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Particulate β-glucan activates early and delayed phagosomal maturation and autophagy within macrophage in a NOX-2 dependent manner

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ABSTRACT

Aims: Macrophage is known to readily engulf any particulate material they encounter, including invading microbes and nano- or micro-particles. While recent studies show that some microparticles (MP) are immunogenic even without drug-cargo, the mechanism underlying this phenomenon is yet unclear. Phagocytosis induces NADPH oxidase-2 (NOX-2) mediated ROS generation that is reported to regulate antibacterial autophagy. We therefore, investigated the role of NOX-2 derived ROS in phagosomal maturation and autophagy induction in response to phagocytic uptake of two kinds of polymeric biodegradable and biocompatible microparticles: yeast-derived β-glucan particles (YDGP) and poly-(D, L-Lactic Acid) microparticles (PMP).

Main methods: J774A.1 macrophage wereas exposed to polymeric particles and the immune responses: ROS, phagosomal maturation and autophagy induction, were examined by assays including NBT, DCFH-DA, NADPH-Oxidase activity, Lysotracker and Acridine Orange. Further, the LC3 and NOX-2 expression were validated by RT-PCR, immunofluorescence assay and Western blotting. Antimicrobial activity of both MP was examined by CFU counting after administration to *Mycobacterium tuberculosis* and *Salmonella typhimurium* infected macrophage.

Key findings: YDGP induces phagosomal maturation and acidic vesicle accumulation at 30 min and 24 h post-exposure, much more proficiently than that by PMP. YDGP exposure also induced NOX-2 dependent expression of light chain 3 (LC3-II), further confirmed as autophagy activation via autophagic flux assay with autophagolysosome inhibitor bafilomycin A1. Additionally, YDGP displayed superior anti-microbial activity than that by PMP.

Significance: The induction of NOX-2-dependent autophagy and antimicrobial activity exhibited by particulate glucans has significant implications in harnessing these drug delivery vehicles as potential 'value-added' autophagy-mediated therapeutics in future.

1. Introduction

Resident tissue macrophage provide one of the first lines of host defence against infection and effectively ingest and digest any particulate matter such as inert particles or microbes. During receptor-mediated endocytosis, such as by Fcγ receptors (FcγR), Toll-like receptors (TLRs), and Dectin-1 receptors, the phagocytic NADPH Oxidase, NOX-2 is assembled on the emerging phagosomal membrane. NOX-2 activation generates superoxide by transfer of electrons from cytosolic NADPH to oxygen in the forming phagosome lumen [1]. Recently, NOX-2 generated ROS have been shown to be linked with rapid translocation of an autophagic protein, LC3, to pattern recognition receptor (PRR)-engaged phagosomes [2].

The generation of ROS in response to uptake of particulate matter (oxidative burst) is considered to be an antimicrobial defence mechanism within macrophage and other phagocytic cells [3,4]. Previous

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studies indicate that even biocompatible and biodegradable polymeric particles can serve, not only as inert carriers for anti-microbial agents but also as potential stimulators of the innate microbicidal responses in macrophages [4–6]. A great deal of research has been carried out on polymers, such as poly(D, L-Lactic Acid) (PLA), poly(D, L-Glycolic Acid) (PGA), and poly(D, L-Lactic-co-Glycolic Acid) (PLGA) for the formulation of drug delivery vehicles, because of their excellent biocompatibility, biodegradability, and mechanical strength.

YDGP are 2–4 μm porous, polymeric particles derived from the yeast cell wall and are composed of glucose units containing β-(1, 3) linkages, with occasional β-(1, 6) branches [3]. β-Glucan acts as a pathogen-associated molecular pattern (PAMP), recognized by a variety of PRRs, such as TLRs, Dectin-1, lactosylceramide receptor, and scavenger receptors on macrophages [7]. Recent studies have demonstrated that these hollow particles allow encapsulation of proteins [8], DNA [9], siRNA [10], and small drug molecules [11,12], and have been thus, considered for use as macrophage targeting vehicles.

Autophagy is a well-known intracellular mechanism activated upon starvation that involves the lysosomal degradation of unnecessary or dysfunctional cellular components [13]. It is also a key component of the innate immune defence against many pathogens and acts by removing them from the cytosol, limiting their escape from phagosomes and promoting phagosome maturation [14,15]. In addition, the LC3 accumulation at early exposure termed LC3-associated phagocytosis (LAP) is reported to be triggered upon phagocytosis of particles that engage specific cell surface receptors, such as TLR, FcγR, and Dectin-1, where the elements of macroautophagy conjugate LC3 to the single membrane phagosomes, triggering autophagy, phagosomal maturation, and degradation of its contents [2,16–18].

In this study, we sought to determine whether the phagocytic uptake of polymeric microparticles: YDGP and PMP, leads to activation of innate responses within macrophages. Specifically, we examined and compared these particles in terms of ROS generation, phagosomal maturation and autophagy activation within murine macrophages and explored the NOX-2-dependence of these events, upon MP exposure, followed by an assessment of their antimicrobial activity.

2. Materials and methods

2.1. ROS measurement

In Nitro blue tetrazolium (NBT) assay, J774A.1 macrophage cells (1×10^6 cells/well) were incubated with 10 μg/ml of YDGP and PMP (supplementary data Fig. S1) in a 6-well plate in the presence of 0.3% NBT (Himedia). After 2 h incubation in 5% CO_2 at 37 °C, the cells were washed twice with PBS and images of cells were captured by phase-contrast microscope (Nikon Eclipse Ti-S).

In the DCFH-DA assay, cells (0.1×10^6 cells/well) were seeded in a 96-well plate and loaded with 10 μM DCFH-DA (Sigma) for 15 min, afterwards to MP exposure. The relative fluorescence units, indicating the intracellular ROS production, were measured using a fluorescence microplate reader (Synergy HT BIO-TEK), with excitation at 485 nm and emission at 530 nm. Further, the assay was repeated using cells pretreated with diphenylene iodide (DPI) (10 μM), rotenone (Rote) (100 μM), and α-tocopherol (Toco) (100 μM) for 30 min.

2.2. NADPH-oxidase activity

The NAPDH-Oxidase activity was determined by superoxide anion production via cytochrome *c* (cyt c) reduction. Macrophage (10^5cells/well) as incubated with phenol red-free DMEM containing 100 μM cyt c (Sigma) and 200 μM β-NADPH (Sigma). They were subsequently exposed to 0.01 mg/ml of MP (YDGP and PMP), with and without 50 μM SOD (Sigma). The experiment was repeated in the presence of NOX-2 inhibitor, DPI (10 μM) to specifically quantitate NOX-2 enzyme activity after MP exposure. The colour formed was read on a plate reader with 550 nm filter, at various time points.

Table 2.1
Details of RT-PCR primers used in the study.

