1, and RIPK3 as potential therapeutic targets for the treatment of these life-threatening disorders. Here, we reveal that necroptosis regulating protein RIPK3 and its downstream substrate MLKL levels were higher in the lungs of human infants with RDS, evolving BPD and established BPD as well as in lungs of neonatal mice exposed to hyperoxia, suggesting the essential role of necroptosis in HALI and the development of BPD. Further, using both genetic and drug-based approaches, we revealed a decisive role of RIPK3 in HALI in neonatal mice. Our mechanistic data showed that RIPK3 inhibitor GW440139B or RIPK3-deficient neonatal mice exposed to hyperoxia showed decreased phosphorylation of MLKL and diminished necroptosis and NLRP3-inflammasome activation in the lung. These findings are similar to the recent studies of Wang et al (20) and Mizumura et al (21), who showed that RIPK3-mediated necroptosis is a major mechanism for lung inflammation and injury in experimental models of ARDS and COPD.

Another important finding of this study is that we illustrated for the first time that the inflammation mediator NLRP3-inflammasome is regulated by RIPK3 mediated necroptosis in HALI in neonatal mice. Recently, NLRP3-inflammasome has been shown to be activated in neonatal mice exposed to hyperoxia (85% oxygen) and in a preterm baboon model of BPD (24). The blocking of NLRP3-inflammasome resulted in decreased lung inflammation and improved pulmonary alveolarization; however, the mechanism for NLRP3-inflammasome activation during hyperoxia has not been explored. We showed that NLRP3-inflammasome activation may be due to increased necroptosis during hyperoxia in neonatal mice. This is supported by our finding that RIPK3 inhibitor GW440139B and genetic deletion of RIPK3 in mice is related to decreased NLRP3-inflammasome activation, caspase-1 and IL-1β in neonatal mice exposed to hyperoxia. These results are further supported by several earlier studies showed that multiple DAMPs (HMGB1, histones, mtDNAs) released by necroptotic cells triggered sustained cytokine release and amplified the inflammatory response in diseases (15, 31-33). Consistent with this line of evidence, we have also previously shown increased levels of HMGB1 in the TA of infants who developed BPD (15) and now we show that there is an increase in HMGB1 and caspase 8 expression in the lungs of human infants with BPD as well as neonatal mice exposed to hyperoxia. Collectively, these data suggest that the activation of NLRP3-inflammasome may be due to increased necroptosis in the lungs of infants exposed to invasive ventilation/hyperoxia which leads to the development of BPD.

Lastly, we demonstrate that the protection conferred by augmenting TREM-1 expression strongly suppressed the activity of RIPK3 and decreased the phosphorylation of its downstream substrate MLKL which is required to execute necroptosis (34, 35). While TREM-1 has been known to be a pro- as well as anti-inflammatory molecule that has contributed to its ability to exacerbate or mitigate inflammation in the lung respectively, we explored a new function of TREM-1 which

confers protection in the hyperoxia-exposed neonatal mice through induction of Ang1 and suppression of Ang2 in the neonatal mice treated with TREM-1 agonist antibody. We have previously reported that Ang2 increases inflammation and necroptotic cell death during hyperoxia in mice (25, 26). These findings, along with the results from the current study indicate that TREM-1 is a functionally important protein in the neonatal lung for improved alveolarization and survival in hyperoxia. In the future, it will be important to clarify the precise mechanism by which TREM-1 regulates Ang1/Ang2 signaling in HALI and BPD in neonatal mice. In summary, our results strongly suggest that RIPK-dependent necrosis is the common denominator between TREM-1 signaling and development of BPD as a consequence of HALI in neonatal mice.

Methods

Human lung TA

Human lung TA pellets were obtained from premature infants being mechanically ventilated in the first PN week with an in-dwelling endotracheal tube. These infants had the final outcomes of having the diagnoses of with or without BPD and/or death. The collection and processing of the lung TA was approved by the Human Investigation Committee (institutional review board) of Yale University (VB). Selected clinical details have been shown in **Supplementary Table 1**.

Immunohistochemistry in human neonatal lungs

Human lung tissue samples were obtained postmortem from premature infants having the diagnoses of RDS: 1-2 days (RDS 1-2), RDS 3-7 days (RDS 3-7), RDS >7 days (RDS >7), BPD and term infants as controls. Collection and processing of the human lung samples were approved by the National Supervisory Authority for Welfare and Health in Finland and the University of Rochester Institutional Review Board. Selected clinical details have been shown in

Supplementary Tables 2-3. Whole lungs from humans were isolated and immediately fixed with 10% neutral-buffered formalin. Sections were stained with antibodies against TREM-1 (R&D Systems, MN). TREM-1 staining was quantified by randomly selecting 5 high-power fields in each slide, counting 200 cells in each area, and expressing the number of TREM-1 positive cells as a percentage.

