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ORIGINAL ARTICLE

Catalase S-Glutathionylation by NOX2 and Mitochondrial-Derived ROS Adversely Affects Mice and Human Neutrophil Survival

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Abstract— Neutrophil survival and oxidative stress during inflammatory conditions are linked to tissue damage. The present study explores less understood role of catalase, the enzyme catalysing hydrogen peroxide decomposition, in neutrophil survival/death. Importantly, inhibition of catalase activity following S-glutathionylation in the PMA, NO, or zymosan-activated neutrophils or treatment with catalase inhibitor led to neutrophil death. On the contrary, introducing reducing environment by TCEP rescued catalase activity and significantly improved neutrophil survival. Furthermore, augmentation in ROS generation by NOX-2 activation or induction of mitochondrial ROS by Antimycin-A induced catalase S-glutathionylation and cell death, which was prevented in the neutrophil cytosolic factor1 (NCF-1-/-) cells or was rescued by MitoTEMPO, a mitochondrial ROS scavenger, thus, suggesting a correlation between catalase S-glutathionylation/activity inhibition and reduced neutrophil survival. Altogether, enhanced NOX2 activation/mitochondrial dysfunction led to reduced survival of human and mice neutrophils, due to H_2O_2 accumulation, S-glutathionylation of catalase, and reduction in its enzymatic activity. The present study thus demonstrated mitigation of catalase activity under oxidative stress-impacted neutrophil survival.

KEY WORDS: Neutrophil survival; NADPH oxidase; Mitochondrial ROS; Catalase S-glutathionylation; MitoTEMPO; Antimycin-A; 3-aminotriazole.

INTRODUCTION

Neutrophils are the crucial player of innate immunity; however, their uncontrolled activation exuberate inflammatory conditions. Short life span of neutrophils with spontaneous programmed cell death endorses efficient resolution of acute and chronic inflammation. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by neutrophils exert robust cytotoxic response against ingested pathogens. In addition, dysregulation of neutrophil production and its turnover resolutely contribute

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to pathological states [1]. Thus, understanding of molecular mechanisms regulating neutrophil survival and death pathways are of high interest for the mitigation of neutrophil-mediated inflammation.

Neutrophil comprises of a well-organised system of oxidants and antioxidants. It is well documented that sustained ROS production can lead to neutrophil cell death through apoptosis and NETosis pathways [2–7]. In neutrophils, NADPH oxidase (NOX-2) generates superoxide that through enzymatic dismutation is converted to H_2O_2, a strong oxidising molecule for most of the biological macromolecule like protein thiols, lipids and DNA. Both enzymatic and non-enzymatic antioxidants are crucial in balancing redox homeostasis, which are critical for ageing, metabolic and degenerative disorders [8, 9]. Interestingly, natural and synthetic antioxidants like α-tocopherol, vitamins (C and E) and N-acetylcysteine modulate cell survival or ameliorate drug and chemical-induced toxicities [10–14]. However, role of the endogenous antioxidant enzyme, catalase, that catalyses the decomposition of H_2O_2 in neutrophils under spontaneous and stress-induced cell death remains relatively less explored. Neutrophils possess diverse antioxidant systems including superoxide dismutase, thioredoxins, peroxiredoxins, glutathione peroxidases, glutathione reductase and catalase; among these, catalase is most abundant in the neutrophils [15, 16].

Enhanced oxidative stress inhibits many important enzymes and also adversely affects mitochondrial integrity, thus promoting cell death *via* mitochondrial independent and dependent permeability transitions. NOX-2 and mitochondrial complex III are the two major sources of ROS generation in neutrophils. Mitochondria in neutrophils are not involved in the ATP production and oxidative phosphorylation rather maintain mitochondrial membrane potential to regulate cell death [17]. In mitochondria, complex I and complex III are the primary sources of superoxide radicals. More recently, inhibition of mitochondrial respiratory complex III by Antimycin-A enhanced mitochondrial ROS generation and reduced mitochondrial membrane potential, demonstrating the role of Complex III in neutrophil apoptosis [18]. Mitochondrial ROS (mtROS) production thus affects mitochondrial membrane potential, metabolic state of mitochondria and cell death [19]. Higher concentrations of mitochondrial ROS adversely affect survival of many cell types, while at modest levels, mtROS is involved in cell proliferation [20–23]. Previous studies have also shown that higher (more polarized) $\Delta\psi m$ is associated with greater mtROS generation, due to the reduced electron transport [24, 25]. Recently, the protective role of MitoTEMPO against oxalate-induced cytotoxicity has been shown by reducing the oxidative stress and mitochondrial dysfunction [26]. Moreover, enhanced oxidative and nitrosative stress also induce S-glutathionylation, a post translational modification (PTMs) of cysteine residues, which is functionally relevant, redox-sensitive and is reversible in nature. Recently, we have demonstrated role of S-glutathionylation in sustained ROS generation by neutrophils [27]; however, role of PTMs, if any, in the neutrophil survival under physiological and pathological conditions remains less defined.

