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Superoxide Induces Neutrophil Extracellular Trap Formation in a TLR-4 and NOX-Dependent Mechanism

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Neutrophils constitute the early innate immune response to perceived infectious and sterile threats. Neutrophil extracellular traps (NETs) are a novel mechanism to counter pathogenic invasion and sequelae of ischemia, including cell death and oxidative stress. Superoxide is a radical intermediate of oxygen metabolism produced by parenchymal and nonparenchymal hepatic cells, and is a hallmark of oxidative stress after liver ischemia-reperfusion (I/R). While extracellular superoxide recruits neutrophils to the liver and initiates sterile inflammatory injury, it is unknown whether superoxide induces the formation of NETs. We hypothesize that superoxide induces NET formation through a signaling cascade involving Toll-like receptor 4 (TLR-4) and neutrophil NADPH oxidase (NOX). We treated neutrophils with extracellular superoxide and observed NET DNA release, histone H3 citrullination and increased levels of MPO-DNA complexes occurring in a TLR-4–dependent manner. Inhibition of superoxide generation by allopurinol and inhibition of NOX by diphenyleneiodonium prevented NET formation. When mice were subjected to warm liver I/R, we found significant NET formation associated with liver necrosis and increased serum ALT in TLR-4 WT but not TLR-4 KO mice. To reduce circulating superoxide, we pretreated mice undergoing I/R with allopurinol and N-acetylcysteine, which resulted in decreased NETs and ameliorated liver injury. Our study demonstrates a requirement for TLR-4 and NOX in superoxide-induced NETs, and suggests involvement of superoxide-induced NETs in pathophysiologic settings.

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INTRODUCTION

Neutrophils are highly specialized innate immune cells and are recognized as critical effectors of the immune response to sepsis and hepatitis, as well as to oxidative stress of the liver. (1,2) A novel mechanism of neutrophil response to perceived threats in the circulation is formation of neutrophil extracellular traps (NETs). (3,4) NETs are extracellular scaffolds composed of nuclear DNA studded with granule proteins, histones and cytoplasmic antimicrobials. (3,4) NETs have been well described in infectious states, where they serve a beneficial role in host defense against bacteria, fungi, and protozoa (5); capture and destruction of circulating pathogens by NETs limits dissemination and minimizes virulence. (3,4) However, NETs have been implicated as harmful contributors to the pathogenesis of methicillin-resistant *Staphylococcus aureus* bacteremia (6) and noninfectious inflammatory conditions including venous thrombosis, (7) transfusion-related acute lung injury, (8) cancer (9) and various autoimmune conditions. (5)

In the liver, reactive oxygen species (ROS) are produced by epithelial cells (hepatocytes and cholangiocytes), resident macrophages (Kupffer cells) and endothelial cells of the hepatic vasculature. ROS are particularly important in the liver, where they may serve physiologic roles in chemoattraction, energy generation, signal transduction and transcriptional regulation. (10,11) However, when it is produced in excess, pathologic effects of ROS predominate, including induction of hepatic stellate cell death, promotion of leukocyte migration, inflammation and fibrosis. (12) Production of ROS in excess of cellular antioxidant defenses is a characteristic of ischemia-reperfusion (I/R)

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injury in the liver, heart and other organs. (13–15) I/R injury is a pattern of damage suffered by tissue due to an initial ischemic period and a subsequent excessive inflammatory response upon restoration of blood flow. (16) It occurs after surgery for resection or transplantation, trauma or hypovolemic shock. Pathologic oxidative stress after liver I/R has been attributed to ROS superoxide (O_2^-), generated by transfer of an electron to molecular oxygen. Mechanisms of superoxide generation include aberrant electron leak across the mitochondrial respiratory chain (17) and electron transfer from NADPH to molecular oxygen by NADPH oxidase (NOX). (18) Hepatocytes and hepatic stellate cells, as well as phagocytic Kupffer cells and neutrophils, express NOX isotypes with different kinetics. (12) The best understood mechanism, however, functions through the enzyme xanthine oxidase (XO), whose expression is highest in the liver, and its substrate hypoxanthine: each enzymatic cycle yields 2 molecules of superoxide. (13,19–21) Superoxide and other ROS are believed to be the principal mediators of I/R injury, (22) and XO is believed to be the principal source of these oxidative intermediates in the liver. (19–21)

High concentrations of superoxide upregulate the expression of endothelial adhesion proteins, (23,24) thereby promoting neutrophil recruitment and accumulation in an ischemic liver lobe. Moreover, superoxide is recognized by neutrophil Toll-like receptor 4 (TLR-4), triggering neutrophil activation, promotion of TLR-4–linked NF-κB effects and production of proinflammatory cytokines. (25) Activation of traditional neutrophil mechanisms in the noninfectious setting of liver I/R produces sterile inflammation that exacerbates tissue injury by generating additional ROS and releasing inflammatory mediators and various proteolytic enzymes. (26,27)

Despite superoxide being among the proximal inciters of sterile inflammation after I/R, its role in inducing neutrophils to form NETs remains unknown. Thus, our study sought to determine whether superoxide can induce NET formation, to resolve the underlying molecular signaling cascade by which this occurs, and to determine the effect of antisuperoxide therapy on NET formation in a model of liver I/R. We hypothesized that extracellular superoxide would induce NET formation through an intracellular molecular cascade common to other NET-inducing agents. Furthermore, we hypothesized that pretreatment with antisuperoxide agents would reduce NETs in livers experiencing oxidative stress after I/R. We found that NETs are induced by extracellular superoxide through a process initiated by neutrophil TLR-4 and propagated by intracellular NOX. Overall, our study reveals an association between acute oxidative stress after I/R established by superoxide and pathophysiologic immune response by NETs.

MATERIALS AND METHODS

Animals

Male wild-type (WT C57BL/6) mice (8–12 wks old) were purchased from Jackson ImmunoResearch Laboratories. TLR4 knockout (KO) mice were provided by Dr. Timothy Billiar (University of Pittsburgh Medical Center). Animal protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh, and the experiments were performed in adherence to the National Institutes of Health guidelines for the use of laboratory animals.