Animals

All experimental WT mice breeding pairs of the C57BL/6J strain were purchased from The Jackson Laboratory (Bar Harbor, ME) while breeding pairs of mice with targeted deletion of TREM-1/3 and RIPK3 genes on a C57BL/6J background were obtained from Genentech, San Francisco, CA, USA. These null mutant mice have been characterized previously (9, 36). All mice were housed and bred in Drexel University animal care facilities and allowed free access to standard food and water. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Drexel University prior to performing any studies.

Neonatal mouse model of HALI

Newborn (NB) mice were used in all studies and litter sizes for each experiment were adjusted to 8-10 pups per treatment group to minimize the effects of differences in nutrition on lung development. For the HALI model, NB WT, TREM-1/3-/- and RIPK3-/- mice were exposed to either RA (21% O_2) or hyperoxia (60% O_2) continuously from PN1 to PN7, as previously described (37). Oxygen levels were constantly monitored inside the chamber and mice were exposed to a 12h light-dark cycle. Nursing mothers were rotated between RA and oxygen-exposed litters daily so as to prevent oxygen toxicity and to ensure sufficient nutrition (milk) for indicated time points. Both male and female pups were pooled together for various analyses.

In separate treatment studies, newborn WT mice were kept in either RA or 60% O_2 and injected with the TREM-1 agonist antibody (1µg/mouse; R&D catalog number MAB1187) or isotype antibody (1µg/mouse; R&D catalog number MAB006) subcutaneously every other day from PN2-7. The mice were monitored twice a day for clinical signs till the experimental end point. The dose of the TREM-1 agonist and isotype antibodies used were based on published literature in mouse models of sepsis (10, 11) and optimized in our study. In the studies to inhibit necroptosis in the lung, NB WT mice were treated with RIPK3 inhibitor GW440139B (GSK 872, 0.5µM) or vehicle alone subcutaneously every other day from PN2-7. The dose of GSK 872 was based on published studies (24).

Analysis of BALF

BAL was performed, as described previously (38). In brief, lungs were lavaged with 0.2 ml of PBS three times and recovered fluid was combined and used to determine the total cell count with TC20 automated cell counter (Bio-Rad Laboratories) while differential counts were performed on cells cyto-centrifuged onto glass slides and stained with Hema3 fixative solution (Fisher Scientific), as described previously (39). For some experiments, the isolated cells pellets from BALF was used to determine TREM-1 positive cells on alveolar macrophage by flow cytometry using CD11c specific antibodies for alveolar macrophage and TREM-1 surface expression as described previously (40, 41).

AM preparation from BALF

To determine the transcript and protein expression of TREM-1 on AM exposed to hyperoxia, we isolated pure AM from BALF of neonatal mice. BALFs were collected, and total cells were counted with TC20 automated cell counter as described above. The cells were adjusted to 5 $\times$

10^5/ml using alveolar macrophage medium and incubated in 6 wells tissue culture dishes for 1h. The nonadherent cells were discarded and adherent cells were washed twice prior to culture in 60% O_2 condition at 37˚C, 5% CO_2 in hyperoxia chamber. The adherent cells were scraped off with a sterile cell scraper and differential staining with Hema3 fixative solution (Fisher Scientific) revealed > 99% pure alveolar macrophages. These cells were stored in RNA-later for RNA isolation for gene transcript analysis and in cell lysis buffer for western blot analysis.

Cell Culture and Reagents

Murine macrophage–like RAW 264.7 cells (TIB-71; American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% FBS (HyClone™, Gibco), 1% penicillin, and 1% streptomycin (Life Technologies, Grand Island, NY) at 37°C in 5% CO_2 till 70–80% confluent. Hyperoxia exposure was performed in sealed, humidified chambers flushed with 85% O_2/5% CO_2 at 37°C, as previously described (37). After experimental time points, cells were scraped off with a sterile cell scraper and stored in RNA-later for RNA isolation and cell lysis buffer for western blot analysis.

Enzyme-linked immunosorbent assay

Cytokines [TNF-α, IL-6, and IL-1β)] and lung myeloperoxidase levels were quantified using commercially available DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions, as previously described (39).