Gene	Primer (5′-3′)	PCR product size (base pair)	Annealing Temp (°C)
LC3-II	5′TGTTACCATACGCCCTTCTGC3′ (F) 5′AACATTGAGGGTTAGCAAAGACAG3′ (R)	195	59.9 °C
NOX-2	5′CTTGGAAATGGATAGTGGGTCCT3′ (F) 5′TGTACCAGACAGACTTGAGAATGGA3′ (R)	201	60 °C
GAPDH	5′GTGTTCCTACCCCCAATGTGTC3′(F) 5′GAAATTGTGAGG GAGATGCTCAG3′ (R)	206	62 °C

2.3. LysoTracker-G and acridine orange staining

After the exposure of MP for 30 min, 4 h and 24 h, the cells were washed with PBS and incubated with 50 nM LysoTracker-G for 15 min at 37 °C. Subsequently, the cells were washed with PBS and visualized under a fluorescent microscope (Nikon Eclipse Ti-S).

Cultured cells exposed to MP were incubated with 1 μg/ml Acridine Orange (AO) (Himedia) for 15 min to detect acidic vesicular organelles (AVOs). MP exposed cells pretreated with DPI, rote, or α-toco were also stained with AO and visualized after illumination with blue light (488 nm excitation) with a fluorescent microscope. Macrophage cultures were induced to undergo autophagy by rapamycin treatment (25 μg/ml) (positive control).

2.4. RNA extraction and RT-PCR

RNA was extracted from cells exposed to MP by using TRI® reagent (Invitrogen) and treated with RNase-free DNase as per the manufacturer's instructions. cDNA was synthesized using one*Taq* RT-PCR kit (NEB, USA), and amplified using gene-specific primers (Table 2.1). Gene expression was confirmed by 1% agarose gel electrophoresis and bands were observed under UV transilluminator. Cells pretreated with DPI, rotenone and α-tocopherol were also exposed to both kinds of MP. RNA isolation and RT-PCR (by using standard conditions) was performed with the same primers. Quantitation of DNA bands was performed by Image J software.

2.5. Immunofluorescence assay for autophagic protein (LC3-II)

Immunofluorescence for visualization of lipidated LC3-II accumulation upon macrophage exposure with YDGP for 30 min and 24 h was performed by overnight incubation at 4 °C with primary antibodies for anti-LC3-II (1:100, CST), as described previously [19], with or without DPI, rotenone, or α-tocopherol pretreatment.

2.6. Western blotting

J774 cells were exposed to YDGP for 30 min and 24 h and subsequently lysed in ice-cold lysis buffer supplemented with protease inhibitor (Sigma). The protein concentration in cell lysates was determined by the Bradford method. Equal amounts of protein (40 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P PVDF membrane (Millipore). The membranes were incubated overnight with primary antibodies for LC3-I/II (1:1000, CST) and β-actin (1:1000, CST) at 4 °C and subsequently with 1:10,000 dilution of secondary anti-rabbit antibody (HRP conjugate) at room temperature for 1 h, after intermittent washing with 0.1% Tween-20 in Tris-buffered saline. Next, the substrate solution was added to the membrane, incubated for 5 min and exposed at room temperature.

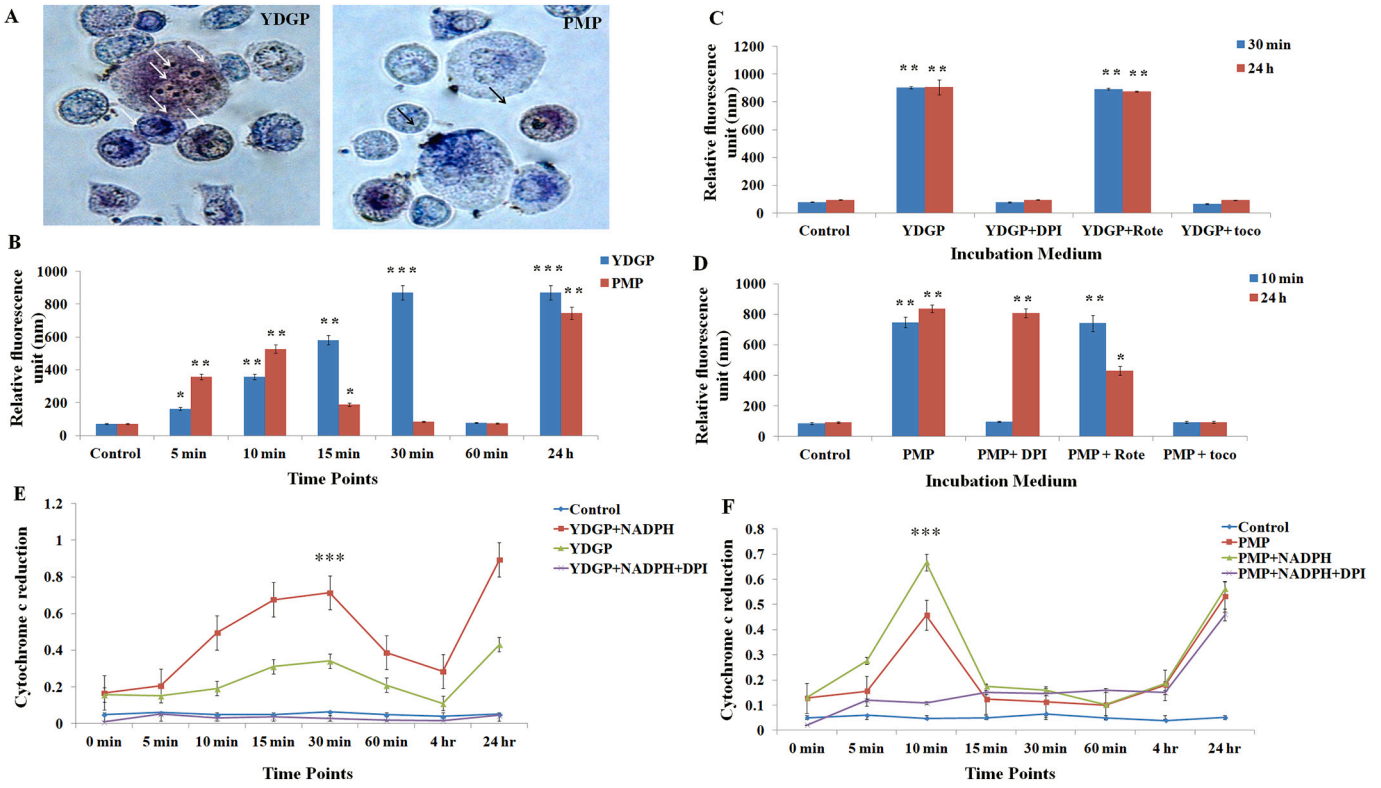

Fig. 1. MP internalization by J774A.1 macrophage cells leads to significant generation of NOX-2 mediated ROS. (A) Qualitative analysis of ROS generation after exposure of YDGP and PMP by NBT assay. Arrows show formazan crystals within the MP exposed cells. (B) Quantitation of ROS generation at different time points after exposure of MP by DCFH-DA assay. (C) DCFH-DA assay in the presence of DPI, rotenone and α-tocopherol after exposure to YDGP and (D) PMP. (E) NOX activity determination in YDGP and (F) PMP exposed cells from 5 min to 24 h by SOD inhibitable-cytochrome *c* reduction, as detailed in Materials and methods. '*', '**' and '***' indicate significant differences in ROS levels, by paired *t*-test at $P < 0.01$, $P < 0.001$ and $P < 0.0001$ respectively, from the control (unexposed) cells. Rote: rotenone; Toco: α-tocopherol.