Neutrophil Isolation and Purification

Mouse neutrophils were isolated from the bone marrow of tibias and femurs as described previously. (9) After filtration through a 70 μm cell strainer (Falcon), erythrocytes were removed from cell aggregates by red blood cell lysing buffer (Sigma-Aldrich). Neutrophils were purified by fluorescent-activated cell sorting.

Neutrophils were sorted on a BD Aria Plus high-speed sorter after incubation with APC-conjugated anti-mouse Ly6G antibody (BD Biosciences) and APC-Cy7 CD11b (BD Biosciences) (purity > 96%). Recovered cells were resuspended in RPMI with 1% Pen/strep and 5% FBS.

In Vitro NET Formation

In all experiments, neutrophils were seeded at a concentration of 3×10^6/mL on poly-L-lysine-coated 6-well cell culture plates for 1 h with polymixin B (10 ug/mL) before stimulation for 8 h with either phorbol-12-myristate 13-acetate (PMA, 100 nM, Sigma-Aldrich), xanthine oxidase (XO, 5, 10, or 20 mU/mL, Sigma-Aldrich) with hypoxanthine (HX, 500 nM, Sigma-Aldrich), LPS (100 ug/mL), or glucose oxidase (100 mU/mL, Sigma-Aldrich). Inhibitors were added 1 h before stimulation at the following final concentrations: diphenyliodoniumchloride 10 uM (DPI, Sigma-Aldrich), (4) allopurinol 100 μM (Sigma-Aldrich).

Quantification of NETs

To quantify NETs in cell culture supernatant or in mouse serum, a capture ELISA myeloperoxidase (MPO) associated with DNA was performed as described previously. (28) Briefly, to show that circulating nucleosomes in mice sera are derived from NETs, we detected myeloperoxidase, a prominent granular component of neutrophils, attached to nucleosomes. MPO-DNA complexes were identified using a capture ELISA. For the capture antibody, Mouse MPO ELISA kit (Hycult Biotech, HK210-01) was used according to the manufacturer's directions. We added 200 μl of sample to the wells and incubated for 1 h. After washing 3 times (300 μl each), 100 μl incubation buffer containing a peroxidase-labeled anti-DNA mAb (component No. 2, Cell Death ELISA^{PLUS}, Roche; Cat. No: 11774424001) was used. Values for soluble NET formation were expressed as fold increase in absorbance above control. Serum nucleosome quantification was performed using Cell Death Kit (Roche).

Immunofluorescence

Neutrophils were seeded atop coated glass coverslips at a concentration of 1×10^6/mL then treated as described

above for 8 h at 37°C. Cells were fixed with 4% PFA, blocked with 1% BSA and incubated with specific primary antibody for citrullinated Histone H3 (Cit-H3 1:300; Abcam), and detected with conjugated secondary antibodies (1:500; AlexaFluor 488 goat anti-rabbit, Invitrogen). F-actin was stained with rhodamine phalloidin (Invitrogen). Cells were then stained with Hoechst for DNA and mounted with Vecta-Shield Mounting media. Slides were viewed with Olympus Provis and Leica TSL-SL immunofluorescent microscopes.

Superoxide Assay

Xanthine oxidase–derived superoxide was measured using a detection assay kit (Abcam). The 2′,7′-dichlorofluorescein diacetate (DCFDA) fluorogenic agent provided measures hydroxyl, peroxyl and other ROS activity. After addition of xanthine oxidase and hypoxanthine at the concentrations identified in the text to RMPI media devoid of cells in 96-well plates, the DCFDA was added. Fluorescence from the DCF was detected by fluorescence microplate reader with maximum excitation and emission spectra of 495 nm and 529 nm, respectively.

Plate Reader Assay

Performed as described by Douda *et al.* (29) Briefly, cells were seeded at 1×10^6 cells per well in a 96-well plate in the culture media in the presence of Sytox Green cell-impermeable nucleic acid stain (Life Technologies) at 5 μM. Fluorescence was measured using a fluorescence microplate reader every 60 min for up to 300 min after treatment of cells. Fluorescence readout obtained from cells lysed with 0.5% (vol/vol) Triton X-100 was considered as 100% DNA release; each data point of the NETotic index represented the percentage of total value.

Liver Ischemia/Reperfusion

A nonlethal model of segmental (70%) liver warm ischemia and reperfusion was used as previously described. (30) Briefly, under anesthesia, a midline laparotomy was followed by occlusion of all structures in the portal triad using a microvascular clamp for 60 min. Removal of the clamp initiated reperfusion, confirmed by immediate color change, prior to closing the abdomen with sutures. Animals recovered for 6 h prior to euthanasia under anesthesia by exsanguination. The temperature during ischemia was maintained at 31°C using a warming incubator chamber. Sham mice underwent anesthesia, laparotomy and exposure of the portal triad without hepatic ischemia. Mice received intraperitoneal injections of allopurinol 18 h and immediately prior to ischemia (50 mg/kg, Sigma-Aldrich) (31) or N-acetylcysteine (150 mg/kg, Sigma-Aldrich) immediately prior to ischemia. (32) Sham animals underwent anesthesia, laparotomy and exposure of the portal triad without liver ischemia.

Liver Damage Assessment

Serum alanine aminotransferase (sALT) levels were measured using the DRI-CHEM 4000 chemistry analyzer system (HESKA). The extent of parenchymal necrosis in the ischemic lobes was evaluated using hematoxylin and eosin (H&E) stained histological sections at 40 × magnification. The necrotic area was quantitatively assessed by using Image J (NIH). Results were presented as the mean of percentage of necrotic area (mm^2) with respect to the entire area of one capture (mm^2).

Immunoblotting

Western blot assays were performed using whole-cell lysates from either liver tissue or neutrophils. Membranes were incubated overnight using an anti-citrulllinated Histone H3 antibody (1:1000 in 1% BSA, Abcam 5103) and detected by goat anti-rabbit secondary antibody (1:5000 in 1% BSA). Incubation with antibody for β-actin (1:1000 in 1% BSA, Abcam) acted as an internal loading control and was detected by rabbit anti-mouse secondary antibodies (1:5000 in 1% BSA).

