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ORIGINAL RESEARCH PAPER

Regulation of oxidized LDL-induced inflammatory process through NLRP3 inflammasome activation by the deubiquitinating enzyme BRCC36

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Abstract

Objective Oxidized Low-Density Lipoprotein (oxLDL) is a well-established pro-inflammatory marker that activates the NLRP3 inflammasome. Ubiquitination plays an important role in modulating the stability and functions of various proteins. BRCC36 is a ubiquitin-modifying enzyme that plays a crucial role in protein stabilization and activation in the cytosol, but its role in OxLDL-induced NLRP3 inflammasome activation is not known. Here, we have investigated the role of deubiquitinating enzyme BRCC36 in regulating NLRP3 inflammasome during oxLDL stimulation.

Methods Raw 264.7 murine macrophages were stimulated with oxLDL and effect of BRCC36 deubiquitination activity was assessed by fluorometric assay, and protein expression was assessed by Western blotting. The level of IL-1β measured by ELISA and LDH activity as pyroptotic cell death marker was assessed by fluorometric assay.

Results The results showed that oxLDL increased the level of NLRP3 in macrophages and also the level of active caspase-1 and IL-1β. It also modulated the expression of deubiquitinating enzymes and caused pyroptotic cell death as indicated by LDH release. Inhibiting the proteasomal activity by MG132 and siRNA-mediated silencing of BRCC36 in macrophages potentially suppressed oxLDL-induced NLRP3 inflammasome activation and IL-1β secretion. Furthermore, the inhibition of proteasomal deubiquitinating activity with specific BRCC36 inhibitor G5 also reduced the inflammatory cell death.

Conclusion Taken together, our study suggests that deubiquitinating enzyme BRCC36 inhibition could potentially suppress oxLDL-induced inflammatory process by inhibiting NLRP3 activation and resultant IL-1β secretion.

Keywords Inflammation · Deubiquitination · BRCC36 · NLRP3 inflammasome · oxLDL · Raw264.7 macrophages

Introduction

NLRP3 inflammasome is primarily activated by indigenous particulate matters including uric acid and cholesterol crystals. Oxidized low-density lipoproteins (OxLDL) has also been implicated in NLRP3 inflammasome activation. Studies have shown that OxLDL-induced secretion of IL-1β in macrophages was involved in foam cells formation, which occurred through NLRP3 inflammasome activation [1]. Our earlier findings have also implicated activation of NLRP3 by oxLDL in monocyte-derived macrophages [2]. NLRP3 inflammasome has a principal role in host defense, and its deregulation causes various inflammatory diseases, including gout, asthma, neurodegenerative diseases, type-2 diabetes, and atherosclerosis [3]. Upon detecting the stress signals, NLRP3 proteins engage procaspase-1 through a C-terminal caspase recruitment domain (CARD) and form a complex proteins assembly called inflammasome [4]. Along with being crucial pathogen remover, excessive inflammasome activation also contributes to many allergic, autoimmune, and inflammatory diseases. Recent studies have extensively investigated NLRP3 inflammasome in various inflammatory diseases; however, little is known about its regulatory mechanisms in macrophages, especially the endogenous mechanisms that stabilize and regulate the NLRP3 proteins and increase their half-life, which is crucial for its activation by oxLDL.

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Several researchers have indicated the independence of NLRP3 inflammasome priming from requirement of new protein synthesis and propose an essential role of ubiquitination in NLRP3 inflammasome activation [4–7]. Ubiquitination principally occurs at lysine residues of substrate proteins, which interacts with C-terminal glycine of incoming ubiquitin creating an isopeptide bond. Ubiquitin itself consists of seven internal lysine residues at locations 6, 11, 27, 29, 33, 48, and 63 that interact with incoming monomeric ubiquitin and form consecutive isopeptide bonds that allow for chains of ubiquitin to be formed. [8]. The most studied ubiquitin linkage types involve lysine 48 (K48) and lysine 63 (K63). K48 linkage targets proteins to the proteasome that explicitly binds with inter-ubiquitin linkages formed by K48 and subsequently degrades them. On the contrary, K63 linkages have been significantly involved in several non-proteolytic-signaling processes [9].

BRCC36, initially reported as BRCA1/BRCA2 containing complex subunit 36, is equipped with deubiquitinating (DUB) action for K63-linked ubiquitin polymers. DUB's enzyme activity of BRCC36 depends on zinc-dependent JAMM/MPN$^+$ domain [10]. DUBs are protein-degrading enzymes that facilitate the elimination or processing of ubiquitins, or ubiquitin-linked proteins by cleaving the isopeptide bonds. Through this process, the half-life of cellular proteins is increased, and molecular signaling is regulated.

DUBs have been lately identified as a critical regulator of multiple cellular events including transcription, protein stability, cell proliferation, and cell death. We hypothesized that DUBs, such as BRCC36, upon activation by oxLDL, a risk factor for many inflammatory diseases, may remove the ubiquitin from target proteins, e.g., NLRP3, leading to enhanced activation of inflammasome and subsequent promotion of inflammation. We report here for the first time that oxLDL-induced NLRP3 inflammasome stabilization and activation occurs due to modulation by BRCC36 activity in murine macrophages.

Materials and methods

Cell culture and stimulation

Mouse macrophages (Raw264.7 cells) were procured from National Centre for Cell Sciences (NCCS), Pune, India. The macrophages were cultured in DMEM containing high glucose medium added with 10% fetal bovine serum (FBS) and 1% antibiotics (p/s) concoction at 37 °C in a humid environment with 5% CO_2. Before stimulation with oxLDL to carry out various experiments, cells were starved with 0.5% FBS containing medium for overnight. In some experiment, PBS and DMSO were used as vehicle control.

Reagents and antibody

FBS and penicillin/streptomycin antibiotic mixture procured from Gibco Life Technologies (Grand Island, NY, USA), BRCC36 mAb, Anti-Ubiquitin (K48 linkage-specific) and (K63 linkage-specific) mAb and G5 Ubiquitin Isopeptidase Inhibitor I from Abcam (Cambridge, United Kingdom), Actin mAb from BD (Franklin Lakes, NJ, USA); NLRP3/NALP3 mAb and Caspase-1 (p20) from AdipoGen Life Sciences (Liestal, Switzerland) Recombinant Human Ubiquitin AFC C-terminal Derivative from Boston Biochem, Inc. (Cambridge, USA), *N*-Acetyl-L-cysteine, Lipopolysaccharides (LPS), Adenosine triphosphate (ATP) and MG-132, supplied by Sigma-Aldrich (St. Louis, MO, USA), Protein G Plus/Protein A Agarose Suspension from Merck Millipore (Burlington, MS, USA), native LDL was supplied by Prospec—Tany Techno Gene Ltd (Israel). Additional chemicals of analytical grade were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd. (Mumbai, MH, India).

LDL Oxidation

Native LDL (5 mg) was dissolved in 1 mL PBS, and subsequently, 1 mL PBS containing 10 μM $CuSO_4$ was added. The mixture was transferred to a 35 mm sterile plastic culture dish and incubate for up to 48 h at 37 °C. After completion of incubation, the oxidized LDL was dialyzed against 12 L of PBS for 48 h at 4 °C [11]. The level of LDL oxidation in different batches was measured through FTIR and found consistent. Protein concentration was also measured through the Bradford assay.

ELISA

The media were collected from different experiments, stored at − 80 °C until used for ELISA. The amount of IL-1β secretion in the culture medium was determined using a mouse-specific ELISA kit according to the manufacturer's protocol (RayBiotech, GA, USA). Colour intensity at the end of ELISA was measured as absorbance at 450 nm with H1 synergy multimode reader (Winooski, VT, USA).