The membranes were developed with enhanced chemiluminescence (ECL) kit by following the manufacturer's (Millipore, USA) instructions.

To examine autophagy flux, cells were preincubated for 2 h with BafA1 (100 nM) before exposure with YDGP (10 μM) for 30 min and 24 h to estimate LC3-II turnover. The bands were detected by western blotting and analysed by ImageJ software.

2.7. Antibacterial activity of YDGP and PMP

The *Mycobacterium tuberculosis (M. tb.)* and *Salmonella typhimurium (S. typhi.)* infected cells (supplementary data 1.5) were exposed to YDGP and PMP at a concentration of 10 μg/ml for 30 min and 24 h. After washing with PBS, the supernatant was discarded from infected and exposed macrophage, and 500 μl of sterile lysis buffer (0.05% SDS, w/v in H_2O) was added to each well. The suspension was vortexed for 10 s to disperse bacteria. The plates were incubated for 5 min at RT and 1:10 dilutions of lysates were prepared. 100 μl of diluted lysates were plated to obtain CFU counts on LJ plates (for *M. tb.*) and NA plates (for *S. typhi.*). The plates were incubated at 37 °C and colonies were examined and counted after 3 weeks (for *M. tb.*) and 24 h (*S. typhi.*) of incubation. Results are presented as the mean of CFU ± standard deviation.

2.8. Statistical analysis

The *P* values were calculated using two-tailed two-sample equal variance Dunnett's *t-test* (compared YDGP exposed vs. control; and treated vs. nontreated groups). A probability value of $P < 0.01$ or less, was considered to be statistically significant. All data are expressed as the mean ± standard deviation of the mean (SD) from three separate experiments.

3. Results

3.1. Microparticle uptake induces significant ROS generation

The microparticles were readily phagocytosed by murine macrophages and were found to be non-cytotoxic at 10 μg/ml concentration (Fig. S1). MP administration led to the formation of a dark purple formazan precipitate within murine macrophages, indicating appreciable ROS production (Fig. 1A). The DCFH-DA assay revealed a significant intracellular ROS generation within 5 min of exposure to both kinds of MPs ($P < 0.01$, $P < 0.001$) (Fig. 1B). YDGP exposure of macrophages was seen to induce significantly higher amounts of ROS after 30 min ($P < 0.0001$) and 24 h exposure ($P < 0.0001$), whereas PMP induced significantly higher amount of ROS after 10 min ($P < 0.001$) and 24 h of exposure ($P < 0.0001$). Based on this data, further experiments involving YDGP were performed at 30 min and 24 h of exposure, whereas those involving PMP were performed at 10 min and 24 h post-exposure time points.

Apart from NADPH oxidase, the mitochondrial complex-1 often serves as a significant source of ROS. We therefore, further examined the precise source of ROS being generated upon the administration of YDGP by pre-treatment with DPI, an inhibitor of NOX2, or rotenone, an inhibitor of mitochondrial complex-I. DPI-pretreatment was seen to inhibit ROS production induced by YDGP administration at both the 30 min and 24 h post-exposure time points. On the other hand, rotenone-pretreatment did not affect ROS generation induced by YDGP at early and delayed time points ($P < 0.001$, $P < 0.001$). An antioxidant α-tocopherol was used as a negative control for ROS generation (Fig. 1C). The ROS generation by PMP administration at the 10 min post-exposure time point was inhibited by DPI pretreatment but, was