Reports in the literature suggest that besides the role in decomposition of hydrogen peroxide (H_2O_2), catalase seems to play a pivotal role in survival/death of many cell types. Inhibition of catalase activity in the rat primary hepatocytes resulted into sustained ROS generation and augmentation of their death [28]. Similarly, parthenolide-mediated cell death was enhanced by catalase inhibition in multiple myeloma cell lines [29]. Further numerous studies have shown the protective role of overexpressed catalase in survival of insulin producing cells [30, 31]. "Acatalasemia", a rare disease is characterised by inherited catalase deficiency, is associated with higher incidence of ulcerative wounds and atherosclerosis [32, 33]. Deficiency of catalase in these patients is also linked with higher incidence of diabetes mellitus due to malfunctioning and death of pancreatic β-cells [34]. These patients also exhibit impaired neutrophil functions; however, death of polymorphonuclear neutrophils (PMNs) was not investigated.

The present study was undertaken to examine the least defined role of catalase in mice and human neutrophil survival. The study demonstrates a specific role of NOX2 and mitochondrial-mediated ROS in catalase inhibition following its S-glutathionylation. Moreover, enhanced oxidative stress, H_2O_2 accumulation as well as death of mice and human neutrophils weres prevented by ROS scavengers. The present study thus demonstrates an inverse relationship between oxidative stress and neutrophil survival which was mediated by S-glutathionylation-dependent reduction in catalase activity.

MATERIALS AND METHODS

Isolation of Neutrophils by Percoll Gradient Density Centrifugation Method

Human neutrophils were isolated from peripheral blood of healthy donor by Percoll density gradient method. C57BL/6 (WT) and their age matched (12–16 weeks) neutrophil cytosolic factor-1 (NCF-1 −/−) knockout mice

[procured from The Jackson Laboratory (Bar Harbour ME, USA)] were used in this study. Studies on human blood and mice were approved by the CSIR-CDRI and KGMU ethics committee.

The isolated human neutrophils and bone marrow-derived neutrophils (BMDNs) were washed with PBS and re-suspended in RPMI1640 containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. CD15 and LY6G labelling was used to assess the purity of the isolated human neutrophils and mice neutrophils respectively by using flow cytometry (FACS Calibur, BD, USA). Microscopic analysis indicated the purity of isolated neutrophils that was ~ 95%, and cell viability as checked by Trypan blue exclusion method was always more than 95% [35, 36].

Treatment

To induce cell death in human PMNs, Phorbol 12-myristate 13-acetate (PMA, 30 nM), Zymosan (2 mg/ml) for 4 h and DETA-NO (300 μM for 12 h), a nitric oxide (NO) donor, were used. MitoTEMPO (50 μM), Antimycin-A (5 μM), 3-aminiotrizole (25 mM), H_2O_2 (500 μM) and Tris (2-carboxyethyl) phosphine (TCEP, 1 mM) were also used in the study.

ROS/Mitochondrial Superoxide Radical and H_2O_2 Generation

Freshly isolated neutrophils (1×10^6cells/ml) treated with PMA, Zymosan or DETA-NO were loaded with dichlorodihydrofluorescein diacetate, (DCF-DA, 10 μM), dihydroethidium, (DHE, 10 μM) or MitoSox Red (MSR, 3 μM) fluorescent dyes for 15 min. Ten thousand events were acquired on FACS Calibur (Becton Dickinson, USA), and subsequently total ROS, superoxide radical and mitochondrial ROS generation were analysed using the Cell Quest Program [37]. Similarly, hydrogen peroxide release was measured by Amplex Red Kit according to manufacturer's instructions (Thermo Fisher scientific).

Catalase Enzymatic Activity

Isolated neutrophils (2×10^5 cells/ml) after respective treatment were lysed in 0.05 M phosphate buffer (pH 7.0). The catalase activity was monitored in a quartz 96-microtiter plate. The supernatant collected, after sonication followed by centrifugation at 13000 rpm, was used to check catalase activity. The substrate, hydrogen peroxide, was diluted and used at working concentration of 5 mM. Quantification of catalase activity in the cell lysates was carried out in the assay buffer following addition of H_2O_2 in each well, and the plate was read in a spectrophotometer at 240 nm in 5 min at 22 °C. Catalase activity as monitored by the rate of decomposition of hydrogen peroxide was proportional to the decrease in O.D at 240 nm [38, 39].

