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Neutrophils Counteract Autophagy-Mediated Anti-Inflammatory Mechanisms in Alveolar Macrophage: Role in Posthemorrhagic Shock Acute Lung Inflammation

Zongmei Wen,*† Liyan Fan,*§ Yuehua Li,*† Zui Zou,* Melanie J. Scott,‡ Guozhi Xiao,* Song Li,** Timothy R. Billiar,‡,# Mark A. Wilson,‡,** Xueyin Shi,* and Jie Fan‡,#,**

Acute lung injury (ALI) is a major component of multiple organ dysfunction syndrome after hemorrhagic shock (HS) resulting from major surgery and trauma. The increased susceptibility in HS patients to the development of ALI suggests not yet fully elucidated mechanisms that enhance proinflammatory responses and/or suppress anti-inflammatory responses in the lung. Alveolar macrophages (AMφ) are at the center of the pathogenesis of ALI after HS. We have previously reported that HS-activated polymorphonuclear neutrophils (PMNs) interact with macrophages to influence inflammation progress. In this study, we explore a novel function of PMNs regulating AMφ anti-inflammatory mechanisms involving autophagy. Using a mouse “two-hit” model of HS/resuscitation followed by intratracheal injection of muramyl dipeptide, we demonstrate that HS initiates high mobility group box 1/TLR4 signaling, which upregulates NOD2 expression in AMφ and sensitizes them to subsequent NOD2 ligand muramyl dipeptide to augment lung inflammation. In addition, upregulated NOD2 signaling induces autophagy in AMφ, which negatively regulates lung inflammation through feedback suppression of NOD2-RIp2 signaling and inflammasome activation. Importantly, we further demonstrate that HS-activated PMNs that migrate in alveoli counteract the anti-inflammatory effect of autophagy in AMφ, possibly through NAD(P)H oxidase-mediated signaling to enhance IκB kinase γ phosphorylation, NF-κB activation, and nucleotide-binding oligomerization domain protein 3 inflammasome activation, and therefore augment post-HS lung inflammation. These findings explore a previously unidentified complexity in the mechanisms of ALI, which involves cell–cell interaction and receptor cross talk. The Journal of Immunology, 2014, 193: 4623–4633.

Hemorrhagic shock (HS) resulting from major surgery and trauma renders patients more susceptible to the development of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome in response to a secondary inflammatory stimulus such as infection. This hyper-susceptibility occurs through a cell priming mechanism (1). Acute lung injury (ALI) is a major component of multiple organ dysfunction syndrome and often serves as a cause of patient death. However, the underlying mechanism by which HS primes for the development of SIRS and ALI has yet to be fully determined. Understanding these mechanisms may lead to improved prophylactic interventions for surgical and trauma patients.

Inflammation is a dynamic self-regulating process governed by interactions between proinflammatory and counterinflammatory factors. Alveolar macrophages (AMφ) are at the center of the development of inflammation. Macrophages are activated via families of related pattern recognition receptors, including TLRs and nucleotide-binding oligomerization domain–like receptors (NLRs) (2–5). We have previously shown that TLR4 and TLR2 signaling plays an important role in the development of ALI after HS through activating AMφ (6–8) and in mediating high mobility group box 1 (HMGB1)–induced activation of NLR protein 3 (NLRP3) inflammasome in AMφ (9).

NOD2, the product of CARD15, is a member of a growing family of NLRs that have been implicated in the regulation of immune responses and cell death in animals and plants (10, 11). NOD2 acts as a cytosolic recognition molecule of bacterial peptidoglycan (PGN), which is found on both Gram-positive and -negative bacteria. The pathogen recognition by NOD2 is believed to be accomplished through interaction of the NOD2 CARD domain with the C-terminal CARD domain of NLRP3, the inflammasome activator (12). As such, NOD2 plays a central role in the initiation of inflammatory responses (13, 14). In addition, NOD2 is required for the development of Crohn’s disease (15). However, its role in the development of ALI is currently unknown.

In this study, we demonstrate that HS-activated PMNs that migrate in alveoli counteract the anti-inflammatory effect of autophagy in AMφ, possibly through NAD(P)H oxidase-mediated signaling to enhance IκB kinase γ phosphorylation, NF-κB activation, and nucleotide-binding oligomerization domain protein 3 inflammasome activation, and therefore augment post-HS lung inflammation. These findings explore a previously unidentified complexity in the mechanisms of ALI, which involves cell–cell interaction and receptor cross talk.
bacteria, through specific detection of the conserved muramyl dipeptide (MDP) structure (12). NOD2 have been shown to associate with RIP2, via caspase recruitment domain (CARD)–CARD interactions, which allow RIP2 to associate with TNF receptor–associated factor/transforming growth factor–β–activated kinase-1 (13). Subsequent signaling leads to activation of NF-kB and upregulation of inflammatory mediators, such as IL-6 (13).