Flow Cytometry Analysis

WT neutrophils were pre-treated with Eritoran (8 ng/mL) or YW4-03 (2 mM) in neutrophil culture with superoxide for 8 h. After incubation, cells were fixed with fixation/permeabilization reagent (eBiosciences) and stained with primary antibody against citrullinated-Histone H3 (1:150) (Abcam) for 30 min. Neutrophils were then stained with secondary antibody (488 goat anti-rabbit) for 30 min. NET formation was observed using FITC channel.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Group comparisons were performed using one-way analysis of variance tests using GraphPad Prism version 6.0h for Mac OSX (GraphPad Software). A $p < 0.05$ was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Superoxide Induces Neutrophil Extracellular Traps

Superoxide released into circulation serves to activate neutrophils to produce proinflammatory cytokines, (25) yet it is unknown whether superoxide stimulates neutrophils to form NETs. To determine the NET-inducing effect on neutrophils, we utilized xanthine oxidase and its substrate hypoxanthine to generate superoxide. (25)

When neutrophils form NETs, decondensed chromatin studded with various proteins is expelled to form distinctive extracellular web-like structures. (3,4) We visualized this process by immunofluorescence after treatment of purified neutrophils in culture with superoxide. NET DNA fiber release was identified by co-localization of extracellular DNA and histones. Significant NET formation was observed by immunofluorescence with superoxide treatment compared to normal media alone (negative control), and was similar to

treatment with the positive control phorbol 12-myristate 13-acetate (PMA) (Figure 1A).

Prior to chromatin extrusion as a NET web, citrullination of histone H3 (cit-H3) by the enzyme peptidyl-arginine-deiminase 4 (PAD4) is required to induce decondensation. (33,34) To further evaluate NET formation to superoxide, we measured this modification specific for NETs by western blot. Superoxide treatment produced cit-H3 equivalent to that of PMA (Figure 1B). Production of cit-H3 from both superoxide and PMA treatments was significantly greater than from negative control–treated neutrophils. When allopurinol, a competitive inhibitor of superoxide generation by xanthine oxidase, was added to culture, cit-H3 levels decreased to control levels (Figure 1C). PMA-induced citrullination of histone H3 was unaffected by allopurinol.

We next examined the relationship between superoxide concentration and NET formation. As the concentration of xanthine oxidase was increased in the presence of excess hypoxanthine, a dose response was observed as significantly more superoxide was generated (Figure 1D). Among the proteins bound to the expelled decondensed chromatin of NETs is myeloperoxidase (MPO). As such, MPO-DNA complexes are indicative of NETs. We detected a dose-dependent increase in MPO-DNA complexes as the extracellular superoxide concentration increased (Figure 1E).

Finally, we evaluated the kinetics of NET formation induced by superoxide with the aid of a plate reader assay. (29) This assay reports NET formation by utilizing the cell-impermeable extracellular DNA dye Sytox Green to detect NET DNA fiber extrusion. The assay shows that superoxide induces neutrophils to form NETs (Figure 1F), and that NET formation induced by superoxide follows different kinetics than that induced by PMA. As expected, addition of allopurinol to inhibit superoxide generation in this assay resulted in a significant decrease in NET formation (Figure 1G). Taken together, these results support NET formation with exposure of neutrophils to extracellular superoxide.

Superoxide-Induced NETs Require NOX

The efficiency of NET release correlates with intracellular ROS generation by NADPH oxidase (NOX) (35) upon neutrophil exposure to PMA, bacteria and immobilized immune complexes, among other physiologic stimuli. (3,35–37) However, others report that an alternative NOX-independent NET-forming cascade exists. (38–40) As xanthine oxidase–derived superoxide is found extracellularly and is membrane impermeable, we hypothesized that NOX activation and intracellular ROS generation is required for superoxide-induced NET formation.

Neutrophils were pretreated with the NOX inhibitor diphenyleneiodonium (DPI) prior to extracellular superoxide stimulation. We found that citrullination of histone H3, a specific marker of NET formation, was significantly decreased with inhibition of NOX by DPI in superoxide-treated neutrophils (Figure 2A). This was consistent with the decreased cit-H3 expressed with DPI pretreatment in PMA-treated neutrophils observed by us and others. (4)

Additionally, we measured the release of MPO-DNA complexes after pretreatment with DPI and found significantly decreased NET formation in both superoxide-treated and PMA-treated neutrophils (Figure 2B). We also evaluated release of MPO-DNA complexes to H_2O_2 treatment, as it is a membrane-permeable ROS and has been reported to induce NET formation in a NOX-independent manner. (4) Treatment with glucose oxidase–derived H_2O_2 resulted in MPO-DNA complex release comparable to superoxide treatment. In contrast to superoxide, and consistent with its reported NOX-independent activity, MPO-DNA complex release was not significantly affected by DPI in H_2O_2-treated neutrophils.

The plate reader assay for extruded NET DNA fibers also reported that DPI treatment to inhibit NOX significantly reduced the NET formation observed with superoxide treatment alone (Figure 2C). Taken together, these results suggest that extracellular superoxide stimulates NETs upstream of NOX and is dependent on NOX to generate intracellular ROS, thereby advancing the NET-promoting cascade.

NET Formation by Superoxide Requires Toll-Like Receptor 4

Neutrophils express most Toll-like receptors (TLRs), and their activation has been reported to induce NETs in response to recognized neutrophil stimuli, such as LPS, (3) HMGB1, (39) bacteria and viruses, (41) and MSU crystals. (42) Yet other familiar NET-inducing stimuli do so independent of surface receptor activation, including PMA, ionomycin and H_2O_2. Of particular note, Lorne *et al.* (25) revealed that neutrophil TLR-4 interaction with superoxide triggers neutrophil activation, promotion of NF-κB effects and production of proinflammatory cytokines. We sought to establish whether NET formation to superoxide occurs through TLR-4 activation.