LDH release assay

The activity of LDH in the cell-culture medium was determined by LDH cytotoxicity detection kit in accordance with the manufacturer's protocol (Cayman Chemical, Ann Arbor, Michigan, USA). Briefly, media supernatant was collected from different experiments, centrifuged, and 100 μL of supernatant was mixed with 100 μL of LDH reaction solution and incubated at room temperature for 30 min at 37 °C.

The amount of LDH in the medium was measured at 490 nm by H1 synergy multimode reader. The cells lysed with 1% Triton X-100 taken as positive control for LDH determination, and plane media were used as a blank. Optical density of blank was subtracted from experimental samples and positive control.

siRNA-mediated gene silencing

For siRNA transfection of Raw 264.7 macrophages manufacturer's instructions (QIAGEN, Hilden, Germany) were followed. Briefly, 6×10^4 cells/wells in 24 well plates, in 100 µL of complete media, were incubated under normal growth conditions. Subsequently, siRNA or scrambled siRNA (25 nM) was added to HiPerfect transfection reagent (3 µL) in 100 µL serum-free media and dropped onto the confluent cells while swirling. After 6 h, 400 µL culture medium was added to the cells. Under normal growth conditions, the cells were incubated with transfection complexes for 24 h, and gene silencing was determined by Western blotting.

Immunoprecipitation

To study the interaction between BRCC36 and NLRP3, cell lysate was prepared in NP-40 buffer containing protease inhibitor cocktail. For immunoprecipitation of NLRP3 to study its ubiquitination, cells lysis was done using RIPA buffer. One µg of antibodies was added to 1 mL of cell lysate containing 500 µg proteins and incubated on ice for 4 h. Afterward, 20 µL of Protein A/G beads were added to 1 mL of the antibody–antigen mixture and gently rocked on a rocking platform for 1 h at 4 °C. The beads were collected by centrifugation at 10,000×*g* for 15 s at 4 °C, and the immune complexes were washed six times using wash buffer to remove the nonspecific binding. The captured antigens were eluted using SDS elution buffer and used for immunoblotting.

Immunoblotting

At the end of experiment, the cells were rinsed with cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors cocktails. Protein estimation was done by Bradford method, and 25 µg of proteins were loaded on 10% SDS–polyacrylamide gel and electrophoresed. Proteins were transferred onto PVDF membrane using semidry method and incubated for 1 h in blocking buffer at RT. The membrane was washed and incubated with specific primary antibodies for overnight at 4 °C followed by washing and incubation with appropriate HRP-linked secondary antibodies for 2 h at RT. The bands were visualised by ECL detection system and developed using X-ray film. The protein bands were scanned using a high-resolution scanner and densitometry was done with Alpha imager (San Jose, CA, USA).

ROS detection

Raw 264.7 cells were starved and treated with 10 µM CM-DCFDA dye for 1 h. The cells were rinsed with Krebs–ringer bicarbonate solution to remove any traces of fluorescent dye and treated with the stimulants. The H1 synergy multimode reader was used to determine the fluorescence intensity of DCF to quantify ROS generation at different timepoints with excitation and emission wavelengths of 485 and 535 nm, respectively.

Statistics

The experimental data, represented as mean ± SD, were analyzed using one-way analysis of variance (nonparametric) followed by Dunnett's test (using graph-pad prism software version 5). To determine the statistical significance among different groups, student's *t* test was used. *P* value at 0.05 or less was statistically significant.

Results

OxLDL stimulates NLRP3 inflammasome via BRCC36 activation and promotes pyroptosis

First of all, to examine the effect of oxLDL on inflammasome activation, we stimulated the murine macrophages, Raw 264.7 cells, with oxLDL for 6 h. As shown in Fig. 1, oxLDL significantly stimulated the release of IL-1β (84.44 ± 7.6 pg/ml) in the media, which was 60 ± 2.2 pg/ml in control, an increase of 1.4-fold, whereas the addition of ATP after oxLDL increased IL-1β secretion to 87.04 ± 7.22 pg/ml. ATP alone also increased IL-1β secretion nonsignificant to 74.81 ± 1.7 pg/ml, an increase of 1.24-fold compared to control (Fig. 1a). As there was no significant difference between oxLDL alone, and activation with ATP, for further studies, ATP was excluded. LPS-induced Raw 264.7 cells were used as a positive control and showed a significant increase (88.5 ± 7.1 pg/ml; 1.45-fold) in IL-1β level. Furthermore, we performed LDH release assay, a pyroptotic cell death marker and found that oxLDL increased LDH level indicating increased cell death by 31.9 ± 0.5 (in percent) compared to control (7.7 ± 0.2), which was an increase of nearly 4-fold (Fig. 1b).

Since NLRP3 activation is an early event upon sensing the danger signal and independent of transcription, we hypothesized that it is critically regulated by deubiquitinating enzymes [5, 12]. Thus, we next analyzed the level of DUB enzyme BRCC36 and components of NLRP3

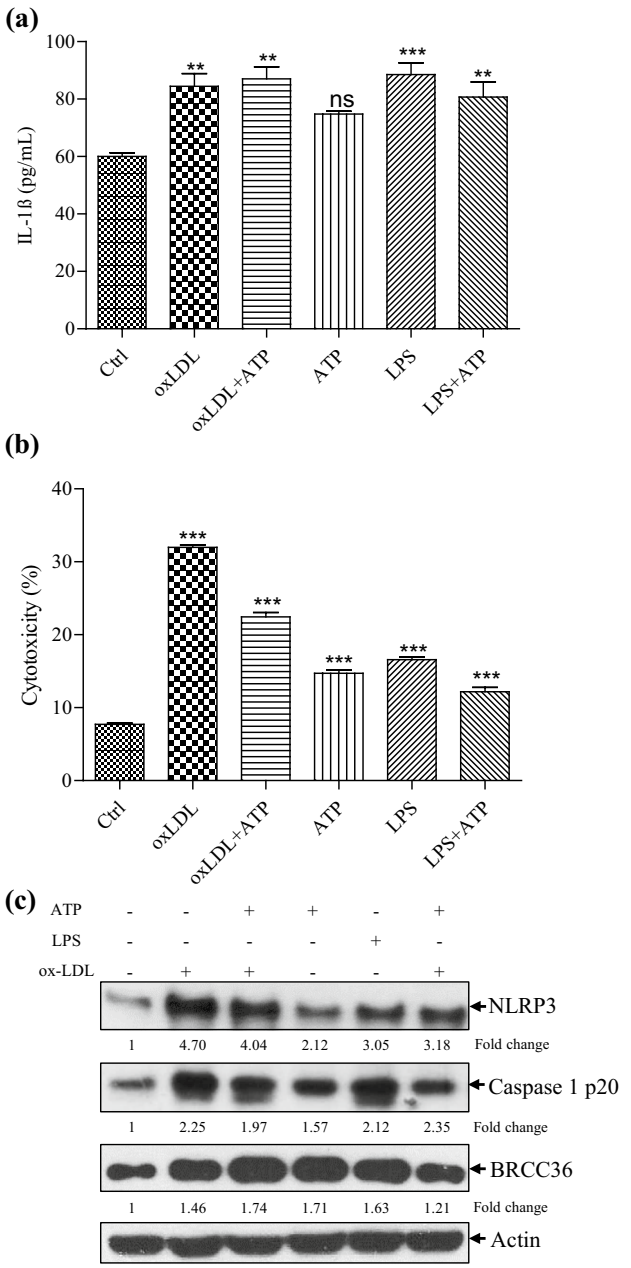

Fig. 1 Raw 264.7 macrophages (~3×10^5) were treated with oxLDL or LPS without or with ATP for detection of inflammatory response. The level of IL-1β through ELISA (**a**) and pyroptotic cell death by LDH release assay (**b**) were assessed. Bars represent average ± SD ($n=3$), $^{**}p<0.001$ and $^{***}p<0.0001$ vs. control. Relative expression of inflammatory markers and BRCC36 was determined by immunoblotting are shown (**c**) ($n=3$)

inflammasome during oxLDL stimulation. There was 1.46-fold increase in the expression of BRCC36 when stimulated with oxLDL compared to control; similarly, NLRP3 proteins increased by 4.7-fold and cleaved caspase-1 expression also increases by 2.25-fold when treated with oxLDL (Fig. 1c).