Cell Death Assay

The cell death was measured by Annexin V PI labelling in neutrophils (1×10^6 cells/ml) pre-treated with vehicle, PMA (30 nM) or Zymosan (2 mg/ml) for 4 h or DETA-NO, (300 μM) for 12 h were pelleted at 2500 rpm for 5 min, washed with ice-cold PBS once and resuspended in 100 μl of binding buffer (10 μM HEPES, pH 7.4, 150 μM NaCl, 5 μM KCl, 1 μM $MgCl_2$ and 2 μM $CaCl_2$). Subsequently, cells were stained with 3 μl of Annexin V-FITC and incubated for 15 min at RT in dark. Further, 2.5 μl propidium iodide was added for 15 min. Finally, 200 μL of binding buffer was added to the stained cells as described in BD biosciences manufacturer protocol and fluorescence of 10,000 cells was acquired by FACS Calibur and subsequently analysed using Cell Quest programme. Percent cell death as depicted in the study involves early apoptotic, late apoptotic and necrotic cells [37].

Mitochondrial Membrane Potential ($\Delta\psi$m) Using JC-1

Neutrophils (1×10^6 cells/ml) were pre-treated with mitochondrial ROS scavenger, MitoTEMPO (50 μM) and Antimycin-A (5 μM) for 60 min followed by the induction with PMA, Zymosan or NO. In these cells, mitochondrial membrane potential was measured using JC-1, a positively charged dye which accumulates in mitochondria in a membrane potential dependent manner. Ten thousand events were acquired and analysed using flow cytometer [40].

S-Glutathionylation, Immunoprecipitation (IP) and Western Blotting

Neutrophils (1×10^7cells/ml) were lysed with lysis buffer after the completion of each treatment in PBS containing 0.1 mM EDTA, 0.1 mM EGTA, 5 mM diisopropylfluorophosphate (DFP), protease inhibitor cocktail, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 mM N-ethylmaleimide (NEM) and 0.5% NP-40. Preclearing of the supernatant was followed by overnight incubation with 1 μg catalase antibody at 4 °C. Subsequently, 20 μl protein-A Agarose beads were added and incubated for overnight at 4 °C. The beads were washed, resuspended in non reducing gel loading buffer,

denatured at 95 °C for 10 min and subsequently probed with anti GSH. Biotinylated glutathione ethyl ester, BioGEE labelling, was also done for the detection of S-glutathionylation of catalase [one BioGEE vial was resuspended in 100 μl of HBSS and incubated for 30 min at 37 °C]. Neutrophils (1×10^7 cells/ml) were loaded with BioGEE for 30 min, and these cells were treated with PMA for 4 h; cells were then lysed with neutrophil lysis buffer (150 mM NaCl, 50 mM Tris, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, protease inhibitor cocktail, 5 mM diisopropylfluorophosphate (DFP), 0.5% NP-40 and 100 mM NEM). S-glutathionylated proteins thereby pulled down with neutravidin agarose beads, resuspended in non reducing gel loading buffer, denatured at 95 °C for 10 min, resolved on SDS PAGE and then were probed with catalase antibody. Input protein was probed with β-actin as loading controls. For monitoring expression level of different proteins (MnSOD, Catalase), cells were lysed with RIPA buffer (PBS containing 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/ml aprotinin, 100 μg/ml PMSF, 20 μg/ml pepstatin, 5 mM DFP (diisopropylfluorophosphate), 1% Triton X-100 and 0.1% SDS) containing Protease Inhibitor Cocktail, 10 mM NaF and 2 mM Na_3VO_4. Equal protein samples were prepared in Laemmli sample buffer, denatured at 95 °C for 5 min and were subjected to reducing 10% SDS-PAGE [41].

Statistical Analysis

Data have been reported as mean ± SEM and represent the results of at least 3–5 independent experiments. The data were analysed by one-way ANOVA followed by Newman-Keul's post-analysis (for multiple group) or using Student's *t* test (for comparisons between 2 groups); p value < 0.05 was considered statistically significant.

RESULTS

Enhanced Oxidative Stress and Neutrophil Survival

Enhanced oxidative stress is associated with reduced survival of human neutrophils. To have a better understanding about the factors that regulate neutrophil survival, cells in suspension were treated for different time intervals with various inducers to achieve significant cell death. Four hours following treatment with PMA, Zymosan and 12 h of NO treatment significantly augmented cell death as confirmed by Annexin V/PI labelling (Fig. 1a and S1A). Relative increase in the oxidative stress was measured by enhanced DCF fluorescence (Fig. 1b). In parallel, superoxide generation was measured by DHE, which was significantly increased as monitored by flow cytometry (Fig. 1c). Moreover, heightened H_2O_2 production was also coupled with enhanced oxidative stress in the neutrophils (Fig. 1d). Further, H_2O_2-stimulated neutrophil death was prevented in the presence of N-acetylcysteine (NAC), as confirmed by Annexin V/PI labelling (Fig. 1e). Involvement of NOX-2 in neutrophil cell death was confirmed by using cells from NCF-1KO, where PMA-induced cell death was significantly prevented as compared to wild type mice neutrophils (Fig. 1f).