We found by flow cytometry that treatment of WT neutrophils with superoxide and eritoran, a TLR-4 antagonist that effectively inhibits downstream TLR-4 signaling, (43) produced significantly less citrullination of histone H3 than superoxide stimulation alone (Figure 3A). Moreover, we found that TLR-4 KO neutrophils did not produce NETs in response to superoxide stimulation. Western blot revealed that TLR-4 KO neutrophils exhibited significantly less citrullinated histone H3 when stimulated with superoxide compared with WT (Figure 3B). Also, in agreement with others, (3) LPS stimulation of TLR-4 KO neutrophils did not increase cit-H3, unlike in WT. TLR-4 KO neutrophils, however, retained the capacity to produce NETs when treated with PMA as expected, because PMA

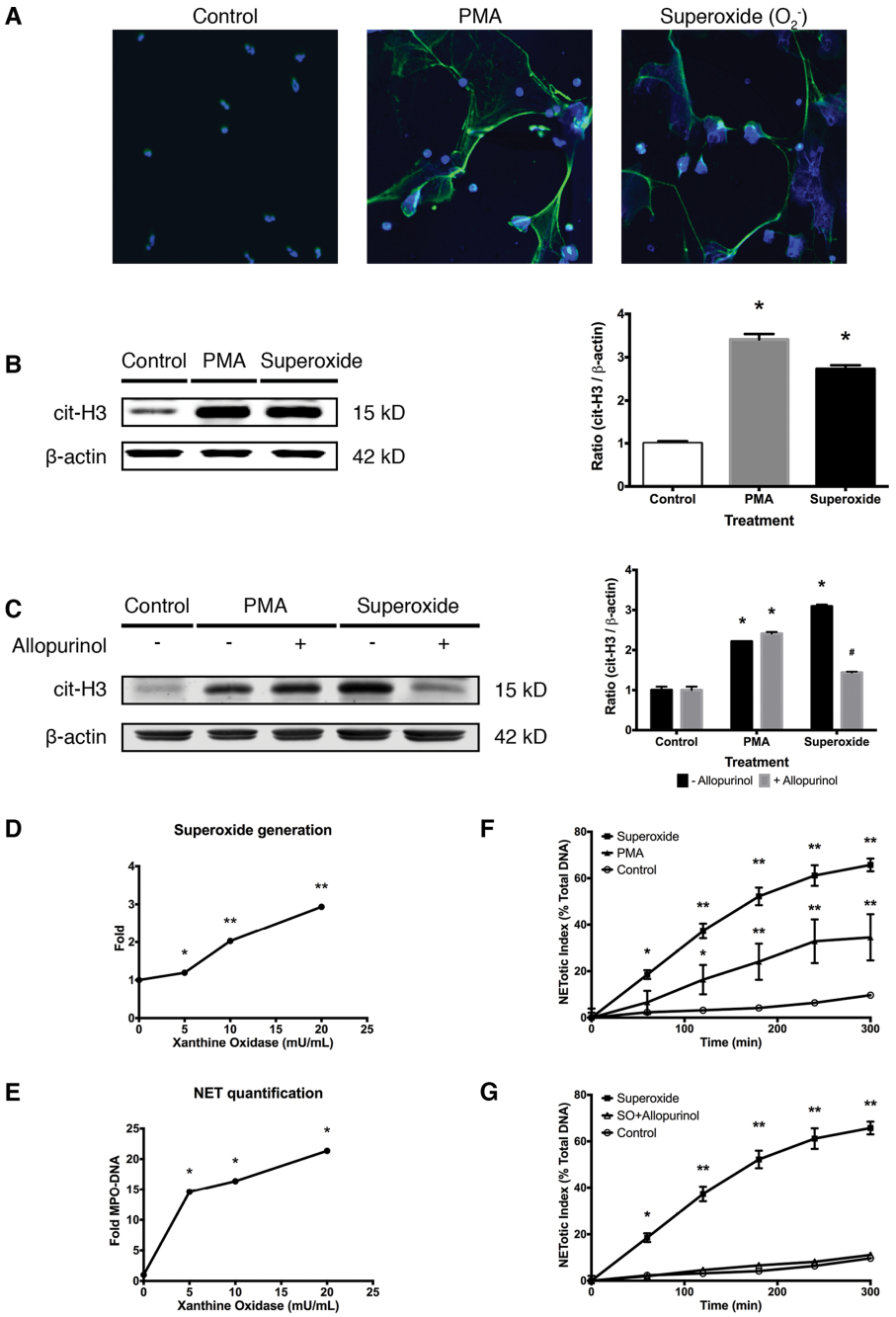

Figure 1. Extracellular xanthine oxidase–derived superoxide induces NETs. (A) Neutrophils were incubated with media alone (negative control), PMA (100 nM), or xanthine oxidase (10 mU/mL) and hypoxanthine (500 nM) to yield superoxide, for 1–8 h at 37°C. Cells were then fixed, immunolabeled with antibody to citrullinated Histone H3, detected with a conjugated fluorescent secondary antibody (green), then stained for DNA with Hoechst (blue). (B) Western blot of citrullinated Histone H3 in neutrophils following treatment as above, or (C) with or without the competitive xanthine oxidase inhibitor allopurinol. β-actin is shown as a loading control. Densitometric quantification charts show mean ± SEM cit-H3 to β-actin, expressed as fold over control. (D) Assay of superoxide generated, and (E) NET MPO-DNA complexes formed in media by xanthine oxidase (0, 5, 10 or 20 mU/mL) and hypoxanthine (500 nM) after 8 h at 37°C. Shown as mean fold over control. (F) NET formation kinetics in response to superoxide or PMA as measured by a fluorescent plate reader assay for extruded DNA fibers (n = 8). (G) Allopurinol inhibits superoxide-induced NET formation. NET release is expressed as percentage of total DNA (n = 8). *$p < .05$ *vs* control; $^{\#}p < .05$ *vs* superoxide treatment; **$p < .001$ *vs* control. All figures represent at least 3 independent experiments for each stimulus.