Proteasome inhibitor MG132 modulates oxLDL-induced inflammation

Since ubiquitin–proteasome system is essential in the stability, activity, and functioning of proteins [13], we next investigated whether inhibition of proteasome by MG132, a well-known proteasomal inhibitor, could regulate BRCC36-mediated inflammatory marker in oxLDL-treated macrophages. Our results show that MG132 inhibited oxLDL-stimulated IL-1β secretion and decreased it from 84.44 ± 7.6 pg/ml to 65.2 ± 2.7 pg/ml, a significant decrease of 0.77-fold (Fig. 2a). However, pre-treatment with MG132 alone did not cause any change compared to control. Interestingly, LDH release as indicated by cytotoxicity was augmented by MG132 and increased from 7.97 ± 0.5 to 35.9 ± 1.1, nearly 4.5-fold compared to control suggesting its toxic effect; however, LDH level induced increased cytotoxicity from 32.45 ± 1 to 63.82 ± 4.78, a change in 1.96-fold when MG132 was given along with oxLDL compared to oxLDL treated group (Fig. 2b). Furthermore, we studied the impact of MG132 on inflammasome proteins and BRCC36 by immunoblotting. The results show that the pre-treatment of MG132 followed by oxLDL stimulation decreased NLRP3 and cleaved caspase-1 to nearly 3- and 0.98-fold, which was 4.65- and 2.3-fold in oxLDL alone induced groups, respectively. Similarly, the level of BRCC36 was also brought down to 1.3-fold by MG132 in oxLDL-treated cells from 1.6-fold in oxLDL alone treated cells. No significant change in BRCC36 level with MG132 alone was observed (Fig. 2c).

Isopeptidase inhibitor G5 modulates oxLDL-induced inflammation

Next, since BRCC36 deubiquitinates and removes ubiquitins through its isopeptidase activity, we inhibited the isopeptidase activity of BRCC36 using a specific inhibitor G5 and examined its effect on NLRP3 accumulation/activation. As shown in Fig. 3a, G5 significantly reduced oxLDL-induced IL-1β secretion to 0.76-fold from 81.85 ± 10.68 to 62.96 ± 3.394; however, G5 alone did not cause any significant changes. G5 also caused significant decrease in oxLDL-induced pyroptotic cell death from 32.75 ± 1 to 15.59 ± 0.94, a decrease of nearly 0.47-fold; however, pre-treatment of G5 alone did not cause any change compared to control (Fig. 3b). Furthermore, the effect of G5 on inflammasome proteins and BRCC36 by immunoblotting found that G5 also reduced the expression of NLRP3 inflammasome to 1.2-fold compared to 4.5-fold in oxLDL-stimulated group. The level of cleaved caspase-1 also decreased from 1.94-fold in oxLDL group to 1.6-fold in G5 pre-treated cells followed by oxLDL stimulation, decreased to nearly 0.82-fold; and BRCC36 level decreased from 1.45-fold in oxLDL group to

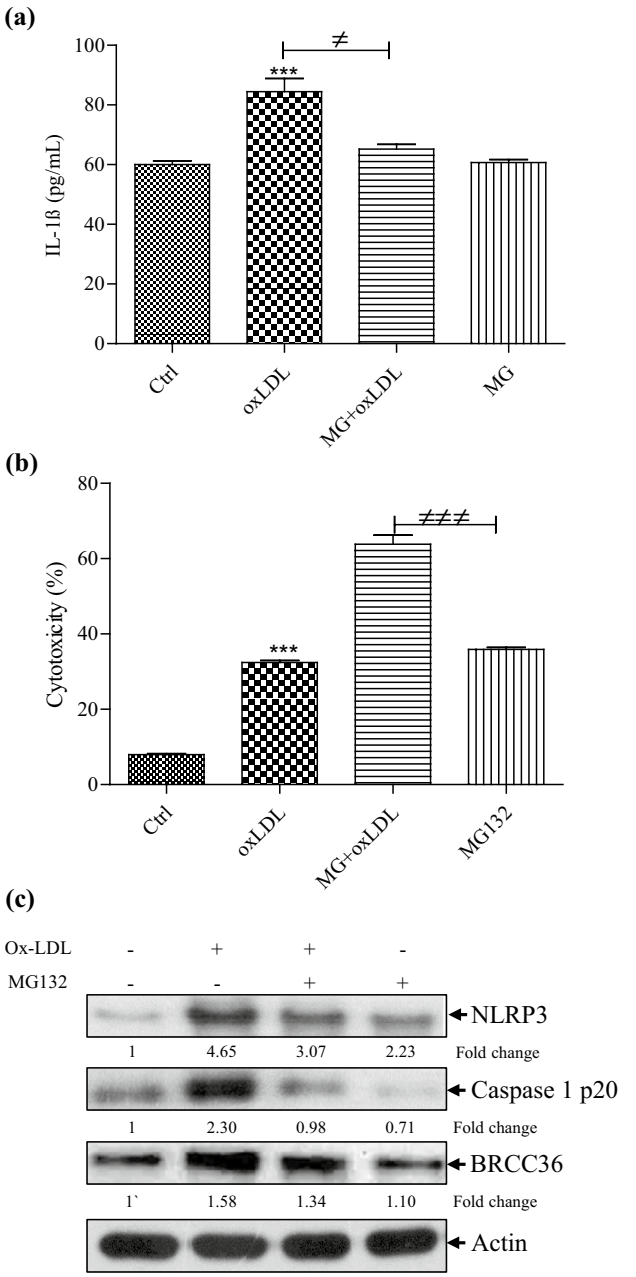

Fig. 2 Raw 264.7 macrophages (~3×10^5) were treated with oxLDL without or with 2 μM MG132 prior to treatment of oxLDL for the detection of inflammatory response. The level of IL-1β through ELISA (**a**), and pyroptotic cell death by LDH release assay (**b**) were assessed. Bars represent average ± SD ($n=3$), $^{*}p<0.01$, $^{***}p<0.001$ oxLDL + MG132 vs. MG132. Relative expression of inflammatory markers and BRCC36 determined by immunoblotting are shown (**c**), ($n=3$)