Recent studies have also shown that NLRs, including NOD2, regulate autophagic processes during bacterial infection, which are now recognized to influence proinflammatory and anti-inflammatory responses in cells (14, 15). Autophagy is a basic cell biological process that occurs under physiologic circumstances in almost all human cell types. One main function of autophagy is to recycle damaged or unneeded cellular proteins to preserve cell function (16). Autophagy is upregulated by various stress conditions including those leading to inflammation (17). However, the role of autophagy in HS-induced inflammation remains unknown.

Emerging evidences have shown that reactive oxygen species (ROS) derived from NAD(P)H oxidase play an important role in mediating organ injury after HS (8, 18–22). NAD(P)H oxidase in polymorphonuclear neutrophils (PMNs) is an important source of ROS mediating organ injury after HS (6, 7, 18, 21–23). NAD(P)H oxidase, a highly regulated membrane-bound enzyme complex, catalyzes the production of superoxide by the one-electron reduction of oxygen using NAD(P)H as the electron donor. Studies have suggested that ischemia-reperfusion primes circulating PMNs for increased ROS production, therefore augmenting neutrophil-mediated lung injury once the PMNs are sequestered in the lung (6, 24, 25). Although NAD(P)H oxidase has classically been thought of as a part of the antimicrobial armamentarium of phagocytes (26), the role of this enzyme (or its isoforms in other cell types) in signaling has been described previously (21, 22, 27, 28).

In this study, we demonstrate that HS upregulates NOD2 expression in AMø through HMGB1/TLR4 signaling. Upregulated NOD2 subsequently sensitizes AMø to respond to NOD2 ligand MDP, which initially leads to augmented inflammation in the lung. NOD2 signaling also induces autophagy in AMø, which, in turn, exhibits a potent anti-inflammatory effect on lung inflammation at later time points, thereby negatively regulating inflammation. However, this anti-inflammatory effect was concealed by HS-activated PMNs that migrated into alveoli and counteract the effects of autophagy in AMø. These findings explore a previously unidentified self-regulatory mechanism within AMø, as well as a role for PMN in counteracting this anti-inflammatory effect.

Materials and Methods

Materials

Recombinant HMGB1 was purchased from R&D Systems (Minneapolis, MN). Stimulating activity of recombinant HMGB1 was confirmed in mouse macrophages by assay of TNF release, with an ED50 of 3–12 μg/ml. In some experiments, to exclude the effects of contaminating LPS, the activated PMNs that migrated into alveoli and counteract the inflammatory mediators, such as IL-6 (13). However, the role of PMN in counteracting this anti-inflammatory effect.

Committee of VA Pittsburgh Healthcare System and University of Pittsburgh. Mice were 12–14 wk of age at the time of experiments and were maintained on standard rodent chow and water ad libitum. The mice were not fasted before experiments. Animals were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylazine via i.p. administration. Femoral arteries were cannulated for monitoring of mean arterial pressure, blood withdrawal, and resuscitation. HS was initiated by blood withdrawal and reduction of the mean arterial pressure to 40 mm Hg within 20 min. Blood was collected into a 1 ml syringe and heparinized to prevent clotting. To exclude the effect of heparin on immune processes, we injected equal amounts of heparin (10 U in 0.1 ml saline) into sham animals through the cannulated femoral artery during the sham operation. After a hypotensive period of 1 h, animals were resuscitated by transfusion of the shed blood and Ringer’s lactate in a volume equal to that of shed blood, over a period of 20 min. The catheters were then removed, the femoral arteries were ligated, and the incision were closed. Sham animals underwent the same surgical procedures without hemorrhage and resuscitation. At 4 h after resuscitation, MDP in a dose of 50 μg/kg body weight was injected intratracheally (i.t.) into the mice (HS-MDP two-hit model). In some animals, autophagy inhibitor 3-MA (0.75 mg/ml in saline, 15 mg/kg body weight) was injected i.t. 10 min before MDP (30, 31).

The animals remained anesthetized throughout the entire experimental period. At various time points after resuscitation (0–18 h), either bronchoalveolar lavage (BAL) was performed and BAL fluid (BALF) was collected or lung tissue was harvested for experimental analysis.

**AMø isolation**

BAL was performed as previously described (32). The immunomagnetic separation system (BD Biosciences Pharmingen, San Diego, CA) was used to isolate AMø from BAL fluid. Magnetic nanoparticle-conjugated Abs (anti-mouse Gr-1, anti-CD4, anti-CD8, and anti-CD45R/B220 Abs; BD Biosciences Pharmingen) were chosen to label and remove PMNs and lymphocytes. The resulting cells consisted of >98% macrophages, and cell viability was >95%.