Western blot analysis

Western blot analysis was performed as described previously (42). In brief, lung tissues (human and neonatal mice) and whole cell extracts were homogenized in RIPA buffer containing protease and phosphatase inhibitors (Roche Complete mini). Lung homogenate and cell lysate were

centrifuged (14,000 × g) at 4 °C for 15 min and the supernatant was collected for further analysis. Thirty micrograms of proteins were loaded onto each well, separated on 10% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane (Bio-Rad) using a Bio-Rad Mini-Blot transfer apparatus. Immunoblotting was performed at 4 °C overnight using primary antibodies directed against TREM-1 (R&D Systems, MN), Angiopoietin1 (R&D Systems, MN), Angiopoietin2 (R&D Systems, MN), RIPK3 (Cell Signaling, Danvers, MA), p-RIPK3 (Cell Signaling, Danvers, MA), Ym1 (Stem Cell Technologies, Vancouver, Canada), Arg1 (BD, San Jose CA), iNOS (Santacruz, CA), KLF4 (Cell Signaling, Danvers, MA), p-MLKL (Abcam, Cambridge, MA), MLKL (Abcam, Cambridge, MA), HMGB1 (Cell Signaling, Danvers, MA), Caspase-8 (Cell Signaling, Danvers, MA), Caspase-1 (Cell Signaling, Danvers, MA), cleaved Caspase-3 (Cell Signaling, Danvers, MA), NLRP3 (Cell Signaling, Danvers, MA), IL-1β (Cell Signaling, Danvers, MA), GAPDH (Cell Signaling, Danvers, MA), Tubulin (Cell Signaling, Danvers, MA) and β-actin (Cell Signaling, Danvers, MA). Membranes were then incubated with a 1:5000 dilution of a secondary antibody (Li-Cor Biosciences, Lincoln, NE) at room temperature for 1 hr. Protein bands were visualized using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Lung Morphometric analysis

At PN7, 6-7 random images per lung and six lungs per experimental group were characterized for measuring lung morphometric analysis. Alveolar size was estimated from the mean chord length of the airspace and radial alveolar count (RAC), as described previously (37). Briefly, hematoxylin-eosin sections images were analyzed using research-based digital image analysis software (Image Pro-Plus 4.0; Media Cybernetics, Silver Spring, MD) and a custom macro written for automated investigations of alveolar morphology.

Intranasal delivery of TREM-1 siRNA

TREM1 siRNA was procured from Ambion and reconstituted to 20µM, following manufacturer's instructions. Briefly, pups were exposed to 60% oxygen immediately after birth following the protocol, as described in Sun et al (43). Three microliter of siRNA (20µM) was administered intranasally to each nostril, on PN2 and PN4, while undergoing hyperoxia exposure. Scr-siRNA (Ambion) was used as control in siRNA experiments.

RNA extraction and PCR

Total RNA was isolated from lung using RNeasy® Mini Kit (Cat. 74106, Qiagen Inc., CA, USA) and cDNA was synthesized from 1 µg of total RNA using iScript™ reverse transcription (Bio-Rad) kit. Relative quantitative RT-PCR was performed using TaqMan RT-PCR Master Mix (Bio-Rad Laboratories) in an iCycler thermocycler (Bio-Rad Laboratories). Primers used were: mouse TREM-1, forward 5′-GAGCTTGAAGGATGAGGAAGGC-3′ and reverse 5′-CAGAGTCTGTCACTTGAAGGTCAGTC-3, mouse IL-6, forward 5′-CCTTCCAGGATGAGGACATGA -3' and reverse 5'-TGAGTCACAGAGGATGGGCTC -3', mouse Ccl2, forward 5'-GTTGGCTCAGCCAGATGCA-3' and reverse 5'-AGCCTACTCATTGGGATCATCTTG-3', mouse β-actin, forward 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and reverse 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. β-actin mRNA expression was used for normalization. Real-time PCR was accomplished in triplicate.

Statistical Analysis

Statistics were performed using GraphPad Prism 7.0 software. Two-group comparisons were analyzed by unpaired Student's *t* test and multiple-group comparisons were performed using one-

way analysis of variance (ANOVA) followed by Tukey post hoc analysis. Statistical significance was achieved with $p < 0.05$.

Author contributions:

Concept and design: DS, MS and VB.

Acquisition of data: DS, MS, PD, SA and GP.

Data analysis and interpretation: DS, MS, and VB.

Drafting, editing and/or critical revision for intellectual content: DS, MS, PD, SA, GP and VB.

All authors have approved the version of the submitted manuscript.

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