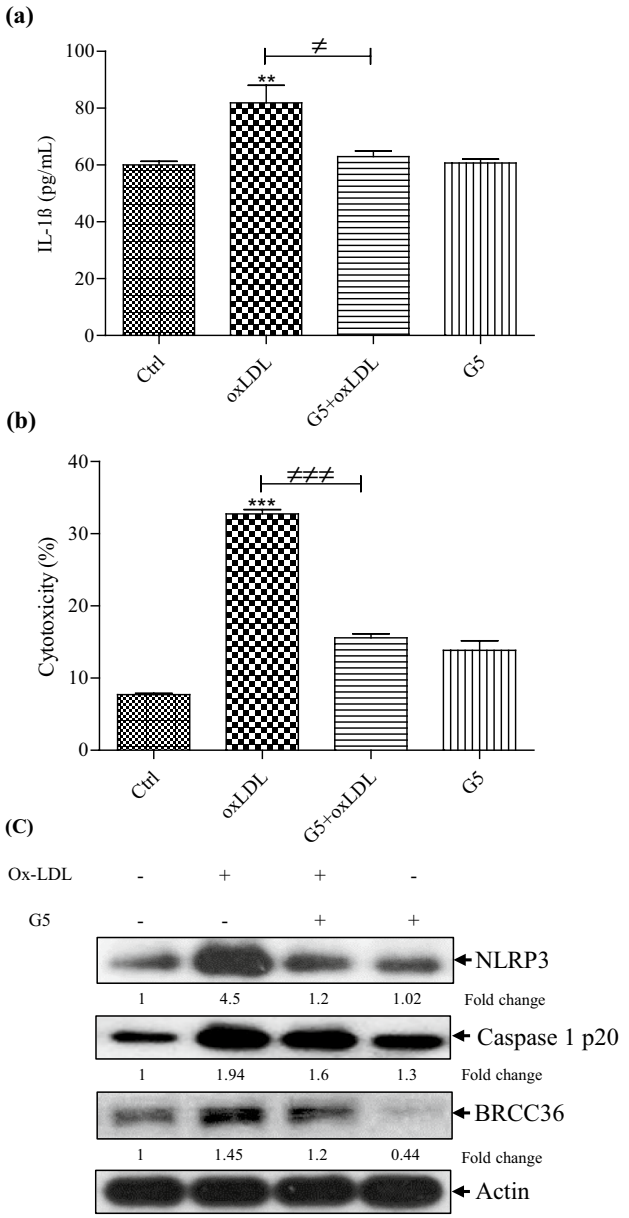

Fig. 3 Raw 264.7 macrophages (~3×10^5) were treated with oxLDL without or with 2 μM G5 prior to treatment of oxLDL for the detection of inflammatory response. The level of IL-1β through ELISA (**a**), and pyroptotic cell death by LDH release assay (**b**) were assessed. Bars represent average ± SD ($n=3$), $^{*}p<0.01$, $^{***}p<0.001$ oxLDL + G5 vs. G5. Relative expression of inflammatory markers and BRCC36 determined by immunoblotting are shown (**c**), ($n=3$)

1.2-fold in G5 pre-treated cells followed by oxLDL stimulation, decreased to nearly 0.82-fold. G5 alone maintained the levels of NLRP3, cleaved caspase-1 near to control and decreased the BRCC36 level to 0.44-fold compared to control. (Fig. 3c).

G5 inhibited in vitro deubiquitinating activity of BRCC36

Next, we measured the effect of isopeptidase inhibitor G5 on the deubiquitinating activity of BRCC36. The cells were induced with or without G5 for 3 h, followed by stimulation with oxLDL for 6 h. The immunoprecipitated BRCC36 was incubated with a substrate Ub-AFC, which

emitted fluorescence upon cleavage of isopeptide bond indicating deubiquitination activity of BRCC36. In the cells treated with oxLDL, the deubiquitinating activity increased significantly at different timepoints (—■—) and isopeptidase inhibitor G5, when given prior to oxLDL stimulation, significantly reduced DUB activity and brought down below control values at all timepoints (—▲—). G5 alone also decreased the overall deubiquitinating activity of BRCC36 (Fig. 4).

Proteasomal- and deubiquitination-dependent regulation of NLRP3 inflammasome activation

To confirm how BRCC36 affects NLRP3 activation in Raw264.7 macrophages, we silenced BRCC36 using siRNA and subsequently examined the levels of inflammatory proteins in media and cell lysate. The siRNA inhibition of BRCC36 inhibited oxLDL-induced IL-1β secretion by 0.76-fold from 81.85 ± 10.7 to 62.22 ± 5 in oxLDL-treated un-transfected cells, which was similar to untreated control (Fig. 5a). BRCC36 siRNA silencing also reduced oxLDL-induced pyroptotic cell death from 32.75 ± 1 to 18.5 ± 0.38 by nearly 0.56-fold (Fig. 5b). The immunoblotting showed that BRCC36 silencing decreased oxLDL-induced NLRP3 to 0.8-fold compared to un-transfected cells. Cleaved caspase-1 level also reduced to 0.4-fold in BRCC36 silenced and oxLDL-stimulated cells compared to un-transfected normal cells (Fig. 5c). Next, we investigated the effect of BRCC36 silencing on proteasomal and deubiquitination activity upon oxLDL induction. In BRCC36 silenced cells, oxLDL stimulation had reduced proteasomal and deubiquitination activity compared to oxLDL-induced normal cells, which in turn reduced the NLRP3 level by 0.79-fold upon induction with oxLDL (Fig. 5d).

OxLDL modulates the ubiquitination pattern of NLRP3 inflammasome

BRCC36 acts in K63-linked deubiquitination, which is linked with protein stabilization [14]. Thus, we further investigated the ubiquitination pattern of NLRP3 protein by subjecting immunoprecipitated NLRP3 proteins to immunoblotting using antibodies against K48 and K63 specific ubiquitinated peptide. As shown in Fig. 6a, unstimulated cells showed more ubiquitinated NLRP3, and oxLDL stimulation resulted in the removal of K48 linked ubiquitin as indicated by faint bands. Proteasome inhibitor MG132 decreased ubiquitination-mediated protein degradation and resulted in accumulation of NLRP3 ubiquitination moiety. BRCC36 silencing did not show any significant effect on K48 linked ubiquitin pattern of NLRP3 (Fig. 6a). When we used K63 ubiquitination-specific antibodies, it was found that stimulation with oxLDL removed K63 ubiquitination from NLRP3, probably through BRCC36 deubiquitinating activity, leading to its accumulation. However, BRCC36 silencing restored K63 ubiquitination level of NLRP3 to the normal level, i.e., near control. BRCC36 silenced cells did not show any effect of MG132 treatment on K63 pattern of NLRP3 (Fig. 6b). Our findings suggest that K63-linked ubiquitination could be important in activation of NLRP3 protein.

BRCC36 restricts NLRP3 inflammasome activation independently of ROS pathway

To examine the effect of oxLDL in ROS generation, Raw 264.7 macrophages were treated with 100 µg/mL of oxLDL for 0, 3, 6, 12, and 24 h and ROS generation was measured. There was increase in ROS generation of 1.5-fold (3 h), 2.08-fold (6 h), 2.54-fold (12 h), and 4.76-fold (24 h) compared to 0 h untreated control cells (Fig. 7a). NAC was used as a negative control, and H_2O_2 was used a positive control. Furthermore, to assess whether BRCC36 regulation

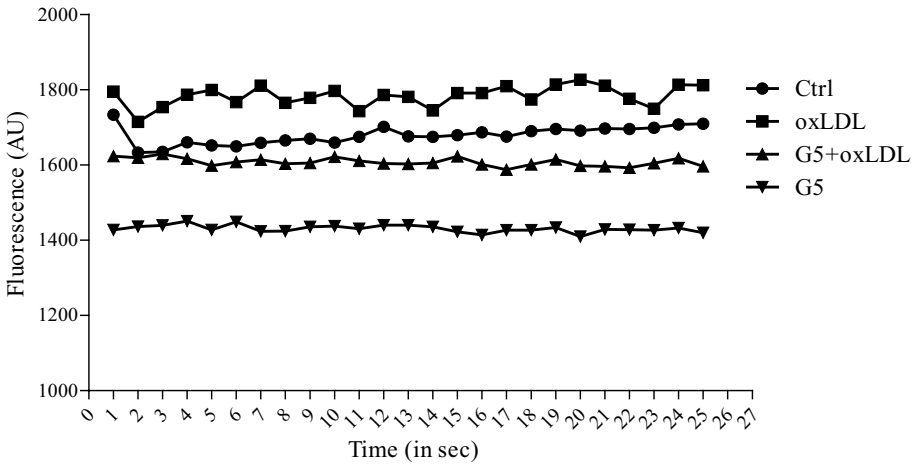

Fig. 4 Raw 264.7 cells (~8×10^5) in 60 mm dish were serum-starved and pre-incubated with 2 µM G5 isopeptidase inhibitor for 3 h followed by oxLDL (100 µg/mL) stimulation. After completion of the experiment, cells were lysed and subjected to immunoprecipitation using BRCC36 monoclonal antibodies. The purified BRCC36 proteins from different groups were used for fluorometric assessment of deubiquitinating activity at different timepoints with Ub-AFC as substrate