**Immunofluorescence confocal microscopy**

AMø were cytospun onto a microscope slide and fixed with 4% paraformaldehyde for 20 min. After washing with PBS, the cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature, followed by blocking with 1% BSA in PBST (PBS with 0.2% Tween 20) for 2 h at room temperature to reduce nonspecific staining. The cells were then incubated with anti-ASC (1:500; Abgent, San Diego, CA) at 4˚C overnight. After washing twice with PBS, the cells were incubated with Alexa Fluor 555–conjugated anti-rabbit IgG (1:500; Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. Hoechst 33258 (1:200; Sigma-Aldrich) was used to stain nuclei. The cells were then washed with PBS three times, followed by confocal microscopy.

**In vivo neutrophil depletion and repopulation**

PMN depletion was induced using RB6-8C5 mAb (Ly-6G/Gr1-specific, eBioscience, San Diego, CA) (33). At ∼16 h before performing shock or sham operation, 10 μg anti-mouse Ly-6G/Gr1 Ab or control Ab (rabbit anti-mouse IgG; Sigma-Aldrich) was administered i.p. to mice in 100 μl saline. Our previous studies have shown that during the period of 16–24 h after injection of anti-mouse Ly-6G/Gr1 Ab, the circulating PMN count in the Ab-treated group was decreased to 0.02 ± 0.02% of total WBCs versus 22.2 ± 1.9% in the control group (28). There were no statistically significant differences in the number of peripheral lymphocytes, atypical lymphocytes, monocytes, or eosinophils between the Ab-treated and control groups (28).

To determine the role of PMN NAD(P)H oxidase in interacting AMø, PMN depletion in neutrophoripemic mice was performed by tail-vein injection of PMNs (∼2 × 10⁶ cells) isolated from the blood of WT or g99δ−/− mice that were subjected to either HS or sham operation. An immunomagnetic separation system (BD Biosciences Pharmingen) (34) was used to isolate PMNs. Viability of the isolated PMNs was >95%, and PMN purity was >95% as assessed by trypan blue exclusion and Wright–Giemsa staining, respectively.

**Coincubination and immunoblotting analysis**

Mouse AMø were lysed (∼1 × 10⁶ cells/ml) in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM Na₂VO₃, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 20 mM PMSF). Protein levels were quantified, and 600 μg total protein for each sample was then immunoprecipitated with anti–muramyl peptide–associated neck-like protein containing a CARD domain (anti-ASC) Ab (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-NOD2 Ab (Santa Cruz Biotechnologies), or anti-p62/SQSTM1 Ab (Sigma-Aldrich). The immunoprecipitated proteins were
separated on a 10% SDS-PAGE gel and were then electrophoresed onto polyvinylidene difluoride membrane and blocked for 1 h at room temperature with TBS containing 3% nonfat dried milk. NLRRP3 and RIP2 protein was detected by probing the membranes with anti-NLRRP3 and anti-RIP2 Abs (Santa Cruz Biotechnologies) at 1:500 dilution, respectively, and detected with Clean-Blot IP Detection Reagent (Thermo Scientific, Rockford, IL) following the manufacturer’s instructions. Blots were then stripped and reprobed with anti-ASC Ab or anti-NOD2 Ab, and detected with Clean-Blot IP Detection Reagent. Caspase-1 cleavage in the AM was measured by detecting its p10 fragment in Western blot using rabbit polyclonal anti-mouse caspase-1 p10 (Santa Cruz Biotechnologies).

**Western blot Analysis**

Aliquots of AM lysates were separated on a 10% SDS-PAGE under nonreducing condition. Equivalent loading of the gel was determined by quantitation of protein, as well as by reprobing membranes for actin detection. Separated proteins were electrophoresed onto polyvinylidene difluoride membrane and blocked for 1 h at room temperature with TBS containing 1% BSA. The membranes were then probed with primary Ab (polyclonal anti-NOD2, -NOD1, –I-B kinase [-IKKγ, –phospho-Ser31], -MIP2, –macrophage migration inhibitory factor [MIF], or -L33 Ab purchased from Santa Cruz Biotechnologies, Santa Cruz, CA) at room temperature for 1 h. After washing, primary Abs associated with the membranes were detected on autoradiographic film by HRP-conjugated secondary Abs and the ECL plus chemiluminescent system (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions. Blots were quantitated using Scion Image software (Scion, Frederick, MD) and normalized to actin. Caspase-1 cleavage in the AM was measured by detecting its p10 fragment in Western blot using rabbit polyclonal anti-mouse caspase-1 p10 (Santa Cruz Biotechnologies).