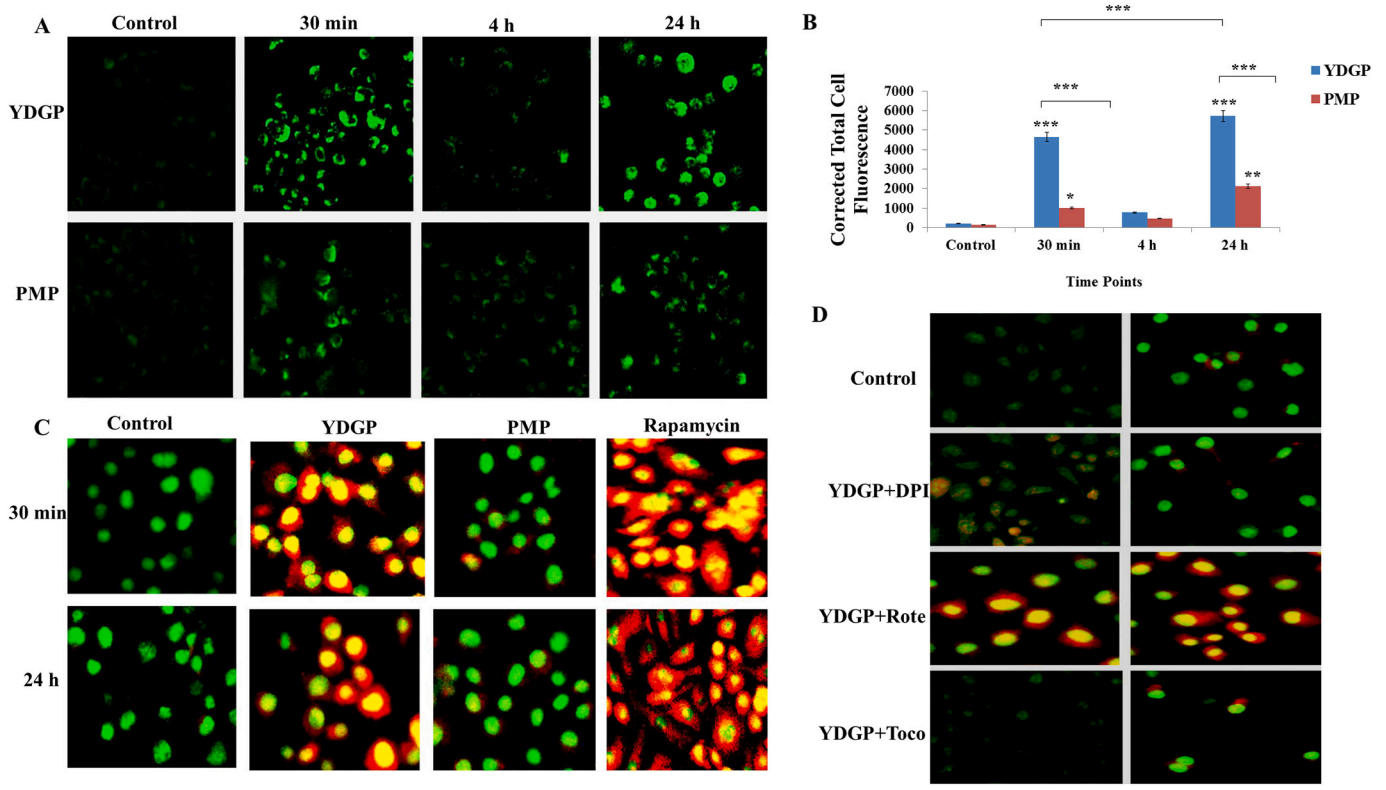

Fig. 2. Time-course of lysosomal activity and phagosomal maturation upon particle exposure. (A) YDGP and PMP exposed LysoTracker-G stained macrophage, showing labeled lysosomal compartments for different time points. (B) Quantitation of lysosomal activity by ImageJ software (C) AO staining to monitor AVO accumulation (red colour) within macrophage exposed to microparticles for 30 min and 24 h. (D) AVO accumulation in DPI, Rote and Toco pretreated cells to YDGP exposure for 30 min and 24 h. '*', '**' and '***' indicate significant differences in fluorescence intensity, by paired *t*-test at $P < 0.01$, $P < 0.001$ and $P < 0.0001$ respectively, from the control (unexposed) cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unaffected by rotenone pretreatment ($P < 0.001$). In contrast, the ROS generation after 24 h exposure to PMP, was inhibited by rotenone pretreatment, but not by DPI pretreatment ($P < 0.001$) (Fig. 1D).

NOX2-specific activity was indicated by a marked increase in SOD-inhibitable cyt c reduction, from 10 min to 30 min of exposure ($P < 0.0001$) within the YDGP-exposed cells, which diminished at 4 h but was re-observed at high levels after 24 h post-exposure ($P < 0.0001$). Cyt c reduction at the 30 min and 24 h post-exposure time points was significantly inhibited by DPI ($P < 0.0001$, $P < 0.0001$), indicating specific activation of NOX2 within YDGP-exposed cells at both time points (Fig. 1E). In contrast, PMP exposed cells exhibited an elevated cyt c reduction at the 10 min and 24 h post-exposure time points ($P < 0.001$, $P < 0.0001$). Upon DPI pretreatment, the cyt c reduction was markedly diminished at 10 min post-exposure, while at 24 h post-exposure, cyt c reduction remained unaffected by DPI (Fig. 1F), indicating the involvement of NOX2 mediated ROS at the 10 min post-exposure time point and of another source of ROS at the 24 h post-exposure time point.

3.2. MPs induce phagosomal maturation and acidic vesicular organelles (AVOs) formation

Next, we examined the intracellular phagosomal maturation upon exposure to these particles. LysoTracker and AO staining were used to monitor lysosomal activity. Macrophage exposure with YDGP microparticles led to induction of prominent lysosomal activity at both 30 min and 24 h post-exposure time points at significantly high levels ($P < 0.0001$, $P < 0.0001$), suggesting rapid phagosomal maturation within the cells (Fig. 2A, B).

The lysosomal activity observed upon PMP exposure for 10 min was very low and similar to that observed at 30 min post-exposure (not shown); thus, subsequent observations for early time points were taken at 30 min of exposure to both the particles. PMP-exposure resulted in attenuated lysosomal activity in comparison to YDGP exposure to macrophage at both the 30 min and 24 h post-exposure time points. Thus, the lysosomal activity provoked within macrophage upon 30 min and 24 h exposure to YDGP was significant and much higher in comparison with that by PMP exposed cells at both the time points ($P < 0.0001$). The sluggish lysosomal activity observed after 4 h exposure to both YDGP and PMP, was observed to be trivial and non-significant. Based on this data, subsequent experiments were performed at 30 min and 24 h post-exposure time points.

Next, we examined phagosomal maturation upon exposure to YDGP and PMP by vital staining of macrophages with AO after 30 min and 24 h of exposure (Fig. 2C). The unexposed, control cells exhibited mainly green fluorescence (staining cytoplasm and nucleolus) with minimal or no red fluorescence. YDGP exposure was seen to induce rapid acquisition of bright red fluorescence indicating accumulation of acidic vesicular organelles (characteristic of autophagy induction) within the cells after 30 min, that slightly increased after 24 h exposure. In contrast, PMP exposure to macrophage did not induce AVO accumulation at both the time points.

AVO formation in the YDGP-exposed cells were found to be completely dependent on ROS generation since α-tocopherol pretreatment led to the abolishment of AO-mediated red fluorescence (Fig. 2D). AO staining of DPI pre-incubated, YDGP exposed macrophage exhibited mainly green fluorescence, along with faint red fluorescence at both the time points. In contrast, pretreatment with rotenone resulted in rapid acidification of phagosomes within YDGP-exposed macrophages, at both the time points.