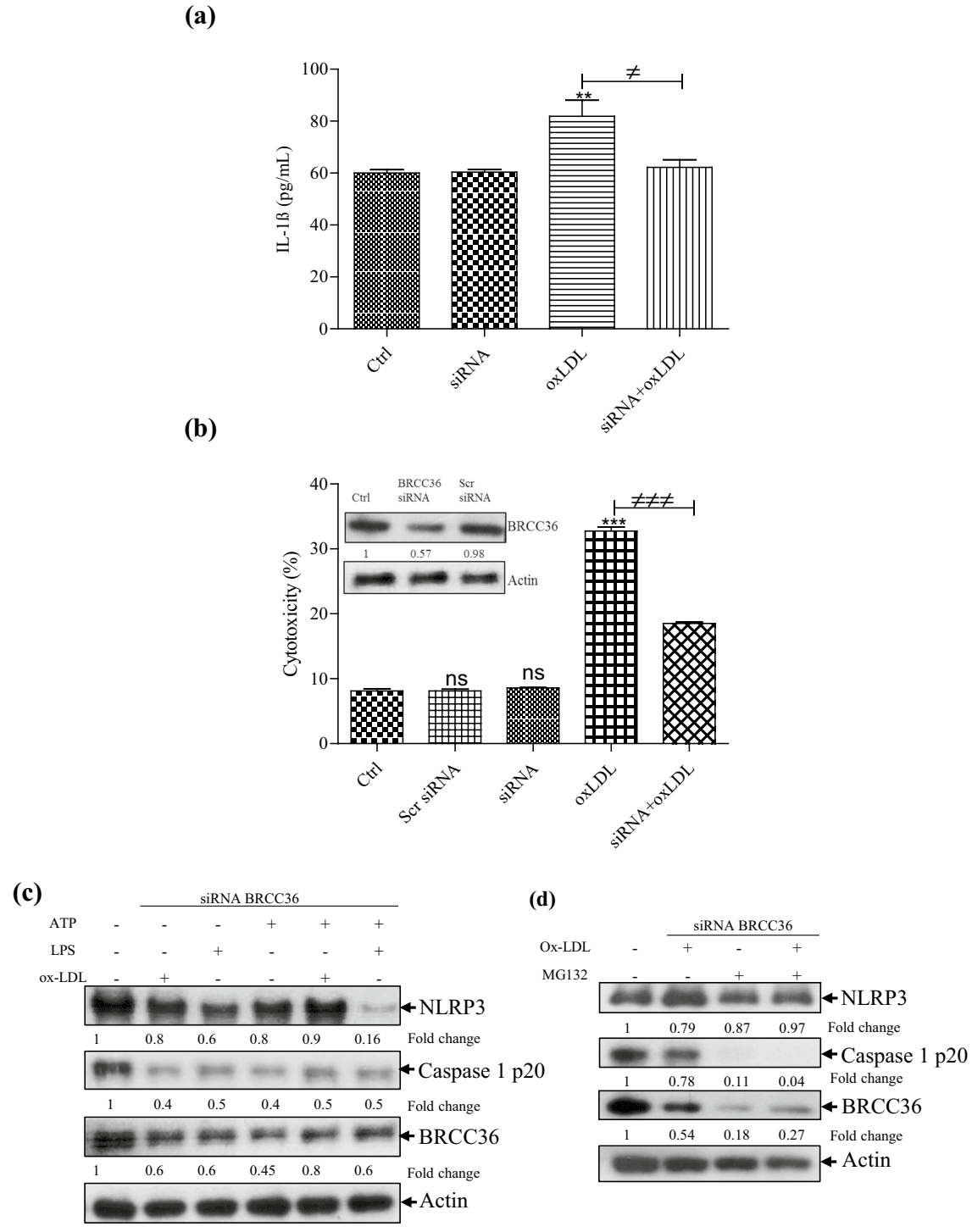

Fig. 5 Raw 264.7 cells without or with siRNA silencing were treated with oxLDL for 6 h and detection of IL-1β through ELISA (**a**), and pyroptotic cell death by LDH release assay (**b**) were performed. The inset image in **b** shows the level of BRCC36 in siRNA silenced cells along with control and scrambled siRNA cells. $^{*}p<0.01$, $^{***}p<0.001$ BRCC36 siRNA + oxLDL vs. BRCC36 siRNA. Bars represent average ± SD ($n=3$). Relative expression of the various inflammatory markers and deubiquitinating enzyme BRCC36 was determined by immunoblotting in siRNA silenced BRCC36 cells (**c**) and in BRCC36 silenced cells treated with or without MG132 (**d**). ($n=3$)

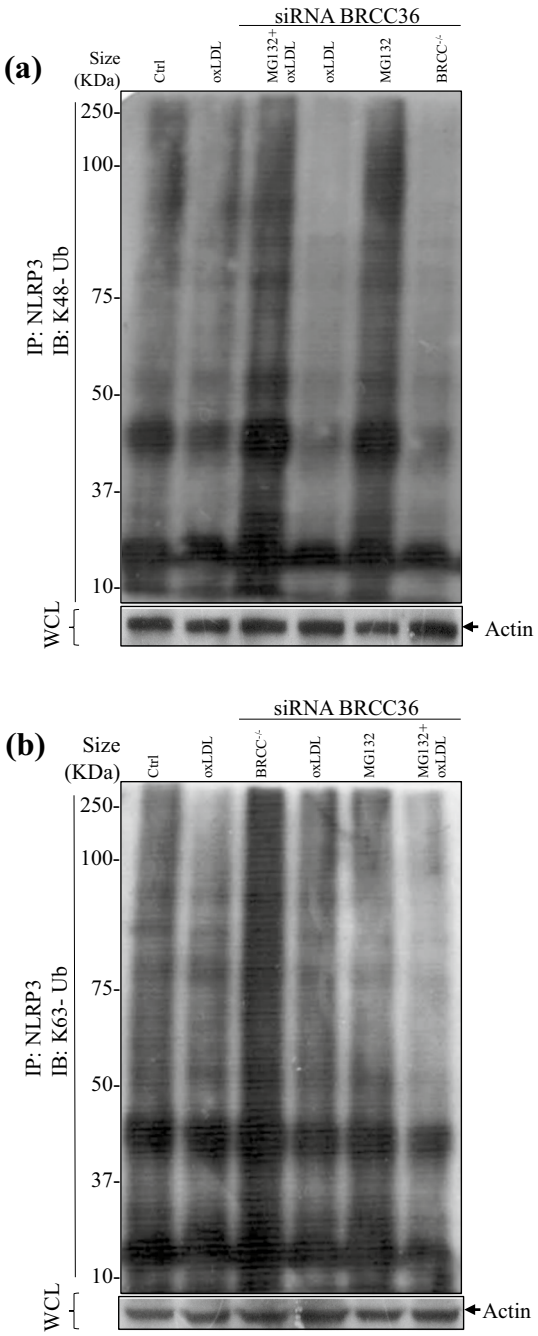

Fig. 6 Raw 264.7 cells (~2.2×10^6) seeded in 100 mm dish followed by overnight starvation and stimulated with MG132 for 3 h followed by oxLDL for 6 h. After completion of treatment, cells were lysed and immunoprecipitated using NLRP3 antibodies. The isolated protein was subjected to immunoblotting using K48 specific (**a**) and K63 specific (**b**) antibodies to determine the level of specific ubiquitination. ($n=3$)

is dependent upon ROS, we pre-treated the cells with ROS quencher NAC and assessed IL-1β secretion. We found that pre-treatment with NAC inhibited oxLDL-stimulated IL-1β secretion by 0.78-fold (Fig. 7b), and it also significantly decreased the level of BRCC36 expression by 0.77-fold (Fig. 7c).