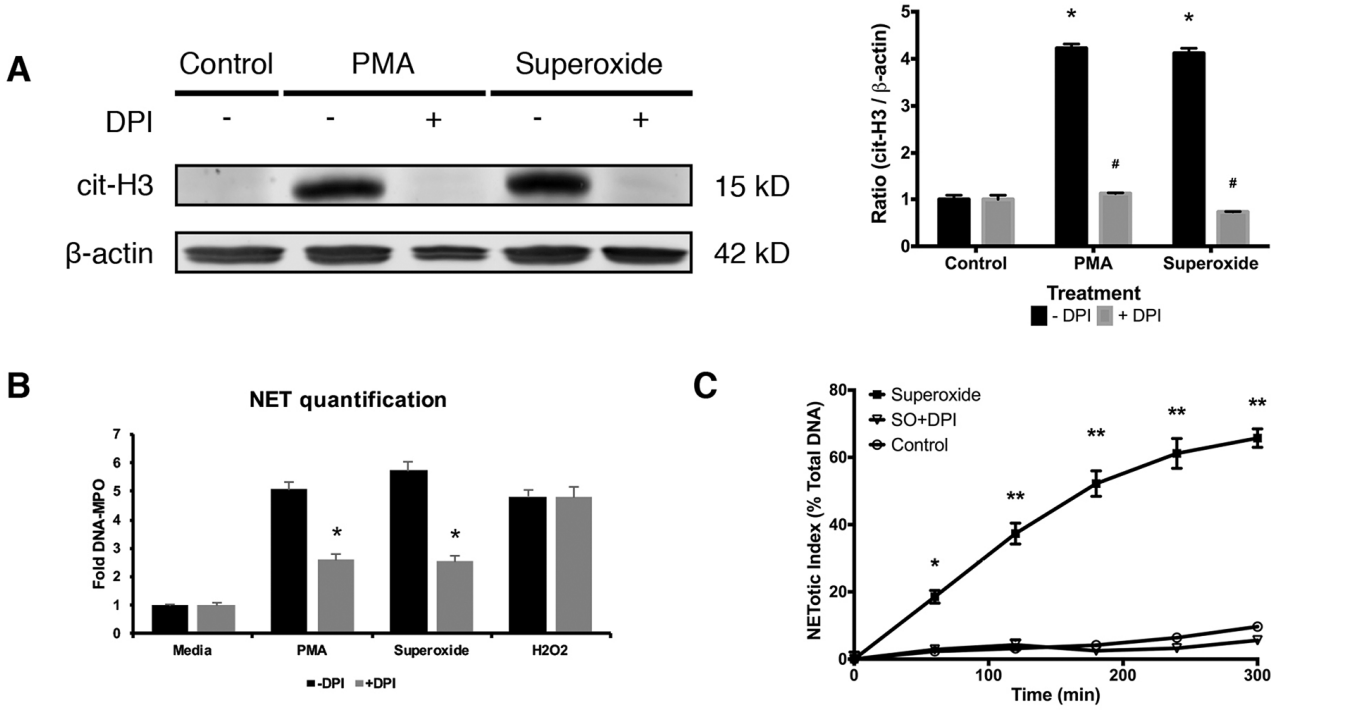

Figure 2. Extracellular superoxide induces NETs via NADPH oxidase. Neutrophils were incubated with media alone (negative control); PMA (100 nM), xanthine oxidase (10 mU/mL) and hypoxanthine (500 nM) to yield superoxide; or glucose oxidase (100 mU/mL) to yield hydrogen peroxide, for 1-8 h at 37°C, in the absence or presence of the NOX inhibitor DPI (10 μM). (A) Western blot of citrullinated Histone H3 levels in neutrophils following treatment as above. β-actin is shown as a loading control. Densitometric quantification charts show mean ± SEM cit-H3 to β-actin, expressed as fold over control. (B) Assay of NET MPO-DNA complexes formed after treatment as above. Shown as mean fold over control. (C) NET formation kinetics in response to superoxide with or without the NOX inhibitor DPI as measured by a fluorescent plate reader assay for extruded DNA fibers (n = 8). NET release is expressed as percentage of total DNA (n = 8). *$p < .05$ *vs* control; #$p < .05$ *vs* respective uninhibited treatment. All figures represent at least 3 independent experiments for each stimulus.

directly activates NOX exclusive of ligand-receptor interactions.

Finally, we quantified NET formation of TLR-4 KO and WT neutrophils by release of MPO-DNA complexes after treatment relative to media alone. We found that superoxide treatment of TLR-4 KO neutrophils resulted in significantly less MPO-DNA complexes released compared with WT and approached the amount observed with media treatment alone (Figure 3C). LPS stimulation of neutrophils demonstrated that MPO-DNA complex release was similarly TLR-4–dependent. Immunofluorescence corroborates these findings. Images illustrate minimal extracellular DNA expelled from neutrophils and the absence of histone co-localization in TLR-4 KO neutrophils stimulated with superoxide and LPS, in contrast to stimulation of WT cells (Figure 3D). Collectively, our findings support a requirement for TLR-4 in superoxide-induced NET formation.

Allopurinol and NAC Reduce NET Formation After Liver I/R

Having shown that xanthine oxidase–derived superoxide induces NETs in culture, we sought to determine whether NET formation was associated with the oxidative stress of liver I/R. The earliest manifestations of I/R occur during a hyperacute phase, characterized by a sharp increase in oxygen consumption and superoxide anion release. (44)

To provide evidence for superoxide-induced NETs *in vivo* after oxidative stress, we measured the quantity of MPO-DNA complexes present in serum. We found significantly greater quantities of MPO-DNA complexes in the serum of WT mice after I/R relative to sham (Figure 4A). Treatment with allopurinol to preclude superoxide generation resulted in significantly decreased levels of MPO-DNA complexes relative to control after I/R. NAC treatment showed a similar decrease in serum MPO-DNA complex level.

We then sought to verify our finding that TLR-4 KO neutrophils did not form NETs in response to superoxide, by subjecting TLR-4 KO mice to the same conditions. We found significantly reduced quantities of serum MPO-DNA complexes in TLR-4 KO after I/R compared with WT. Allopurinol and NAC treatment conditions demonstrated similarly