**Measurement of cytokines**

IL-1β, TNF-α, and IL-6 in AM, BALF, cell supernatant, and cell culture media were measured using ELISA Ready-Set-Go kit for mouse IL-1β, TNF-α, and IL-6 (eBioscience), respectively, following the manufacturer’s instructions.

**RNA extraction and quantitative real-time PCR**

Total RNA was isolated from AM by TRI Reagent (Molecular Research Center, Cincinnati, OH) following manufacturer’s instruction. Real-time RT-PCR was done using a PrimerPCR SYBR Green Assay kit (Bio-Rad, Hercules, CA) in a Bio-Rad iQ5 real-time PCR machine (Bio-Rad Laboratories, Hercules, CA) using SYBR Green detection protocol. The specific primers for mouse NOD2, TNF-α, IL-6, and IL-1β real-time RT-PCR were also purchased from Bio-Rad, and the assays were performed following the manufacturer’s instruction. After amplification protocol was over, PCR product was subjected to melt curve analysis using Bio-Rad iQ5 software. Fold change was calculated using the ΔΔCt method (35) and the value for the GAPDH gene, which was normalized to untreated mouse AM. 

**NF-κB p65 assay**

NF-κB activation in AM was measured by detecting p65 in cell nuclear extracts using TransAM NF-κBp65 assay kit obtained from Active Motif (Carlsbad, CA), following manufacturer’s instruction. Nuclear protein extracts were prepared from AM by the method of Deryckere and Gannon (36).

**Data presentation and statistical analysis**

The data are presented as mean ± SEM of the indicated number of experiments. Statistical significance among group means was assessed by ANOVA. Student–Newman–Keuls post hoc test was performed. Differences were considered significant at p < 0.05.

**Results**

**HS upregulates NOD2 expression in AM through HMGB1/TLR4 signaling**

We observed a TLR4-dependent upregulation of NOD2 expression in AM after HS. C57BL/6 (WT) mice and TLR4–/– mice were subjected to HS, and at 1–8 h after resuscitation, BAL fluid was collected and AM were isolated using an immunomagnetic separation system (7). NOD2 mRNA and protein levels in the AM were measured using real-time RT-PCR and Western blotting, respectively. As shown in Fig. 1A and 1B, NOD2 expression was increased in AM isolated from WT/HS mice by 4 h, but not in AM from TLR4–/–/HS mice.

Our previous studies have shown that HMGB1 is an important mediator of AM activation during HS (37, 38). To determine whether extracellular HMGB1 is responsible for the HS-induced NOD2 expression, we administered neutralizing Ab to HMGB1 to mice (600 µg/mouse) 10 min before HS. Treatment with anti-HMGB1 Ab (Ab) or nonspecific IgG was injected into mice 10 min before HS. As shown in Fig. 1C, HMGB1 (Ab) decreased NOD2 expression by 76% in WT/HS mice, but not in TLR4–/–/HS mice.

**NF-κB activation in AM**

NF-κB activation in AM was measured by detecting p65 in cell nuclear extracts using TransAM NF-κBp65 assay kit obtained from Active Motif (Carlsbad, CA), following manufacturer’s instruction. Nuclear protein extracts were prepared from AM by the method of Deryckere and Gannon (36).

**Figure 1.** HS upregulates NOD2 expression in AM. NOD2 mRNA (A) or protein (B) in AM in WT and TLR4–/– mice at 1–8 h after HS or sham surgery. In some experiments, neutralizing Ab to HMGB1 (Ab) or sham surgery. In some experiments, neutralizing Ab to HMGB1 (Ab) or nonspecific IgG was injected into mice 10 min before HS. n = 4/gp, *p < 0.01 or **p < 0.01 versus the indicated groups. NOD2 expression in WT AM stimulated with increasing concentrations of HMGB1 (C) or 1.0 µg/ml HMGB1 for up to 4 h (D). WT AM were also treated with heated HMGB1 (100°C, 5 min) (Heat) or PmB-treated HMGB1 (PmB) for 4 h (E). NOD2 expression (F) in WT and NOD2–/– AM after sham (SM) or HS surgery of HS up to 8 h. Images are representative of three independent experiments. Graphs depict the mean ± SEM of the % changes in NOD2 or NOD1 expression from three to four experiments. (C, D, and E) *p < 0.01 compared with the groups with no asterisk.
Upregulated NOD2 signaling mediates HS-primed AM\(\phi\) activation

To determine the pathophysiological significance of NOD2 upregulation in AM\(\phi\) activation, we applied the HS-MDP two-hit mouse model. MDP is a NOD2 ligand and constituent of PGN from both Gram-positive and -negative bacteria. Mice were first subjected to HS for 2 h, and at 4 h after resuscitation, MDP (50 \(\mu\)g/kg body weight) was injected i.t. to stimulate the upregulated NOD2 in AM\(\phi\). AM\(\phi\) were recovered from BAL fluid at 4 h after MDP stimulation for measurement of cytokine expression. The results demonstrate that in WT AM\(\phi\), but not in TLR4\(^{−/−}\) and NOD2\(^{−/−}\) AM\(\phi\), antecedent HS significantly enhanced the expression of the mRNA and protein of TNF-\(\alpha\) (Fig. 2A) and IL-6 (Fig. 2B), as well as MIP2 and MIF (Fig. 2C) in response to MDP, suggesting that NOD2 upregulation may contribute to hyperinflammation after HS.