Role of Mitochondrial ROS During Neutrophil Cell Death

Mitochondrial oxidative stress seems to be important factor in enhancing the sensitivity of neutrophils to oxidative insult. Mitochondrial ROS is recently shown to activate NADPH oxidase that subsequently affects cell death. Decrease in membrane potential in vehicle-/agent-treated cells was confirmed by using JC1 dye. In addition, mitochondrial ROS was also augmented in the activated neutrophils as demonstrated by MitoSOX Red (Fig. 2a). Involvement of mitochondrial ROS was evaluated by using cells from NCF1-KO, where pre-treatment with Antimycin-A (AA) in PMA-treated PMNs showed enhanced cell death suggesting towards the role of mitochondria (Fig. 2b). In addition, pre-treatment with MitoTEMPO (MT, 50 μM), a mitochondrial ROS scavenger, prevented the loss in mitochondrial membrane potential and cell death in activated cells. (Fig . 2c, d). Similarly, treatment with antimycin-A augmented mitochondrial ROS, PMNs death and also loss in mitochondrial membrane potential (Fig. 2c, d).

Status of Antioxidants Enzymes SOD and Catalase During Neutrophil Cell Death

ROS/RNS and antioxidant proteins collectively intricately regulate the intracellular redox homeostasis. Enhanced oxidative stress is accompanied by change in the activity, expression level of compensatory enzymatic and non-enzymatic antioxidants. To assess the effect of enhanced oxidative stress, expression of superoxide dismutase 2 (SOD2) and catalase was checked in the control and activated cells. SOD2, catalyses dismutation of superoxide to H_2O_2 and its expression, was found to be augmented in the apoptotic cells as compared to the control vehicle-treated cells (Fig. 3a). Moreover, SOD2 activity was also enhanced in PMA-treated neutrophils (data not shown).

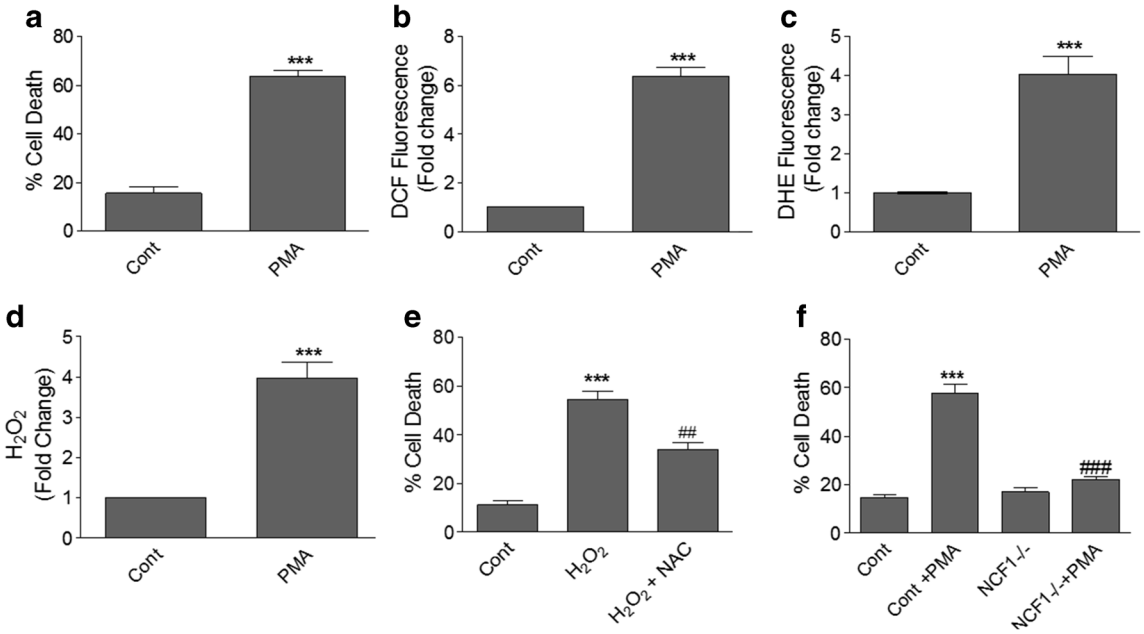

Fig. 1. Oxidative stress in human neutrophils and their survival. **a** Cell death was monitored in control and PMA-treated neutrophils for 4 h by using Annexin V-PI labelling. **b** ROS/RNS generation as checked by DCF-2DA in PMA-treated human neutrophils. **c** Superoxide radical production was measured by DHE using flow cytometry in PMA-treated human neutrophils. **d** H_2O_2 production represented in fold change as checked by Amplex RED Kit in PMA-induced PMNs. **e** H_2O_2-induced cell death was checked by using Annexin V-PI labelling. **f** ROS or oxidative stress-mediated cell death in PMA-induced neutrophils was confirmed in NCF1 -/- mice. Data are represented as mean ± SEM from minimum three different experiments. ***$p < 0.001$versus vehicle-treated control neutrophils. $^{\#\#}p < 0.01$, $^{\#\#\#} < 0.001$ *versus* PMA or H_2O_2-treated neutrophils.