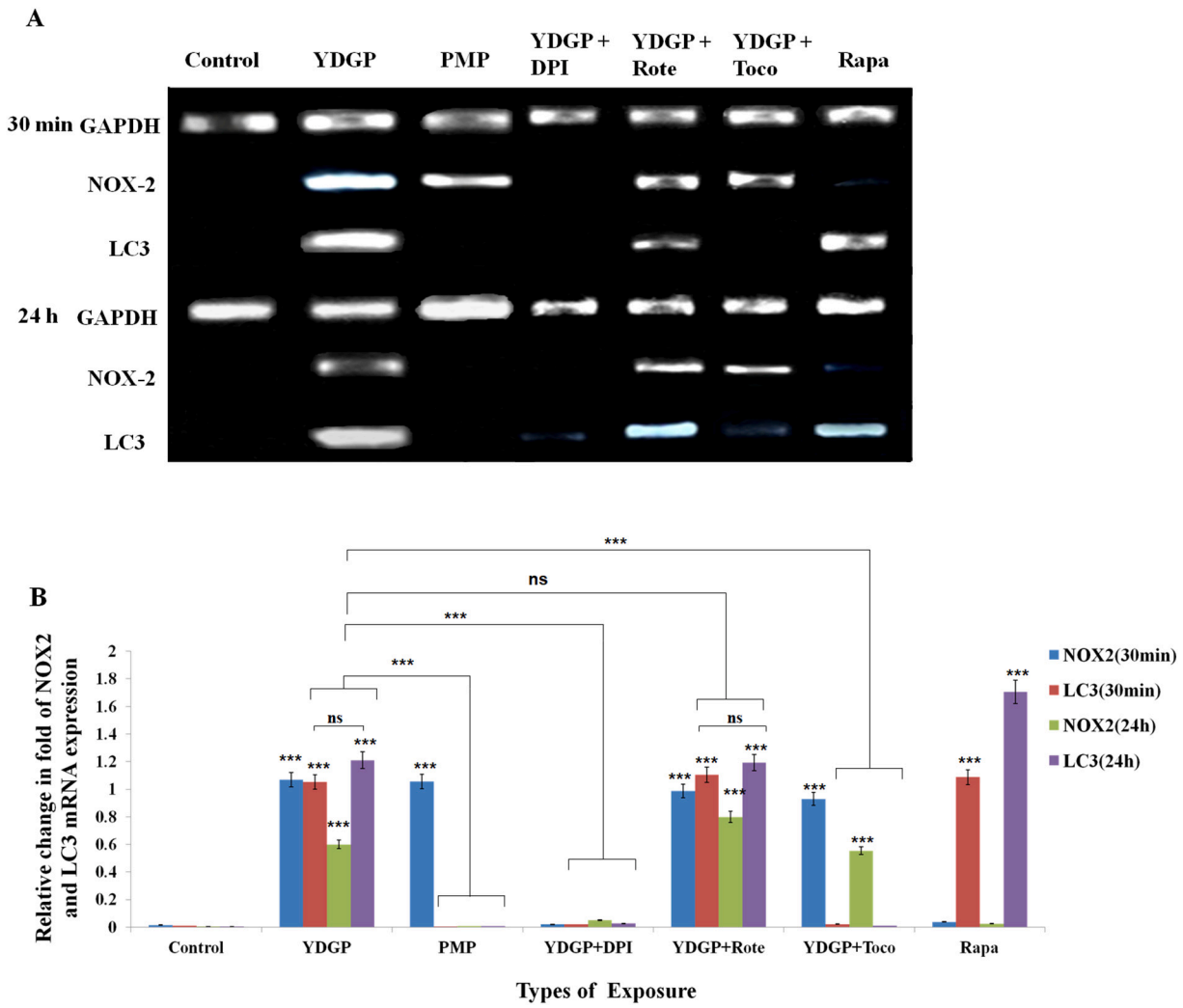

Fig. 3. Effect of YDGP and PMP exposure on the expression of *NOX-2* and autophagy mediators within J774A.1 macrophage. (A) *NOX-2* and *LC3* expression after YDGP and PMP exposure for 30 min and 24 h. (B) Quantitation of *NOX-2* and *LC3* mRNA expression after YDGP exposure in the presence of DPI, rotenone and α-tocopherol at 30 min and 24 h, expressed as the ratio of densitometric measurement of the sample to the corresponding internal control GADPH. '***' indicate significant differences in band intensity, by paired *t*-test at $P < 0.0001$, ns (nonsignificant).

3.3. YDGP exposure activates NOX2-dependent LC3 gene expression at the early and delayed time points

Further we examined the intracellular expression of *NOX2* and autophagy marker gene, *LC3* after 30 min and 24 h exposure to these particles. Cells exposed to YDGP exhibited *NOX2* and *LC3* expression at both 30 min and 24 h (Fig. 3A), that was compared with the steady expression of *GAPDH*, a housekeeping gene. PMP exposed cells did not show *LC3* expression at both the time points, but exhibited *NOX2* expression after 30 min exposure to PMP. Quantitative analysis revealed significant induction of *LC3* expression in YDGP exposed cells at both time points ($P < 0.0001$, $P < 0.0001$), in comparison with control and PMP exposed cells (Fig. 3B).

At both, the time points, *LC3* expression in YDGP-exposed cells was completely inhibited by DPI pretreatment ($P < 0.0001$, $P < 0.0001$) in comparison with the DPI untreated cells; on the other hand, rotenone pretreatment did not affect *LC3* expression (Fig. 3C, D). This suggests a key role of *NOX2* in *LC3* expression at both the time points, and over-rules the involvement of mitochondrial ROS in *LC3* expression induced upon YDGP uptake. The antioxidant α-tocopherol pretreatment completely abolished the expression of *LC3* at both the time points, but not that of NOX2 ($P < 0.0001$, $P < 0.0001$).

3.4. YDGP signaling recruits autophagy protein LC3-II to phagosomes in NOX2 dependent manner

After the gene expression analysis, immunofluorescence studies to assess the intracellular spatial arrangement of LC3-II (the processed form of LC3 associated with autophagosomes) were performed, that exhibited LC3-II protein accumulation after 30 min as well as 24 h (Fig. 4A) of YDGP exposure. DPI pretreatment of YDGP exposed cells at both the time points, led to significant decline in immunofluorescence in comparison to control cells, indicating that YDGP signaling recruits autophagy protein LC3-II to phagosomes in a NOX2 dependent manner. α-Tocopherol was seen to suppress the accumulation of LC3-II protein at both the time points, thereby indicating that ROS plays a major role in autophagy induction mediated by LC3-II translocation to phagosomes at both the time points.

Analysis of LC3-II protein (16 kDa) expression by Western blotting depicted significant expression at both the time points within YDGP exposed cells in comparison with control cells (Fig. 4B). Next, the YDGP-induced autophagic flux was further investigated in the presence and absence of vacuolar ATPase inhibitor bafA1 [20,21]. While the 2 h pretreatment with 100 nM bafA1 led to slightly enhanced LC3-II expression levels, the exposure to both bafA1 and YDGP resulted in additionally increased LC3-II levels (Fig. 4B and C). These results