BRCC36 physically interacts with NLRP3 for its DUB activity

To perform deubiquitination on NLRP3 proteins, BRCC36 is anticipated to have protein–protein interaction with NLRP3. To test this possibility, we immunoprecipitated NLRP3 protein after 6 h of oxLDL stimulation and performed immunoblotting using BRCC36 antibodies. As shown in Fig. 8, immunoprecipitated NLRP3 pulled down BRCC36 protein with it, and the level of BRCC36 was more in oxLDL-treated group suggesting an interaction between NLRP3 and BRCC36 upon oxLDL stimulation. LPS as a positive and non-immunized mouse serum as a negative control used in this study (Fig. 8). Unexpectedly, this experiments also revealed NLRP3 association with BRCC36 in unstimulated cells.

Discussion

OxLDL is known to activate inflammasome and implicated in atherosclerosis and diabetes as this endogenous soluble ligand is intra-lysosomally converted into insoluble crystals [15]. It plays a crucial inflammatory role in atherosclerotic plaque formation. OxLDL exhibits epitopes which share similarity with phosphorylcholine residue found in *Streptococcus pneumoniae* [16]. These danger-associated molecular patterns (DAMP) are recognized by different receptors present on immune cells and consequently activate NLRP3 inflammasome that leads to secretion of IL-1β and results in various inflammatory pathology [17].

NLRP3 inflammasome expression is a rate-limiting step for inflammatory activity [18]. Thus, its expression must be tightly controlled to sustain immune homeostasis and avoid detrimental effects. To help with maintaining a basal level, cells have ubiquitination-mediated degradation of proteins which determines their half-life in the cell. Ubiquitination of protein mainly occurs at one of the seven lysine residues or N-terminal methionine of previous ubiquitin molecules. The ubiquitin linkage at K48 residue marks the proteins for the proteasomal degradation, while K63 ubiquitin linkages have been implicated in several non-proteolytic-signaling processes [9].

The mechanisms that control NLRP3 activation in oxLDL-induced diseases are not well understood. The scientists are working on the ubiquitin–proteasome system to understand these molecular systems [19]. Here, we show importance of BRCC36 deubiquitinating activity in NLRP3 activation and promotion of inflammation and cell death mechanisms. Our results show that the inhibition of K63

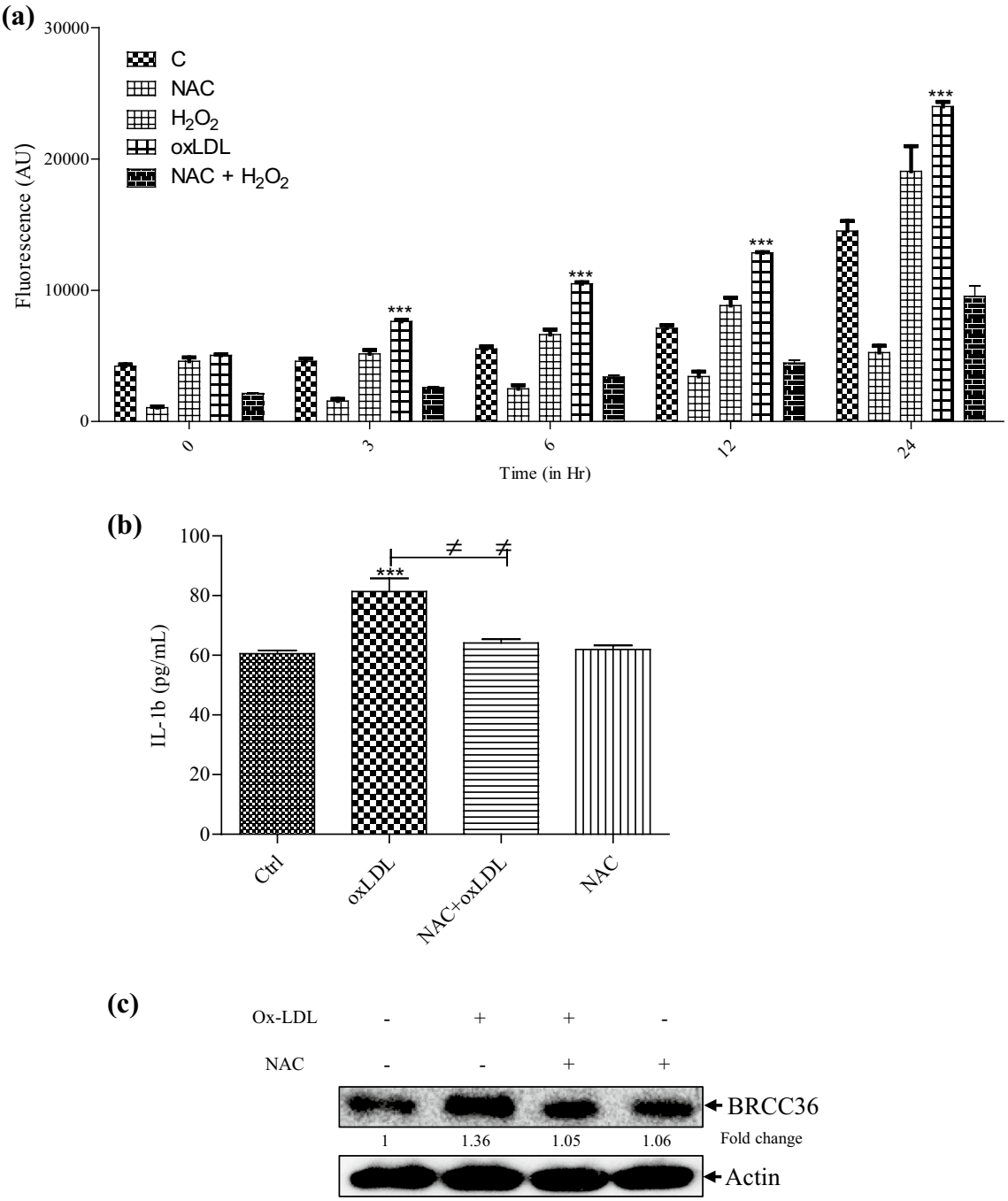

Fig. 7 Raw 264.7 cells (1×10^4) were seeded in 96-well plate followed by overnight serum starvation. Raw 264.7 cells were pre-treated with of NAC (5 mM) for 3 h and induced with H_2O_2 (1 mM) or oxLDL (100 µg/mL) and DCF fluorescence was measured at different timepoints. Bars represent average ± SD ($n=3$), $^{***}p<0.0001$ vs. control (**a**). Raw 264.7 cells (3×10^5) were seeded in 6-well plate followed by overnight serum starvation. Cells were pre-treated with of NAC (5 mM) for 3 h and induced with oxLDL (100 µg/mL) for 6 h for the detection of IL-1β through ELISA. Bars represent average ± SD ($n=3$), $^{***}p<0.0001$ vs. control, $^{\neq\neq}p<0.001$ (**b**). In similar condition, cell lysates were prepared for immunoblotting of BRCC36, and beta-actin was used as a loading control (**c**)

deubiquitinating activity of BRCC36 enzyme could limit NLRP3 inflammasome activity in the presence of inflammatory molecules such as oxLDL, and decrease the resultant inflammation.