Interestingly, the catalase expression remains unchanged in both control and PMA-treated neutrophils (Fig. 3b). However, catalase enzymatic activity was significantly reduced in the activated neutrophils. Moreover, reduction in the catalase activity of AA pre-treated activated neutrophils was further significantly increased while rescued by the pre-treatment of activated cells with MitoTEMPO (Fig. 3c). Cell death was also checked in the presence of catalase inhibitor, 3-aminotriazole (3-AT). Pre-treatment with 3-AT prior to the PMA-induced neutrophil activation further enhanced cell death as compared to the PMA-treated neutrophils (Fig. 3d, e). In addition, catalase activity was checked in 3-aminotriazole-treated neutrophils in the presence of PMA. There was significant decrease in the catalase activity in 3-aminotriazole pre-treated cells (Fig. 3f), suggesting an inverse correlation between catalase activity and cell death.

Involvement of S-Glutathionylation in the Decrease of Catalase Activity and Neutrophil Cell Death

The accumulation of H_2O_2 and the decrease in catalase activity implied the association of oxidative stress with reduced cell survival. Studies from our lab and others have shown enhanced S-glutathionylation of several neutrophil proteins under oxidative stress condition. Decrease in the catalase activity prompted us to monitor S-glutathionylation of catalase, by immunoprecipitation (Fig. 4a) and BioGEE labelling (Fig. 4b). S-Glutathionylation of catalase was significantly enhanced during neutrophil cell death. The effect of catalase S-glutathionylation on its enzymatic activity was checked by treating catalase protein *in vitro* with GSSG, which significantly reduced the catalase enzyme activity (Fig. 4c). Further, treatment with reducing agent, Tris (2-carboxyethyl) phosphine, (TCEP) significantly normalised the catalase activity (Fig. 4d) and also rate of cell death in human neutrophils (Fig. 4e, f).

DISCUSSION

Intracellular redox status regulates cell survival, which is defined by an intricate balance between the generation and scavenging of ROS. Low amount of ROS acts as signalling molecules and are continously