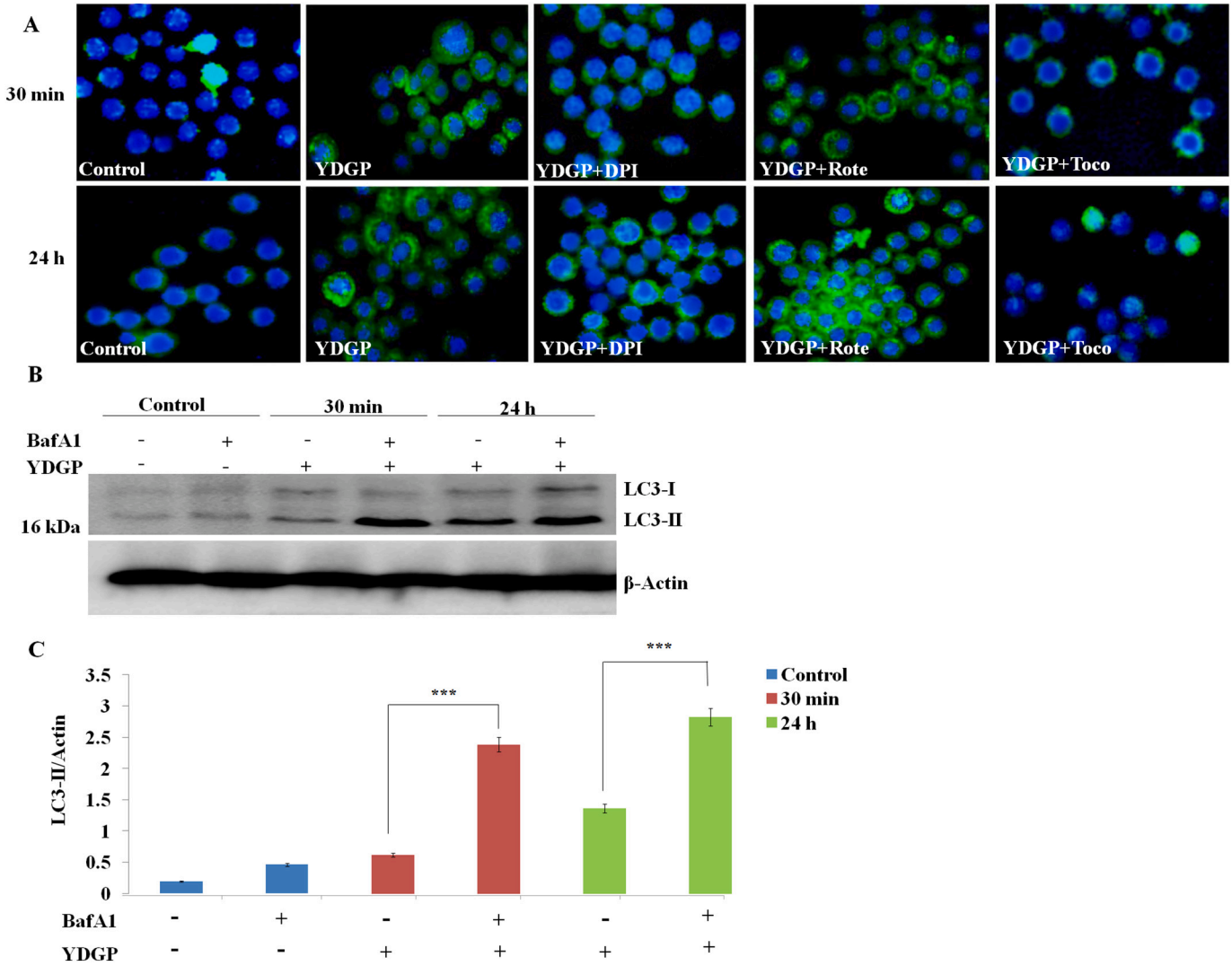

Fig. 4. YDGP exposure induces NOX-2 dependent autophagy activation at 30 min and 24 h post-exposure time points. (A) Cells exposed with YDGP for 30 min and 24 h, with and without inhibitors, were subjected to immunofluorescence staining for lipidated LC3-II (green colour), Hoechst (blue colour) counterstaining and thereafter, visualized under a fluorescent microscope. (B) LC3 levels in YDGP exposed macrophage subjected to BafA1 pretreatment. Western blot of J774 cell lysates treated with YDGP (10 μM) for 30 min or 24 h, in the presence or absence of 100 nM BafA1 (2 h pre-incubation) using anti-LC3 antibody. (C) Relative band intensity of LC3-II was calculated after normalization by load control β-actin. '***' indicate significant differences in band intensity, by paired *t*-test at $P < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

indicate intracellular turnover of new autophagosomes, thus confirming autophagy induction in response to YDGP administration, at both 30 min and 24 h post-exposure time points.

3.5. *In vitro anti-mycobacterial activity of YDGP and PMP against M. tb. and S. typhi*

Since the microparticles (specifically YDGP) were seen to activate macrophage, we reasoned that they might also have an impact on clearance of intracellular bacterial infections such as *M. tb.* and *S. typhi*. To investigate whether these particles can restrict the growth of intracellular bacteria within macrophage, cells were infected with aforementioned bacteria and then administered 10 μg/ml of either YDGP or PLA microparticles for 30 min and 24 h, followed by CFU counting. The CFU counting of mycobacterial colonies obtained after 28 days of plating the lysates of infected and treated cells, indicated that even blank glucan particles (YDGP) at a concentration of 10 μg/ml were capable of killing intracellular mycobacteria (Fig. 5A). A significant inhibition in the intracellular survival of mycobacteria ($P < 0.01$) as well as of *S. typhi.* ($P < 0.001$) was observed in cells exposed to YDGP for 24 h, in comparison with the infected, unexposed control cells (Fig. 5A and B). In contrast, exposure to PMP for 30 min and 24 h did not inhibit *M. tb.* and *S. typhi* growth ($P > 0.01$, $P > 0.01$) (Fig. 5A and B).

4. Discussion

Phagocytosis is a complex biochemical process involving two phases: firstly, passive particle recognition by cell-surface receptors, remodeling of actin cytoskeleton in membrane around the particle, and an active second phase beyond half-engulfment, in which cup boundary is extended, receptors move into engulfed region and the surface tension energy is twice as high as at the beginning of engulfment due to membrane folding back onto itself [22]. If this energy cannot be provided, then cup progression stalls and the particle remains incompletely engulfed [23].

Phagocytosis of particulate matter is generally construed as an activation signal. Both the biodegradable and biocompatible particles: Yeast derived glucan and PLA particles widely used as biocompatible drug delivery vehicles, were readily phagocytosed by macrophage (Supplementary Fig. S1) owing to their appropriate size [24] and led to