IL-1\(\beta\) importantly contributes to the development of post-HS SIRS (21, 39–41), and the production of active IL-1\(\beta\) is tightly controlled by the formation and activation of the inflammasome (42–44). Thus, the effect of upregulated NOD2 signaling on Nlrp3 inflammasome activation in AM\(\phi\) was also evaluated. Using the HS-MDP two-hit mouse model, we found that at 4 h after MDP, AM\(\phi\) from WT/HS animals exhibited a noticeable increase in the association between Nlrp3 and ASC, cleavage of caspase-1, and IL-1\(\beta\) level in BAL fluid as compared with that in the AM\(\phi\) from...
WT/sham animals (Fig. 2D, 2E). In contrast, genetic deficiency of TLR4 and NOD2 in AMΔΔ significantly decreased inflammasome activation and IL-1β release into BAL fluid compared with WT in response to HS-MDP (Fig. 2D, 2E).

The alterations in IL-1β expression and secretion from AMΔΔ were further recapitulated in ex vivo experiments. Mice were first subjected to HS model, and at 4 h after resuscitation, AMΔΔ were then isolated from BAL fluid collected from these mice and treated with MDP (1 µg/ml) in vitro for 4 h. IL-1β in cell culture media and in AMΔΔ lysates was measured by ELISA. As shown in Fig. 2F, sequential treatments of HS-MDP induced a significant IL-1β secretion into the medium from WT/HS AMΔΔ, but not from TLR4Δ/Δ Δ/Δ or NOD2Δ/Δ Δ/Δ AMΔΔ, although HS-MDP increased intracellular IL-1β expression in all of the WT, TLR4Δ/Δ Δ/Δ, and NOD2Δ/Δ Δ/Δ AMΔΔ. In vivo administration of neutralizing Ab against HMGB1 in WT mice before HS attenuated IL-1β secretion from the AMΔΔ in response to in vitro MDP stimulation (Fig. 2F).

Moreover, PMN infiltration in the lung was evaluated by counting PMNs in BAL fluid at 4 and 8 h after HS-MDP. Fig. 2G shows that antecedent HS primed for an accelerated and enhanced PMN infiltration in the lung in response to MDP, as compared with sham animals. However, genetic deficiency of TLR4 or NOD2 significantly attenuated PMN infiltration in the lung in response to MDP, as compared with WT/sham animals. However, genetic deficiency of TLR4 or NOD2 prevented this HS-MDP–induced PMN pulmonary infiltration. Taken together, these results indicate an important role for HMGB1/TLR4-mediated upregulation of NOD2 in mediating HS-primed lung inflammation.

**NOD2 signaling induces autophagy in AMΔΔ**

Because NOD2 signaling regulates autophagic processes (14, 15, 45), we therefore further determined the effect of upregulated NOD2 on autophagy formation in AMΔΔ. We observed in the HS-MDP two-hit model that upregulated NOD2 signaling induced autophagy in AMΔΔ. AMΔΔ were recovered from BAL fluid at 1–8 h after i.t. MDP or saline. Microtubule-associated protein 1 LC3 puncta, a marker of autophagy induction, and LC3-II, an activated form of LC3, were detected in the AMΔΔ by confocal microscopy and Western blot, respectively. As shown in Fig. 3A, HS induced LC3 puncta formation in up to 15% of AMΔΔ by 8 h after i.t. saline. By contrast, HS primed for increased LC3 puncta formation in 55% of WT AMΔΔ in response to MDP, and this increase was significantly attenuated by giving i.t. autophagy inhibitor 3-MA (15 mg/kg body weight) 10 min before MDP. NOD2 deficiency prevented HS-MDP–induced AMΔΔ increases in autophagy (Fig. 3A). Fig. 3B shows that at 8 h after MDP, LC3-II, an indication of LC3 activation, was detected in the AMΔΔ from WT/HS mice.