Initially, to examine NLRP3 inflammasome activation in our experimental model, the cells were stimulated with oxLDL as a priming signal and ATP as an activating signal. We found that oxLDL alone could induce IL-1β secretion,

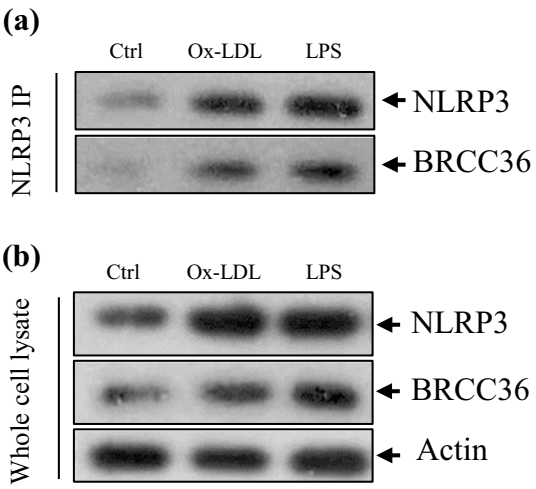

Fig. 8 Raw 264.7 cells (8×10^5) were plated in 60 mm dish followed by serum starvation and stimulation with oxLDL (100 µg/mL) or 1 µg/mL of LPS for 6 h. The cell lysates were subjected to immunoprecipitation using NLRP3 antibodies followed by immunoblotting (**a**), or whole-cell lysates were subjected to immunoblotting (**b**). The representative images from both experiments are shown ($n=3$)

and activation signal of ATP was not causing significant difference (Fig. 1a). Furthermore, we observed that oxLDL exposure resulted in increased cell death (Fig. 1b). The cell death caused by oxLDL was pyroptosis in nature as indicated by LDH release and accompanied by inflammation as evident by increased inflammasome activation and secretion of IL-1β. Although deubiquitinating enzymes have been implicated in the stabilization and activation of various proteins, the exact mechanism and its involvement in NLRP3 activation is not precisely clear. Thus, to understand this, we examined a deubiquitinating enzyme BRCC36, which is implicated in deubiquitination and activation of many proteins.

BRCC36 is a member of the JAMM/MPN family of zinc metalloproteases that precisely act on K63-linked polyubiquitin chains and cleave it [14]. Originally, BRCC36 was discovered as a part of BRCA-1A macromolecular complex, where its activity contributes to DNA repair [20]. Its deubiquitinating activities are controlled by two scaffold proteins localized in cytosol and nucleus [21]. Its higher order assembly with KIAA0157, that makes dimer and interact with target proteins SHMT2 and RAP80, is necessary for deubiquitinase action and biological function [22]. It is evident that K63-specific deubiquitinating activity of BRCC36 was co-fractionated with the 19S subunit of the 26S proteasome, the COP9 signalosome (CSN) complex [14]. We thus examined the role of proteasomal activity in NLRP3 inflammasome activation in oxLDL-treated macrophages. We found that a general proteasome inhibitor MG132 inhibited NLRP3 inflammasome activation and IL-1β secretion (Fig. 2a, c). However, it also caused toxicity to cells and led to enhanced cell death (Fig. 2b). It is likely that it also induces various cell death receptors via different pathways as reported earlier [23, 24].

Since BRCC36 is a part of BRISC (BRCC36 isopeptidase) complex [25], next, we inhibited the isopeptidase activity through cell-permeable synthetic compound G5. We found that G5 significantly inhibited oxLDL-induced BRCC36 at protein level (Fig. 3c) and activity level (Fig. 4). We further assessed NLRP3 inflammasome assembly and found that G5 not only inhibited NLRP3 inflammasome-induced Caspase-1 activation and IL-1β secretion, but also prevented oxLDL inflammatory cell death, i.e., pyroptosis (Fig. 3).

Next, to ascertain whether the oxLDL-induced increase in NLRP3 inflammasome activity is through the BRCC36, we knocked down BRCC36 by siRNA transfection. BRCC36 knockdown in macrophages showed a significantly low level of NLRP3 inflammasome, caspase-1 activation, and IL-1β secretion (Fig. 5a, c) suggesting the role of BRCC36. The siRNA-mediated silencing of BRCC36 also inhibited oxLDL-induced pyroptotic cell death. These results confirmed that BRCC36 could be an essential player for NLRP3 inflammasome activation in the presence of stressor molecule such as oxLDL. Furthermore, the inhibition of proteasome activity and deubiquitinating enzyme BRCC36 together severely inhibited the level of cleaved caspase-1, suggesting that the BRCC36 could be an important anti-inflammatory target.

BRCC36 contains K63-linked ubiquitin-specific deubiquitinating activity [26] Therefore, we analyzed ubiquitination pattern of NLRP3 molecule and found that in unstimulated cells, NLPR3 molecules were ubiquitinated with both K48- and K63-linked ubiquitin molecules which could have helped maintain the turnover and half-life of NLRP3 protein. However, oxLDL treatment could trigger BRCC36 deubiquitinating activity and removed K63-linked ubiquitination. The process helped in decreased proteasomal degradation of NLRP3, resulting in its accumulation and activation and subsequent release of IL-1β and pyroptosis. BRCC36-silenced cells did not respond toward the K48 ubiquitination, as its level remained unchanged. These findings suggested that the inhibition of BRCC36 subsequently inhibited K63-linked ubiquitination of NLRP3, and hence, it suppressed the NLRP3 inflammasome activation (Fig. 6a, b).

It has been shown that oxLDL promotes ROS-induced NLRP3 inflammasome activation and subsequently release of IL-β in the media [1]. To test this in our system, we induced the murine macrophages with oxLDL at different timepoints and found that it increases the ROS generation and secretion of IL-β in the media. ROS quencher NAC could successfully suppress oxLDL-induced IL-β in the media (Fig. 7a, b). Furthermore, our immunoblotting studies showed that NAC treatment also significantly decreased