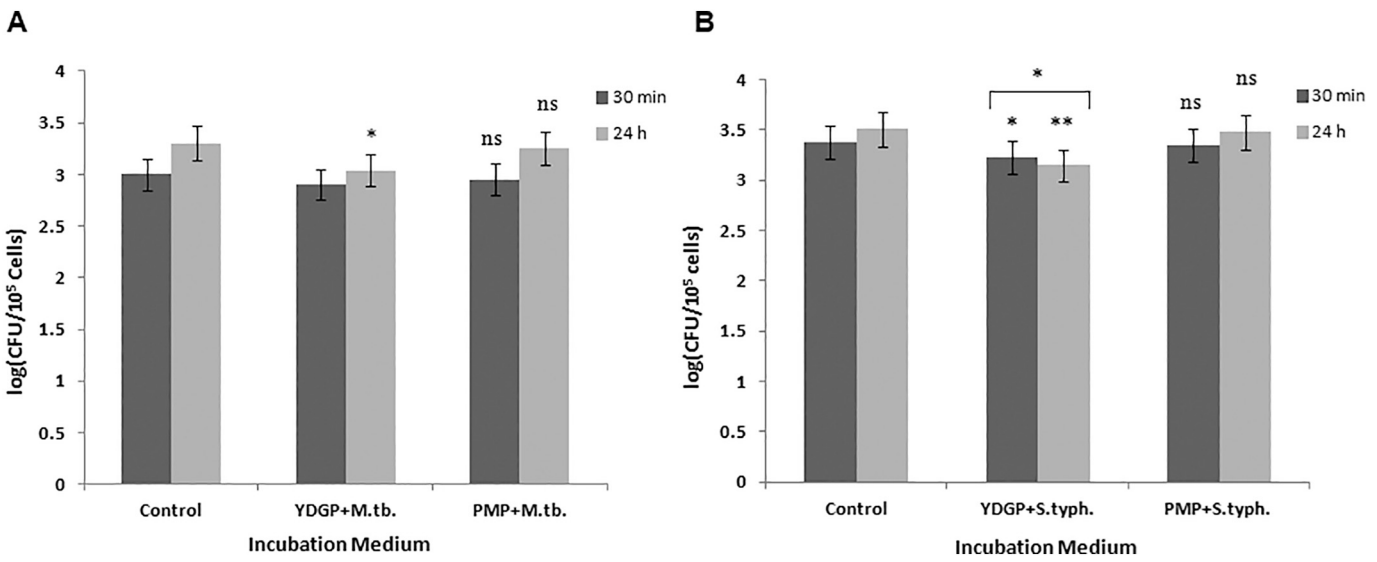

Fig. 5. CFU of (A) *M. tb.* and (B) *S. typhi* recovered from lysates of 10^5 infected cells receiving no treatment (control), or treatment with YDGP or PMP for 30 min or 24 h. '*' and '**' indicate significance by paired *t*-test at $P < 0.01$ and $P < 0.001$ on comparing bacterial growth inhibition in infected group exposed to MPs with the unexposed infected control groups.

rapid induction of ROS.

Macrophage Dectin-1 is specifically known to get activated by particulate but not by soluble β-glucans [25] and is the main phagocyte receptor for glucan particles. The binding to Dectin-1 has been shown to signal through the Try kinase syk and activate NADPH Oxidase, leading to a release of ROS into the phagosome [26,27]. In accordance with these studies, our data exhibited that ROS generation by macrophage upon exposure to YDGP was mediated by NOX-2 activation at both the time points showing significant ROS generation. In YDGP exposed cells, the NOX-2 activity gradually increased from 5 to 60 min (maximum activity at 30 min), diminished at 4 h, and then again increased at 24 h. On the other hand, PMP exposure induced NOX-2 activation at 30 min post-exposure time point only, indicating the involvement of other sources of ROS at the delayed time point of exposure (24 h), such as mitochondrial electron transport chain or xanthine oxidase, rather than NOX-2.

ROS is known to activate lysosomal degradation [2,28] and thus, we assessed these MP for induction of lysosomal activity and subsequent acidification of vesicles. YDGP exposure to cells induced vigorous lysosomal activity, suggesting rapid phagosomal maturation within cells at both the time points of exposure showing significantly high levels of ROS generation (Fig. 2A). While our prior study on PMP targeting to murine macrophage, exhibited Golgi vesicles interacting with phagosomal membranes, and releasing contents into MP containing phagosomes [4], we observed a much lower lysosomal activity upon PMP exposure, as compared to that by YDGP exposure. The fact that Dectin-1, a β-glucan receptor (PRR), specifically controls internalization [7] and subsequent phagolysosomal maturation of β-glucan [29] but not PLA containing phagosomes, explains the lower lysosomal activity upon exposure to PMP. Also, no lysosomal activity was observed at 4 h of exposure (selected arbitrarily in the mid of early and delayed time points) for both particles. The increased detection of AVOs viz. autophagic vacuoles and lysosomes at both the time points upon exposure to YDGP but not to PMP, indicate potential of YDGP for promotion of both lysosomal activity and phagosomal maturation.

The uptake of particulate glucan was seen to rapidly trigger the translocation of LC3 to YDGP containing phagosomes and promote phagosomal maturation at both the early and late time points. NOX-2-generated ROS molecules have been reported to be required for the translocation of LC3 to phagosomes that eventually fuse with lysosomes and degrade the cargo or invading pathogens [17]. LC3 is an active partner in the phagosome maturation process and directly correlates with autophagosome numbers [30]. Endogenous LC3 expression at both RNA and protein levels confirmed autophagy induction upon YDGP exposure at both early and late time points in a ROS and NOX-2-dependent manner (Fig. 4). Furthermore, these effects were completely abrogated by treatment with the NADPH oxidase inhibitor DPI, indicating the predominant role of NADPH oxidase activity in phagosomal maturation and LC3-II accumulation. This is expected because the NOX-2–NADPH complex that gets internalized from PM during phagosome formation is the major source of ROS that serves as a key signal for LC3 recruitment. The decline observed in NOX-2 expression from 30 min to 24 h post-YDGP exposure, with a concomitant increase in LC3 expression indicate that autophagy is triggered at early time point via NOX-2 activation within macrophage, and further continues at later time point while NOX-2 activation starts waning (Figs. 3E and 4C). Additionally, BafA pretreatment prior to YDGP exposure, to block cargo-degradation in autolysosomes, further increased LC3-II turnover, implying that YDGP induces dynamic autophagic flux at both time points.

Besides NADPH oxidases, mitochondrial complex-1 is also reported to be a major source of ROS production and plays a role in autophagy activation during starvation and stress conditions [31]. The mitochondrial complex-1, on the other hand, seemed to have no involvement in ROS generation, AVO accumulation and LC3 expression observed upon macrophage exposure to YDGP at both the time points. Though PMP induced mitochondria-mediated ROS generation after 24 h exposure, the ROS generation here does not seem to be sufficient to induce AVO accumulation and autophagy activation.

The NOX-2 expression observed after 30 min exposure to PMP, did not induce LC3 expression and AVO accumulation within the macrophage. Moreover, despite the similarity in lysosomal activity observed at 10 min and 30 min of exposure to PMP, the ROS generation by PMP at this time point was seen to be low and non-significant (Fig. 2B). The low lysosomal activity observed at the 4 h post-exposure time point for both the particles (Fig. 3A), possibly indicate the completion of phagosomal maturation at early (30 min exposure) time point, and later re-activation upon particle exposure up to 24 h.

This study is thus, in agreement with other reports in the last few years showing a direct connection between phagocytosis and autophagy induction [16]. In accordance with Sanjuan et al. [32], we speculate the internalized GP to induce rapid recruitment of LC3-II to single-

membrane phagosomes at the early time point (30 min), via 'LAP', starting from 15 min up to 60 min within macrophage upon zymosan uptake. On the other hand, the accumulation of LC3-II positive autophagosomes in the cytosol by conventional autophagic pathway occurs much later than LAP, activating after 6 to 24 h of exposure [32,33]. The activation of autophagic pathways induces accelerated phagosome maturation, degradation of phagocytosed cargo and reduced survival of phagocytosed pathogens [2,32–34]. Ma et al. [17] show that LC3 recruitment to phagosomes facilitates recruitment of MHC class II molecules to phagosomes (indicating rapid degradation of phagosomal cargo) and promotes efficient antigen presentation to CD4 T cells.