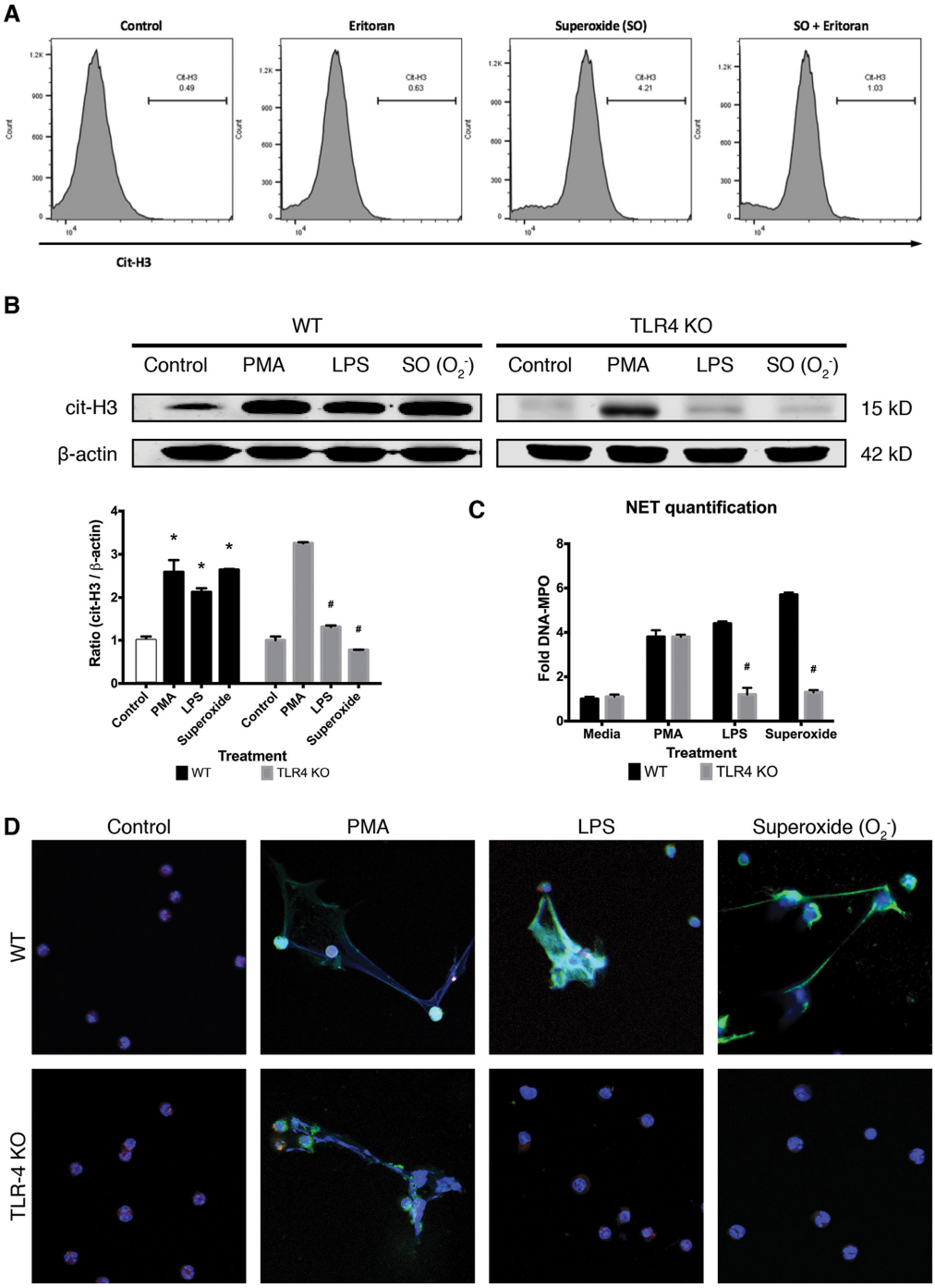

Figure 3. TLR-4 mediates NET induction by extracellular superoxide. (A) Flow cytometry analysis of citrullinated Histone H3 in WT neutrophils after treatment with media alone (control), eritoran TLR-4 antagonist (8 ng/mL), xanthine oxidase (10 mU/mL) and hypoxanthine (500 nM) to yield superoxide, or superoxide and eritoran, for 8 h. (B) Western blot of citrullinated Histone H3 in WT or TLR-4 KO neutrophils after treatment with media alone (negative control), PMA (100 nM), LPS (10 ug/mL), or xanthine oxidase (10 mU/mL) and hypoxanthine (500 nM) to yield superoxide, for 1–8 h at 37°C. β-actin is shown as a loading control. Densitometric quantification chart shows mean ± SEM cit-H3 to β-actin, expressed as fold over control. (C) Assay of NET MPO-DNA complexes formed after treatment as above. Shown as mean fold over control. *#p* < .05 *vs* respective uninhibited treatment. (D) Neutrophils were fixed after treatment as above, immunolabeled with antibody to citrullinated Histone H3, detected with a conjugated fluorescent secondary antibody (green), then stained for DNA with Hoechst (blue). All figures represent at least 3 independent experiments for each stimulus.