To confirm that the MDP/NOD2-mediated autophagy is secondary to HMGB1 signaling, AMΔΔ isolated from nonsurgically treated WT mice were sequentially treated with HMGB1 for 4 h and then with MDP for 8 h. As shown in Fig. 3C, sequential treatment of HMGB1-MDP induced LC3 puncta formation in ∼50% of the AMΔΔ, which was decreased to 10% of the AMΔΔ by adding 3-MA (5 mmol/L) 10 min before MDP. Fig. 3D shows that sequential treatment of HMGB1 and MDP induced formation of LC3-II, which diminished by 3-MA. Collectively, the results indicate an NOD2-dependent activation of autophagy in AMΔΔ at a late phase of HS-MDP–induced onset of inflammation.

**Autophagy inhibits inflammatory processes in AMΔΔ, and these effects are counteracted by PMNs**

Next, we addressed the significance of autophagy induction in the post-HS inflammation. We observed an important anti-inflammatory role of autophagy in regulating MDP-induced inflammation in AMΔΔ in vitro. As shown in Fig. 4A–C, at 12 h after MDP treatment, the expression of TNF-α and IL-6 mRNA and protein, and IL-1β mRNA in HMGB1 pretreated AMΔΔ were markedly decreased compared with levels at 4 h after MDP. These decreases in cytokine expression at 12 h were prevented in LC3ΔΔ ΔΔ AMΔΔ. These data suggest a novel function of autophagy in downregulating cytokine expression at a transcriptional level. Autophagy has also been shown previously to reduce inflammasome activation (46, 47). Similarly, in our model, Nlrp3 inflammasome assembly and caspase-1 cleavage also declined at 12 h after MDP as shown by immunoprecipitation (IP) with inflammasome component ASC (Fig. 4D).

We have previously shown that HS-activated PMNs sensitize AMΔΔ and lung endothelial cell (EC) to bacterial products by upregulating TLR2 signaling in AMΔΔ and ECs, as well as NAD(P)H oxidase activation in ECs, and thereby promote lung inflam-
Autophagy inhibits inflammatory processes in AMφ, and these are counteracted by PMNs. AMφ isolated from WT or LC3\(^{-/-}\) mice were sequentially treated with HMGB1 for 4 h and then with MDP for 4 or 12 h. The expression of mRNA and protein of TNF-\(\alpha\) (A) and IL-6 (B), and mRNA of IL-1β (C) in AMφ were then measured by real-time RT-PCR and Western blot, respectively (A–C). The association of Nlrp3 and ASC was detected by IP with anti-ASC Ab followed by immunoblotting (IB) for Nlrp3 and ASC; caspase-1 cleavage product p10 fragments were detected by Western blot (D). AMφ from WT mice were pretreated with HMGB1 for 4 h and then cocultured with PMNs isolated from HS or sham (SM) mice 2 h after resuscitation in the presence or absence of MDP (1 \(\mu\)g/ml) for 4 and 12 h. In some groups, methoxy-polyethylene glycol–coupled catalase (PEGC; 1000 U/ml) was added to the cocultures. In some other groups, H\(_2\)O\(_2\) (250 \(\mu\)M), instead of PMNs, was added to AMφ. The expression of mRNA and protein of TNF-\(\alpha\) (E) and IL-6 (F), and mRNA of IL-1β (G) in AMφ were then measured by real-time RT-PCR and Western blot. The association of Nlrp3 and ASC was detected by IP with anti-ASC Ab followed by IB for Nlrp3 and ASC; caspase-1 cleavage product p10 fragments were detected by Western blot (H). *\(p < 0.01\) versus other groups at 12-h time point; \(n = 3\). Images are representative of three independent studies.

The in vitro observations led us to further investigate the interaction of PMN and AMφ in vivo. As shown in Fig. 5A and 5B, in vivo treatment of WT mice with HS-MDP increased TNF-\(\alpha\) and IL-6 expression in AMφ, which reached a peak by 12 h after MDP. However, depletion of circulating PMNs in WT mice before HS-MDP resulted in significantly lowered TNF-\(\alpha\) and IL-6 expression in AMφ, reaching a peak at 4 h after MDP, followed by continuous decline (Fig. 5A, 5B). These results suggested an in vivo role for PMNs in counteracting autophagy-derived anti-inflammatory effects in AMφ.