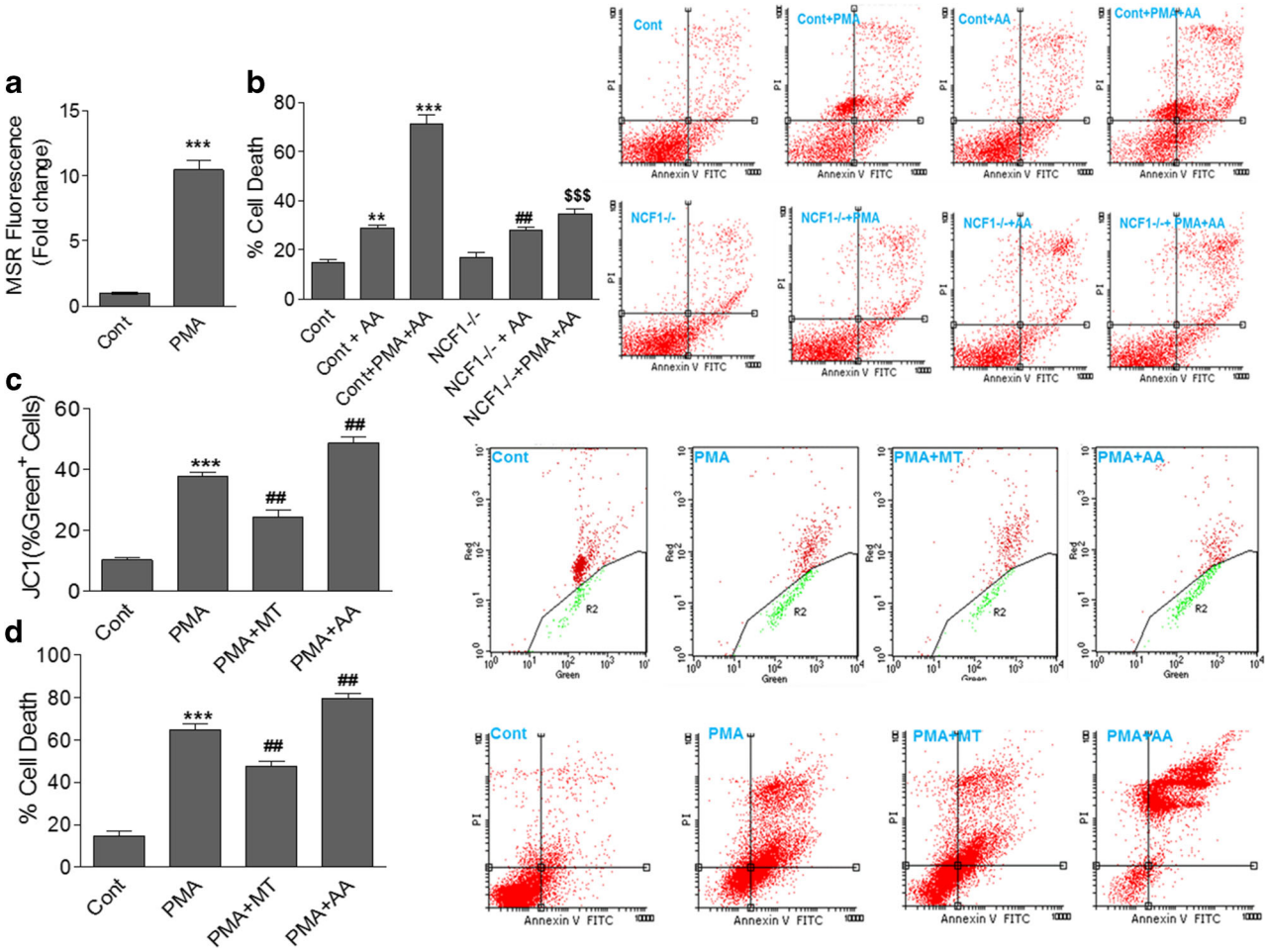

Fig. 2. Mitochondrial ROS and neutrophil survival. **a** PMA-induced mitochondrial ROS generation was monitored by MitoSOX Red by flow cytometry. **b** Mitochondrial ROS dependent cell death was confirmed in PMA-induced NCF1-/-mice neutrophils pre-treated with Antimycin-A. Human PMNs were pre-treated with MitoTEMPO (50 μM) and Antimycin-A (5 μM) for 60 min, washed with HBSS. **c** PMA-induced mitochondrial dysfunction was measured in the presence of MitoTEMPO and Antimycin-A by JC1 labelling. **d** PMA-induced cell death was measured in the presence of MitoTEMPO; mitochondrial scavenger and mitochondrial complex III inhibitor by Annexin V-FITC/PI labelling. Data are represented as mean ± SEM from minimum three different experiments. $^{***}p < 0.001$, $^{**}p < 0.01$ *versus* control mice and human neutrophils. $^{\#\#}p < 0.01$ versus PMA-treated mice and human neutrophils. $^{\$\$\$}p < 0.001$ versus PMA and antimycin-A-treated control mice neutrophils.

produced in the cells. Conversely, involvement of high ROS is demonstrated in passive as well as induced cell death by the oxidative modification of intracellular proteins. In the present report, crucial role of catalase, an enzyme involved in the scavenging of H_2O_2, has been demonstrated for the first time in the death of neutrophils. PMA, NO or zymosan-induced cell activation led to the accumulation of H_2O_2, which was further enhanced by 3-aminotriazole, a catalase inhibitor. S-Glutathionylation of catalase following neutrophil activation by PMA, NO or zymosan, also led to the reduction in its enzymatic activity and enhanced cell death.

The probable involvement of intracellular oxidation of proteins in cell death has engrossed greater attention recently. Mounting evidences have suggested role of ROS/oxidative stress-mediated cell death [42–46] that is equally supported by the protective role of antioxidants in the cell survival [47]. More precisely, hydrogen peroxide was found to play a key role in death of various cell types. Interestingly, SOD/catalase mimetic platinum nanoparticles significantly prevent apoptosis of human lymphomas [48]. Moreover, arsenic trioxide-induced death of K562 cells was sensitive to modulation of catalase [49]. In addition, pivotal role of catalase in the cell survival has been