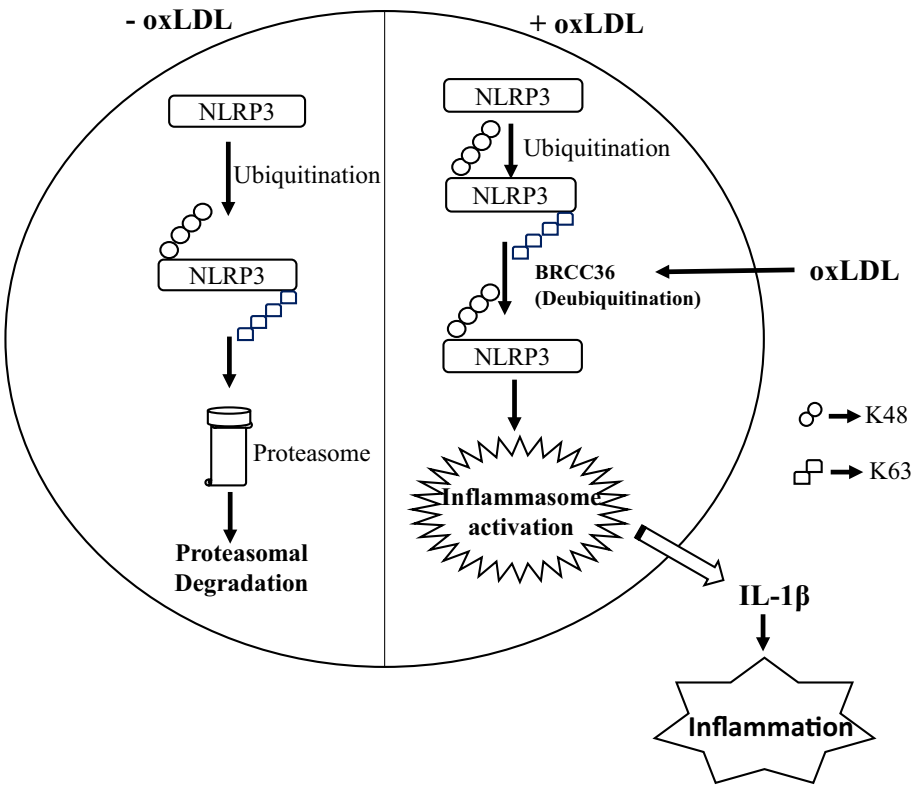

Fig. 9 Schematic diagram showing NLRP3 inflammasome activation through BRCC36 deubiquitination activity in the presence of oxLDL. In normal condition (− oxLDL), NLRP3 proteins get ubiquitinated regularly and sent for proteasomal degradation maintaining its half-life in the cells. However, in the presence of oxLDL (+ oxLDL) in cells, the deubiquitinating enzyme BRCC36 gets activated which removes K63 ligated ubiquitins from NLRP3 proteins which starts accumulating and form NLRP3 inflammasome assembly leading to release of IL-1β and promotes inflammation

BRCC36 expression (Fig. 7c), which indicated that BRCC36 promoted inflammasome activation and subsequently release of IL-β in media could be via ROS-induced pathway. This, however, requires further studies for its validation.

Our observations that BRCC36 inhibition restricted oxLDL-induced NLRP3 inflammasome activation suggest that BRCC36 may be directly interacting with NLRP3. We confirmed this through immunoprecipitation of NLRP3 proteins followed by immunoblotting using BRCC36 antibodies, which showed that BRCC36 coprecipitated with NLRP3, suggesting its interaction and association with NLRP3 protein (Fig. 8). Interestingly, the two proteins could also be present in the unstimulated cells, which may suggest that although both proteins may be present together in unstimulated cells and BRCC36 remains inhibited, which upon stimulation could get activated. The stimulus-dependent activation of BRCC36 helps in K63 specific deubiquitination process, which results in NLRP3 inflammasome complex formation and activation, leading to increased inflammation.

Conclusions

In conclusion, our study reveals that oxLDL-induced K63-linked deubiquitination of NLRP3 inflammasome protein leads to the secretion of IL-1β and potentially enhanced inflammation. K63-linked ubiquitination of NLRP3 inflammasome is regulated by deubiquitinating enzyme BRCC36, which interacts with NLRP3 proteins for optimum activity (summarized in Fig. 9). Inhibition of BRCC36 deubiquitinating activity suppresses NLRP3 inflammasome activation, which could be a potential approach to control excessive inflammation.

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References

1. Liu W, et al. OxLDL-induced IL-1 beta secretion promoting foam cells formation was mainly via CD36 mediated ROS production leading to NLRP3 inflammasome activation. Inflamm Res. 2014;63(1):33–43.
2. Varghese JF, Patel R, Yadav UCS. Sterol regulatory element binding protein (SREBP) -1 mediates oxidized low-density lipoprotein (oxLDL) induced macrophage foam cell formation through NLRP3 inflammasome activation. Cell Signal. 2019;53:316–26.
3. Mangan MSJ, et al. Targeting the NLRP3 inflammasome in inflammatory diseases. Nat Rev Drug Discov. 2018;17:588.
4. He Y, Hara H, Núñez G. Mechanism and regulation of NLRP3 inflammasome activation. Trends Biochem Sci. 2016;41(12):1012–21.
5. Juliana C, et al. Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. J Biol Chem. 2012;287(43):36617–22.

6. Shim D-W, Lee K-H. Posttranslational regulation of the NLR family pyrin domain-containing 3 inflammasome. Front Immunol. 2018;9:1054.
7. Yang J, Liu Z, Xiao TS. Post-translational regulation of inflammasomes. Cell Mol Immunol. 2017;14(1):65–79.
8. Heride C, Urbe S, Clague MJ. Ubiquitin code assembly and disassembly. Curr Biol. 2014;24(6):R215–20.
9. Varadan R, et al. Structural properties of polyubiquitin chains in solution. J Mol Biol. 2002;324(4):637–47.
10. Ng HM, et al. The Lys63-deubiquitylating enzyme BRCC36 limits DNA break processing and repair. J Biol Chem. 2016;291(31):16197–207.
11. Shintani H. LDL isolation and copper-catalysed oxidation. Pharm Anal Acta. 2013;4:247.
12. Py BF, et al. Deubiquitination of NLRP3 by BRCC3 critically regulates inflammasome activity. Mol Cell. 2013;49(2):331–8.
13. Pickart CM, Eddins MJ. Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta. 2004;1695(1–3):55–72.
14. Cooper EM, et al. K63-specific deubiquitination by two JAMM/MPN + complexes: BRISC-associated Brcc36 and proteasomal Poh1. EMBO J. 2009;28(6):621–31.
15. Sheedy FJ, et al. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. Nat Immunol. 2013;14(8):812–20.
16. Binder CJ, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between Streptococcus pneumoniae and oxidized LDL. Nat Med. 2003;9(6):736–43.
17. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood. 2011;117(14):3720–32.
18. Song H, et al. The E3 ubiquitin ligase TRIM31 attenuates NLRP3 inflammasome activation by promoting proteasomal degradation of NLRP3. Nat Commun. 2016;7:13727.
19. Drews O, Taegtmeyer H. Targeting the ubiquitin-proteasome system in heart disease: the basis for new therapeutic strategies. Antioxid Redox Signal. 2014;21(17):2322–43.
20. Ng H-M, et al. The Lys63-deubiquitylating enzyme BRCC36 limits DNA break processing and repair. J Biol Chem. 2016;291(31):16197–207.
21. Feng L, Wang J, Chen J. The Lys63-specific deubiquitinating enzyme BRCC36 is regulated by two scaffold proteins localizing in different subcellular compartments. J Biol Chem. 2010;285(40):30982–8.
22. Zeqiraj E, et al. Higher-order assembly of BRCC36-KIAA0157 is required for DUB activity and biological function. Mol Cell. 2015;59(6):970–83.
23. Yoshida T, et al. Proteasome inhibitor MG132 induces death receptor 5 through CCAAT/enhancer-binding protein homologous protein. Cancer Res. 2005;65(13):5662–7.
24. Möller Westerberg C, Hägglund H, Nilsson G. Proteasome inhibition upregulates Bim and induces caspase-3-dependent apoptosis in human mast cells expressing the Kit D816V mutation. Cell Death Dis. 2012;3:e417.
25. Yan K, et al. The deubiquitinating enzyme complex BRISC is required for proper mitotic spindle assembly in mammalian cells. J Cell Biol. 2015;210(2):209.
26. Shao G, et al. The Rap80-BRCC36 de-ubiquitinating enzyme complex antagonizes RNF8-Ubc13-dependent ubiquitination events at DNA double strand breaks. Proc Natl Acad Sci USA. 2009;106(9):3166–71.