**PMN NAD(P)H oxidase regulates inflammatory response in AMφ**

Previous studies suggested that PMN NAD(P)H oxidase is critical in mediating PMN interaction with other cells (7, 8, 20, 21, 28). To elucidate the role of PMN NAD(P)H oxidase in counteracting the effect of autophagy in AMφ, we depleted circulating PMNs before subjecting mice to HS-MDP, and in some cases, we repleted mice made neutropenic with PMNs isolated either from WT or gp91\(^{-/-}\) mice. As shown in Fig. 5C and 5D, depletion of...
PMNs resulted in a decrease in expression of TNF-α and IL-6 in AMφ at 12 h in WT mice, but not in LC3<sup>−/−</sup> mice, indicating an autophagy-derived anti-inflammatory role. Repletion of WT PMNs in WT neutropenic mice restored expression of TNF-α and IL-6 in AMφ at 12 h, whereas replenishing neutropenic mice with gp91<sup>phox</sup>−/− PMNs failed to do so (Fig. 5C, 5D). The maintained increases in IL-1β in BAL fluid in LC3<sup>−/−</sup> mice, as shown in Fig. 5E, demonstrate a role of autophagy in suppressing IL-1β release at 12 h after MDP. Similarly, a role for PMN NAD(P)H oxidase counteracting the effects of autophagy is suggested, because replenishing the neutropenic mice with WT PMNs, but not gp91<sup>phox</sup>−/−-deficient PMNs, increased IL-1β release in response to HS-MDP at 12 h. Fig. 5F demonstrates the lung histological changes at 18 h after HS-MDP. HS-MDP induced PMN infiltration in alveoli and interstitial edema in WT mice. LC3 deficiency worsened the HS-MDP–induced lung inflammation. PMN deple...
found that PMN depletion and repletion did not alter AMβ autophagy formation in response to HS-MDP (Fig. 6A). This finding excludes a direct role for PMN in suppressing autophagy formation.

NOD2 through RIP2–IKK–NF-κB signaling induces inflammatory cytokine expression (48–52). We measured the association of NOD2 and RIP2, as well as IkK phosphorylation, in AMβ after in vivo HS-MDP treatments by IP. We found that in WT and LC3-/- animals, the association of RIP2 and NOD2 could be detected at 4 h after MDP, and decreased at 12 h after MDP in WT mice but remained increased in LC3-/- mice (Fig. 6B). PMN depletion and repletion did not affect the HS-MDP-induced association of RIP2 and NOD2. PMN depletion in WT mice decreased IkK phosphorylation and NF-κB p65 activity, whereas repletion of WT PMNs, but not gp91<sup>phox</sup>−/− PMNs, restored IkK phosphorylation and NF-κB p65 activity. These results suggest that AMβ autophagy downregulates inflammation through suppression of association of RIP2 and NOD2, whereas PMN NAD(P)H oxidase acts through a different pathway to induce IkK phosphorylation and NF-κB activation.

To address whether autophagy-suppressed association of Nlrp3 and ASC is caused by p62-induced degradation of Nlrp3, we measured the physical binding between Nlrp3 and p62. We found that at 12 h after MDP treatment in WT AMβ, but not in LC3-/- AMβ, there was a binding between Nlrp3 and p62, and this binding associated with a decrease in total Nlrp3 protein (Fig. 6C). These alterations were not affected by PMN depletion and repletion (Fig. 6C), suggesting that PMNs are not involved in autophagy regulation of Nlrp3 amount. Nonetheless, the decrease in total Nlrp3 protein at 12 h after MDP did not consequently reduce the association of Nlrp3 and ASC, as well as caspase-1 cleavage, the markers of inflammasome activation, unless in animals that underwent PMN depletion or PMN depletion followed by repletion of gp91<sup>phox</sup>−/− PMNs (Fig. 6C). Taken together, these results suggest a p62-mediated decrease in Nlrp3 availability and suppression of inflammasome activation after autophagy development, whereas PMNs promote inflammasome assembly and activation through a mechanism other than affecting Nlrp3 abundance.

**Discussion**

After survival from resuscitation, HS patients often face a risk for the development of SIRS and ALI. We have previously reported that augmented activation of innate immunity is an important mechanism underlying HS-primed ALI (7, 32, 53). In this study, we explored a novel role for PMN in augmenting post-HS lung injury through counteracting AMβ anti-inflammatory mechanisms. This study supports a model, as illustrated in Fig. 7, that augmented activation of innate immunity is an important mechanism underlying HS-primed ALI (7, 32, 53). In this study, we explored a novel role for PMN in augmenting post-HS lung injury through counteracting AMβ anti-inflammatory mechanisms.

HS lung inflammation.

Previous reports have shown that HMGB1 contributes to augmented lung injury by increasing the release of chemokines and ligands of NOD2 may be derived from a wound or the gut after hemorrhage (54–56) or exist in the surrounding environment.