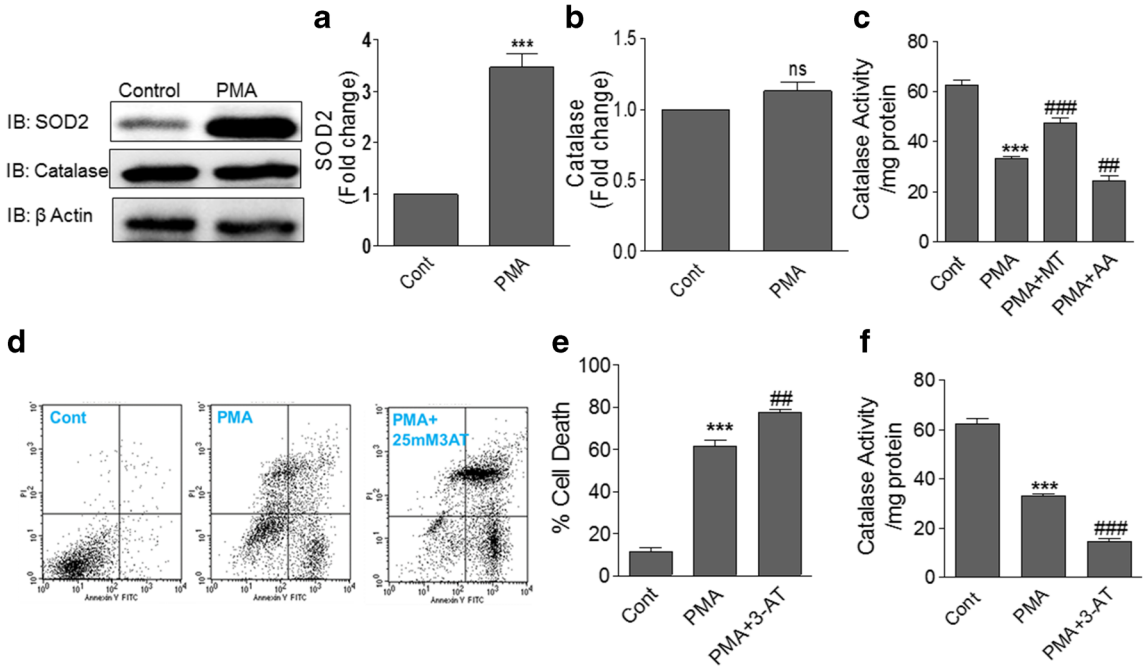

Fig. 3. SOD2, catalase expression and catalase enzymatic activity in the resting and activated neutrophils. **a** SOD2 expression as checked by Western blotting, probed with anti-SOD2 antibody in activated neutrophils. **b** Catalase expression as checked by Western blotting, probed with anti-catalase antibody in activated neutrophils. **c** Human PMNs were pre-treated with MitoTEMPO, antimycin-A and then induced with PMA. Catalase activity was checked in control and treated-neutrophils. Role of catalase in cell survival. **d**, **e** Percent cell death was measured by Annexin V/PI labelling in PMA-activated neutrophils pre-treated with catalase inhibitor 3-aminotriazole. **f** Catalase activity was checked in the control and 3-aminotriazole pre-treated PMA-stimulated neutrophils. Data has been represented as mean ± SEM from minimum three different experiments. $^{***}p < 0.001$ *versus* untreated control neutrophils. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *versus* PMA-treated human neutrophils.

demonstrated in acatalasemic, diabetes mellitus, hypertension and vitiligo patients [50]. In most cell types, antioxidant enzymes work in tandem to protect the cell from oxidative damage [51–54]. The role of catalase in cell survival/ death was suggested by experiments conducted to examine the effect of RBCs on neutrophil activation and cell death [55, 56]. Ajitvarki et al. observed that neutrophil death was protected by RBCs in a co-culture, while inhibition of RBC catalase by 3-aminotriazole partially suppressed the protective effect of RBCs on neutrophil apoptosis. RBCs that are bestowed with high catalase activity would probably account for the decomposition of H_2O_2 release by neutrophils, thereby supressing consequential cell death. This prompted us to investigate the role of endogenous catalase within neutrophils as activated cells generate high amount of H_2O_2 and also exhibit enhanced cell death. Results obtained from the present study showed increased expression of MnSOD (SOD2) that regulate H_2O_2 generation by dismutation of superoxide radicals. Decomposition of H_2O_2 in human neutrophils is regulated by catalase as well as peroxiredoxins. Hyper-oxidation of peroxiredoxins correlated with the augmented level of H_2O_2 in the activated neutrophils. Moreover, catalase activity was also reduced due to the oxidative modification of cysteine residues. Pivotal importance of antioxidants in the prevention of cell death has also been delineated [57–59]. So far, role of H_2O_2 in the death of U937 and HL60 cells has been shown, which was further enhanced in the presence of catalase inhibitor (3-aminotriazole) [60–62]. Further, overexpression of catalase protected HepG2 cells from cell death [63]. Together, these studies support our finding that catalase has an important role in the oxidative stress-mediated cell death.

In addition to NADPH oxidase in human neutrophils, mitochondrial complex III also has a key role in the superoxide generation and is an important regulator of cell death. The purpose as well as abundance of mitochondria in the neutrophils has remained controversial. Initially, it was believed that only a few mitochondria were present in the human neutrophils. Recent studies have, however, found that mitochondria have a unique role in neutrophils as they are not involved in the ATP production and oxidative phosphorylation, rather maintain mitochondrial membrane potential and regulate cell death [17]. Low to moderate level of