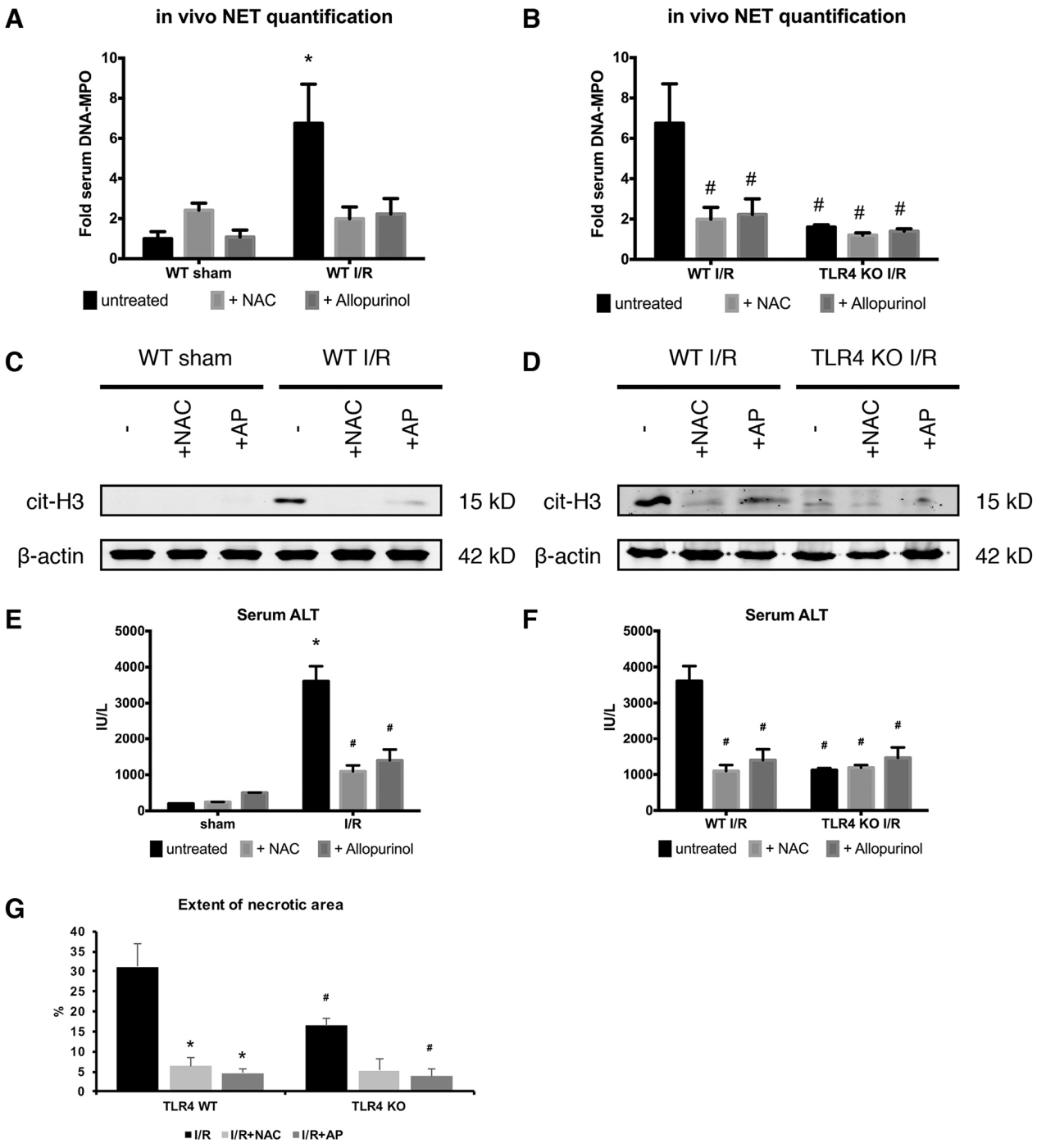

Figure 4. Superoxide- and ROS-inhibitors reduce NETs *in vivo*. WT and TLR-4 KO mice underwent either sham laparotomy or 1 h of ischemia and 6 h of reperfusion, and received control, N-acetyl-cysteine (NAC; 150 mg/kg) or allopurinol (50 mg/kg) treatment. (A) ELISA assay of circulating *in vivo* NET MPO-DNA complexes present in the serum of WT mice, or (B) TLR-4 KO mice after I/R and treatment as above. Shown as mean fold over control. *$p < .05$ *vs* respective uninhibited treatment. (C) Western blot of *in vivo* citrullinated Histone H3 levels after sham or I/R and treatment as above from WT liver protein lysates, or (D) WT and TLR-4 KO after I/R and treatment as above. β-actin is shown as a loading control. (E) Assay of serum ALT levels in WT mice, or (F) TLR-4 KO mice after I/R and treatment as above. Data represent the mean ± SEM (n = 6 or more mice per group). *$p < 0.05$ vs sham. #$p < .05$ vs untreated WT I/R group. (G) Quantification of necrotic hepatocytes in H&E stained liver sections from control-, NAC- or allopurinol-treated mice 6 h after reperfusion. *$p < 0.05$ *vs* WT control group after liver I/R; #$p < .05$ *vs* respective WT inhibitor–treated group after liver I/R.

reduced quantities of serum MPO-DNA complexes after I/R compared with WT (Figure 4B).

We also evaluated liver protein isolates for evidence of NET formation by detecting cit-H3. Western blot revealed that WT mice subjected to I/R exhibited marked cit-H3, unlike those subjected to sham procedure. Treatment with allopurinol and NAC resulted in significant reduction of cit-H3 after I/R compared with I/R + vehicle (Figure 4C). Consistent with our serum MPO-DNA results, TLR-4 KO mice did not exhibit NET formation, as evidenced by significantly diminished cit-H3 after I/R alone or with allopurinol and NAC treatments (Figure 4D).

In agreement with previous studies, (14,21,45) both allopurinol and NAC treatments independently resulted in significant protection from organ damage after I/R, as measured by reduced serum ALT levels, compared with WT mice receiving I/R + vehicle treatment (Figure 4E). Serum ALT from TLR-4 KO mice subjected to I/R was significantly lower than in WT, as were allopurinol and NAC treatment groups (Figure 4F). Histologic evaluation corroborated this finding, as liver sections from allopurinol- or NAC-treated mice exhibited markedly less severe sinusoidal dilatation and confluent pericentral hepatocellular necrosis in liver tissue than untreated I/R mice (Figure 4G). In summary, these results confirm NET formation in response to the superoxide predominant oxidative stress of liver I/R, and provide evidence for NET involvement in the hyperacute injury phase.

DISCUSSION

The pathogenesis of excessive superoxide generation and states of oxidative stress is largely attributable to pathophysiologic inflammatory responses. Neutrophils are key effectors of the resultant sterile inflammation and have recently been shown to worsen noninfectious disease by forming NETs. (5) Our recent findings show that NET formation is stimulated by proteins released from necrotic hepatocytes as sequelae of I/R (46); however, the more proximal effects of oxidative stress itself on NET formation remains unexamined. Lorne *et al.* demonstrated that superoxide does activate neutrophils to produce proinflammatory mediators. (25) Therefore, we hypothesized that NETs form in response to the high concentrations of circulating superoxide typical of oxidative stress. We found in this study that extracellular superoxide also stimulates neutrophils to form NETs, and that this process requires TLR-4 and NOX (Figure 5). We also demonstrated that treatment with the antioxidants allopurinol and NAC results in less NET formation than is associated with amelioration of liver injury after I/R.