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**FIGURE 6.** Autophagy and PMNs target NF-κB signaling and inflammasome via different pathways. (A) PMN depletion and repletion were performed in WT mice, which were then subjected to HS-MDP two-hit model. The PMNs for repletion were isolated from WT mice. AMβ were isolated from BALF at 8 h after MDP. LC3 puncta in AMβ was detected using immunofluorescence confocal microscopy (original magnification ×600), and LC3-I and LC3-II in AMβ were measured by Western blot. (B and C) PMN depletion and repletion was performed in WT and LC3-/- mice, which were then subjected to HS-MDP two-hit model. At 4 and 12 h after MDP, AMβ were isolated from BALF. (B) The association of RIP2 and NOD2 was detected by IP with anti-NOD2 Ab followed by immunoblotting (IB) for RIP2 and NOD; the phosphorylated IKK was measured by detecting p65 in cell nuclear extracts using TransAM NF-κB p65 assay kit. *p < 0.01 versus other groups at the same time point. n = 3/7 per group. (C) The association of Nlrp3 and p62 was detected by IP with anti-p62 Ab followed by IB for Nlrp3 and p62. Association of Nlrp3 and ASC was detected by IP with anti-ASC Ab followed by IB for Nlrp3 and ASC. Total Nlrp3 as well as caspase-1 cleavage product p10 fragments were detected by Western blotting. Images are representative of three independent studies.
FIGURE 7. Model of PMN counteraction of autophagic anti-inflammatory mechanisms to augment ALI after HS. HS increases HMGB1/TLR4 signaling and upregulates NOD2 expression in AMφ, with a subsequent sensitization of AMφ to NOD2 ligand MDP, which leads to augmented inflammation in the lung. In addition, upregulated NOD2 signaling induces autophagy in AMφ, which, in turn, negatively regulates lung inflammation by suppressing NOD2-RIP2 signaling and inflammasome activation. PMNs counteract the anti-inflammatory effect of autophagy, possibly via NAD(P)H oxidase–derived ROS, and therefore enhance post-HS lung inflammation.

proinflammatory cytokines, as well as subsequent PMN sequestration after HS and resuscitation (7, 8, 37, 57). In this study, we further observed that HMGB1/TLR4 signaling upregulated NOD2 expression in AMφ, with a subsequent sensitization of AMφ to NOD2 ligand MDP, which leads to augmented inflammation in the lung. In addition, upregulated NOD2 signaling induces autophagy in AMφ, which, in turn, negatively regulates lung inflammation by suppressing NOD2-RIP2 signaling and inflammasome activation. PMNs counteract the anti-inflammatory effect of autophagy, possibly via NAD(P)H oxidase–derived ROS, and therefore enhance post-HS lung inflammation.

We also investigated the mechanisms by which autophagy negatively regulates AMφ inflammatory responses. NOD2 through interaction with RIP2 activates IKKγ and subsequently NF-κB, which transcriptionally promotes inflammatory cytokines expression (48–52). The data from this study demonstrated that in the late phase (12 h after MDP), induction of autophagy in AMφ decreased the association of NOD2 and RIP2, and this was prevented by LC3 deficiency. Interestingly, these changes were not affected by PMN depletion and repletion, which suggests separate mechanisms of regulation. However, with the decrease in the association of NOD2 and RIP2, the corresponding decrease in IKKγ phosphorylation and NF-κB activation were observed only when PMNs were depleted, or PMNs depleted and then repleted with gp91<sub>phox</sub><sup>−/−</sup> mice. These results support a notion that PMNs promote IKKγ activation, and this counteracts the role of autophagy in suppression of NOD2–RIP2 interaction.

The p62 protein, also called sequestosome 1 (SQSTM1), is an ubiquitin-binding scaffold protein that is able to polymerize via an N-terminal PB1 domain and interact with ubiquitinated proteins via the C-terminal UBA domain (58). Also, p62 binds directly to LC3 via a specific sequence motif and undergoes degradation by autophagy, and thus serves to link ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome (58). NLRP3 protein deubiquitination has been suggested as an important mechanism for NLRP3 inflammasome priming and activation (61, 64). Our present study suggests that autophagy suppresses inflammasome activation by promoting degradation of ubiquitinted Nlrp3 via p62-mediated pathway. However, we have previously shown that in HS, PMNs promote inflammasome activation in lung ECs through PMN NAD(P)H oxidase–derived...
ROS, a mechanism other than affecting Nlrp3 abundance (21). We also showed that ROS promote the association of thioredoxin-interacting protein with Nlrp3 to subsequently induce inflammasome activation and IL-1β secretion from the ECs. Further study will be needed to confirm whether this mechanism is also valid in AMöD.

In summary, this study identifies a previously unrecognized HMGB1/TLR4-NOD2-autophagy axis that serves as a macrophage self-regulatory mechanism governing post-HS inflammatory responses to bacterial products. The study also explored a novel function of PMN NAD(P)H oxidase−derived oxidant signaling in enhancing HS−primed lung injury. PMN NAD(P)H oxidase activates transcellular oxidant signaling through its ability to counteract the autophagy−induced anti−inflammatory mechanisms, and therefore enhancing post−HS lung inflammation and injury. In the broadest sense, these findings may also be valid in other human diseases in which macrophages play a role, including diseases associated with acute and chronic inflammation.

Disclosures

The authors have no financial conflicts of interest.

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