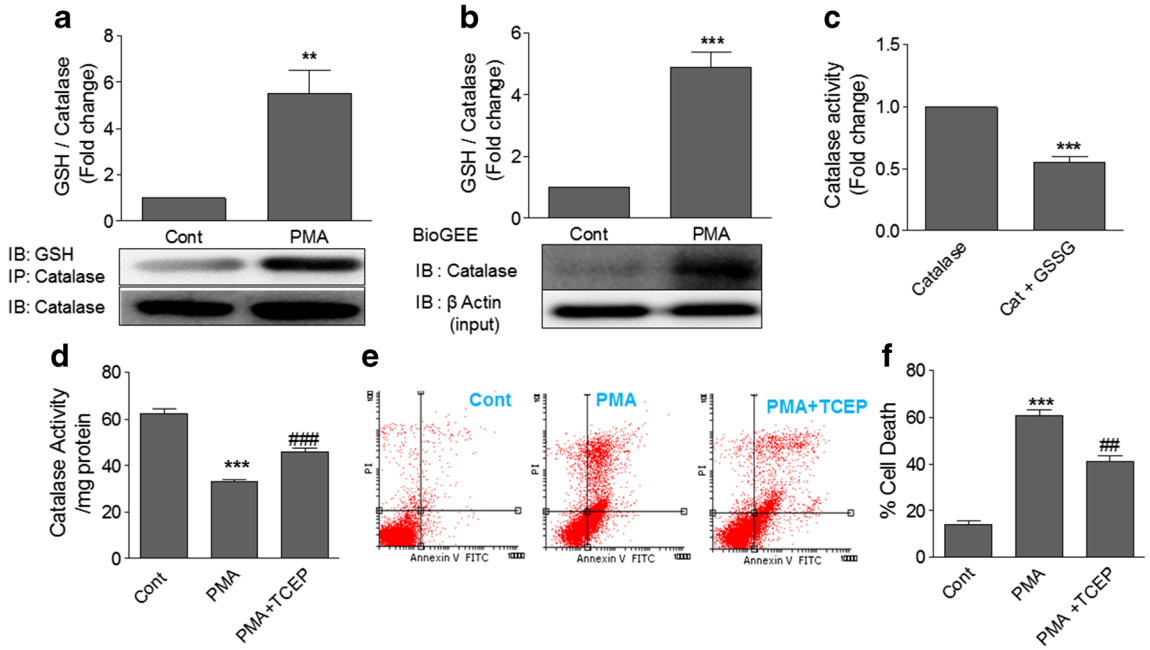

Fig. 4. S-Glutathionylation of catalase and its enzymatic activity. PMA-induced protein S-glutathionylation in human neutrophil lysates was probed with anti-GSH antibody. **a** Neutrophil lysate was immuno-precipitated with catalase antibody and probed with anti-GSH monoclonal antibody. **b** Protein S-glutathionylation also detected by probing with anti-catalase in BioGEE-labelled PMNs stimulated with PMA. **c** Catalase protein was treated with 2 mM GSSG for 1 h at 37 °C and catalase catalytic activity was measured. **d** Catalase activity was measured in TCEP-treated PMA-induced cells. **e**, **f** Cell death was measured by Annexin V/PI labelling in TCEP-treated PMA-induced cells. Data are represented as mean ± SEM from three different experiments. $^{***}p < 0.001$ versus untreated control neutrophils, $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$ versus PMA-treated neutrophils.

mtROS is coupled with cell proliferation and metabolic adaptation during hypoxia and regulates inflammatory response while higher levels have been linked with cell death in many cells types [64]. Impairment of oxidative phosphorylation or any disturbance in the expression profile of antioxidant enzymes can result into overproduction of mitochondrial ROS. Enhanced mtROS production has been linked with instability of genome in chronic myeloid leukaemia stem cells [65]. Mitochondrial O_2 production exhibits positive correlation with oxygen concentration inside the cells [66]. Thus, it is crucial for neutrophil survival, at the inflamed or infected sites, where local O_2 tensions are in general very low. Altogether, negative impact of enhanced mtROS on mitochondrial membrane potential reduction and cell survival is suggested [67]. Neutrophil suspension following activation with PMA or Zymosan for 4 h or 12 h with NO produced large amount of ROS, as monitored by DHE and DCF. Further, mitochondrial ROS was also augmented as demonstrated by MitoSOX; thus, oxidative stress is being contributed both by NOX2 and mitochondria, to impact neutrophil survival. Therefore, we examined the effect of MitoTEMPO, a mitochondrial ROS scavenger, which enhanced neutrophil survival by protecting the loss of mitochondrial membrane potential in activated cells. Similarly, treatment with antimycin-A, which augmented MSR through inhibition of Complex III further enhanced PMN death and also exhibited a loss of mitochondrial membrane potential. Thus, results obtained support the hypothesis that the axis of mitochondrial dysfunction/enhanced ROS production negatively impact neutrophil survival.

Accumulated evidences in the recent past clearly demonstrated that ROS generated from NOX and mitochondria are involved in cell signalling *via* various mechanisms among which reversible oxidation of active cysteine residue of diverse proteins is crucial [68]. Recently we showed the role of NO in neutrophils differentiation [69] and as neutrophils are terminally differentiated cells, post translational modification of protein might be a more efficient way to regulate cellular functions than gene transcription in these short-lived cells. Oxidative stress-mediated S-glutathionylation of several proteins is already documented in neutrophils [41, 70]. Here in the present study, we demonstrated a novel and key role of S-glutathionylated catalase in neutrophil survival.

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AUTHOR'S CONTRIBUTION

S.N performed most of the experiments, and was involved in writing of the manuscript. M.DY, S.S and D.A provide experimental help. T.C provided the buffy coat samples. K.J provided the knock-out mice. S.K was involved in the writing of manuscript and also gave suggestions during the experiments. M.D., the corresponding author, guided, designed and conceptualized the work and was involved in finalising the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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