The central role of neutrophils as early responders of the innate immune system means infectious pathogens, sterile proteins and autoimmune factors alike have been described to stimulate NETs. Early work on NETs even implicated the reactive oxygen species hydrogen peroxide (H_2O_2) as a stimulus and revealed the requirement for intracellular ROS generated by NOX in propagating a NET-promoting cascade. (4) By virtue of its uncharged nature, H_2O_2 was found to induce NET formation despite NOX inhibition by crossing the cell membrane to act as a surrogate NOX product. In our study, we examined extracellular superoxide (O_2^-) and found that it readily induces NETs. Consistent with most stimuli, NOX is required to propagate the process by generating intracellular ROS second messengers. In contrast to H_2O_2, superoxide does not cross the cell membrane due to its negative charge,

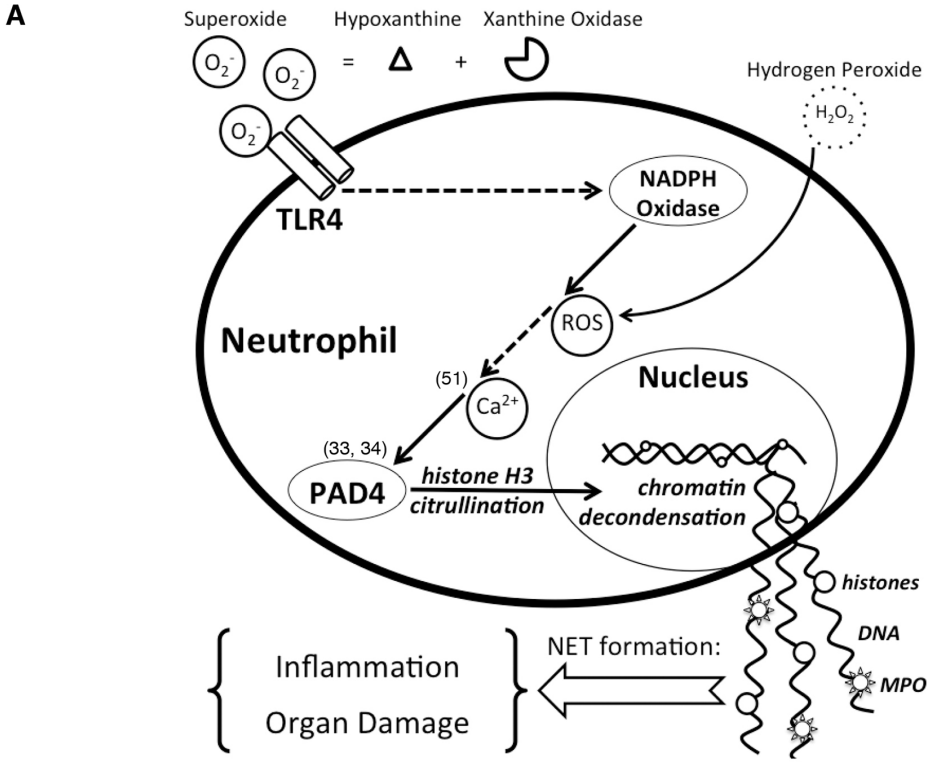

Figure 5. Proposed model of NET induction by extracellular superoxide. (A) Schematic representation of superoxide (O_2^-) generation during and elements of the NET-promoting cascade. During liver I/R, membrane-nonpermeable superoxide functions to activate TLR-4 signaling pathways, which subsequently activates NOX, leading to induction of NETs. Membrane-permeable hydrogen peroxide (H_2O_2) propagates NET formation without activation of NOX.

and therefore does not form NETs with NOX inhibition. In the context of neutrophil-mediated immunity, superoxide is commonly cited as a product of NOX essential to the phagocytic burst. Our finding that superoxide induces NETs not only provides evidence for a role in conditions of oxidative stress, but also raises the possibility of neutrophils' phagocytic activities initiating a cycle of inflammation involving NETs.

Neutrophil activation occurs through pattern recognition receptors, particularly members of the TLR family, upon detection of extracellular infectious or sterile threats. Neutrophils express most TLRs; their activation generates diverse effects on cell survival, chemotaxis and gene regulation. (15,47,48) In the context of NETs, various TLRs have been found to mediate NET formation, including TLR-2, (41,42) TLR-7 and TLR-8, (49) TLR-4 (39,46) and TLR-9. (46) The association of TLR-4 with xanthine oxidase was previously shown to result in neutrophil activation by superoxide. (25) In our study, we found that superoxide-induced NET formation does not occur in TLR-4 KO neutrophils. Our finding builds upon the previous discovery that oxidants enhance TLR-4 signaling in innate immune cells, specifically macrophages. (50) Oxidants accomplish this by altering lipid raft composition, increasing surface receptor density, and affecting transcriptional and translational regulation of proinflammatory genes. Another effect of oxidants on these cells is an increase in intracellular calcium. This has significant implications for neutrophils, as calcium participates in the NET-promoting cascade by activating nuclear PAD4 to catalyze histone citrullination, chromatin decondensation and NET fiber expulsion. (33,51) With the knowledge that oxidants enhance innate immune cell response to subsequent challenges, our finding that superoxide induces NETs via TLR-4 suggests that superoxide may prime neutrophils for NET formation in settings where an oxidative stress is closely associated with infection or necrosis, such as liver I/R.

Oxidative stress is a defining feature of liver I/R; excess generation of ROS, such as superoxide, by liver epithelial cells, Kupffer cells and vascular endothelium results in a proinflammatory extracellular milieu. The detrimental effects of ischemic insults on organs are worsened by increased generation of extracellular superoxide by xanthine oxidase, while enhanced removal or dismutation of superoxide is associated with lesser injury. (52) Previous work using animals with liver I/R have consistently shown that antioxidant therapy reduces inflammatory injury. (19,20,45) Consistent with this previously published work, our finding that *in vivo* NET formation and consequent liver injury are reduced by treatment with the xanthine oxidase inhibitor allopurinol provides evidence not only that superoxide promotes a NET-mediated inflammatory response, but also that antioxidant therapy has therapeutic potential in liver I/R beyond reducing direct ROS cytotoxicity.

CONCLUSION

In summary, our study demonstrates that extracellular superoxide stimulates neutrophils to release NETs through TLR-4 and NOX signaling. Prevention of excess superoxide generation and a state of oxidative stress induced by allopurinol reduces NET formation and associated liver injury. Therefore, to achieve the greatest efficacy, therapies targeting NETs should be designed to limit activation during the hyperacute, ROS-predominant phase common to I/R of all organs.

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DISCLOSURE

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