Further, in this study we have shown that treatment of infected macrophages with YDGP significantly reduces the intracellular burden of *M. tb.* and *S. typhi.* within 24 h, while PMP of a similar size do not. Previous studies have shown that particles made up of inert biological material such as PLA augment the bactericidal activity of macrophages by activating innate immune pathways in macrophages [4,35,36]. Particulate glucan have been reported to act as immunomodulatory agents, capable of eliciting a strong immune response within cells [3,19,36] via interaction with Dectin-1 and TLRs. Similarly, a recent study reports increased activation of NFκB, autophagy and inhibition of *M. tb.* H37Ra and H37Rv growth by 2.2 μm biodegradable PLGA particles, but not by non-biodegradable polystyrene MPs [37]. Interestingly, the polystyrene MPs also activate NFkB and trigger LAP in macrophages, but do not significantly affect intracellular growth of *M. tb.*

Apparently, the internalization of particles and subsequent intracellular immune responses are related to different biophysical properties of these materials such as chemical composition and by the receptors that recognize them. While poly(lactic) acid microparticles are internalized by macrophage, in an opsonin-independent fashion, via the macrophage receptor with collagenous structure (MARCO) [38], particulate glucan is known to be recognized mainly by Dectin-1 and TLR2 receptors [25,26]. At the molecular level, β-glucan ligands on particles engage the extracellular domains of phagocytic Dectin-1 receptors; receptor signaling then triggers intracellular signaling pathways that control NOX-2 activation, thereby generating ROS [26], and autophagy that culminates into slow lysosomal degradation of glucan cargo within phagosomes. The activation of autophagy and phagosomal maturation by YDGP indicates the ligation and signaling in response to glucan recognition through Dectin-1 and TLR2 receptors [2,16,17,39]. Our data on NOX-2 mediated rapid recruitment of LC3-II to phagosomes by particulate glucans at early time point (30 min post-exposure) is in agreement with previous studies [2,27,32]. On the other hand, the autophagy induction observed at the delayed (24 h) post-exposure time point can be attributed to the energy deprived (exhausted and starved) state of phagocytes with the Dectin-1 receptors engaged with particulate glucan and still signaling. This is likely as some studies also exhibit incomplete particle uptake, a mechanical bottleneck around half-engulfment [23,40] that may be the result of particle attachment on cell-membrane areas, unable to phagocytose due to low energy, resulting in engaged glucan specific PRRs. The Dectin-1 receptor is reported to be recruited to phagosomes containing zymosan particles but not to phagosomes containing immunoglobulin G–opsonized particles [26]. Dectin-1 phagosome has also been shown to serve as a scaffold for the assembly of the downstream signaling complex [25]. We thus speculate that the presence of Dectin-1 receptors bound to particulate glucan within early and late endosomes containing partially degraded glucan particles, would continue signaling for autophagy induction via NOX-2, until the particles are completely degraded into soluble glucan slowly, in a time dependent manner. Our data shows the NOX-2 activity to be quite low at 24 h post-exposure time point within YDGP exposed cells, but appeared to be necessary and sufficient to induce LC3 recruitment. NOX2 membrane component, cytochrome b_{558} (comprising $p22^{phox}$ and $gp91^{phox}$), is well documented to be recruited to phagosomes from Rab11 and Rab5 positive, recycling endosomal and lysosomal vesicles so as to replenish oxidatively damaged NOX2 at phagosomes [41,42]. A key role for Rab11 has been provided in autophagy induction by its translocation from recycling endosomes to autophagosomes, facilitating fusion of late endosomes with autophagosomes, thereby promoting autophagic flux [43]. Additionally, NOX oxidases have been recently shown to be positive regulators of ER stress and autophagy [44] and have been shown to be activated in response to stress and contribute to cell survival [45,46].

Our results show slightly higher levels of autophagy induction by YDGP at the delayed 24 h post-exposure time point in comparison with that at the early 30 min post-exposure time point. A possibility in line with this observation is that autophagy can be expected to be elevated under the stressful energy depleting conditions created at cellular level [47], due to prolonged presence of partially degraded particulate glucan and proton pumping by vacuolar ATPase across endosomal membranes [48], thereby adding to signaling for promotion of autophagy pathway within YDGP exposed cells at the delayed time point.

In the present study, we demonstrate for the first time that purified particulate β-glucans from yeast cells (YDGP) trigger autophagy within macrophage at two different time points. The study shows the activation of NADPH Oxidase and ROS accumulation within the forming phagosome, leading to autophagy induction at both early and delayed post-exposure time points. In line with recent studies showing various kinds of autophagy, our data suggests that YDGP activate two discrete pathways for autophagy [33] at two different post-exposure time points: an early induction (at 30 min post-exposure) time point coinciding with non-canonical autophagy (LAP), and a concomitant delayed induction of macro-autophagy at 24 h post exposure time point [2,32,33]. Thus the response induced by YDGP in terms of autophagy pathway activation seems to depend upon the duration of stimulation with particulate glucan. The data presented here is preliminary and further studies are planned in our lab to delineate these pathways. Nevertheless, it demonstrates an interesting insight into the NOX-2 dependent phagosomal maturation and autophagy induction by YDGP that 'adds on value' to their use as particulate delivery system.

ROS are highly reactive molecules that activate physiological signaling pathways; apart from activating immunological events such as cytokines and chemokines induction, RNS, Th1 responses (classical activation of macrophages), they can also directly bind to the pathogen [49–51] and degrade them. Present data also indicates that YDGP alone (without any drug cargo) are capable of inducing significant antimicrobial activity within *M. tb.* and *S. typhi.* infected macrophage, after 30 min and 24 h exposure. We speculate that this reduction in intracellular bacterial load by exposure to YDGP may be probably due to NOX-2 mediated ROS [52] and autophagy induction via Dectin-1 [17] and TLR [2,32] receptor signaling, and has implications in terms of autophagy mediated-host directed therapeutics. However, further experiments for affirmation of the NOX-2 dependence of autophagy induction and its role in anti-microbial activity of these particles are planned in future. Also, whether early or late autophagy targets intracellular microbes more efficiently, or these autophagy pathways can be subverted during certain infections, remains to be seen. Also, because both these autophagy pathways are regulated by NOX-2 activation as a consequence of phagocytosis, how these pathways interrelate will be an important question for future studies.

The anti-microbial effects of YDGP particulate β-glucans have been reported for various models of diseases such as upper respiratory tract infections [53], anthrax and cancer [54]. The particulate form of glucan (YDGP) assumes added significance as it not only allows drug delivery to phagocytes, but also induces NOX-2 activation upon phagocytosis, and thereby activates a cascade of anti-microbial innate immune responses. The need to develop new immunotherapeutics is necessary in light of the emergence and rise of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains, particularly in reference to TB. Our recent studies on YDGP loaded with nanosized anti-TB drug Rifabutin (RB) [12], demonstrated this delivery system to activate host phagocytes, and to confer enhanced protection against intracellular *M. tb.* than that by

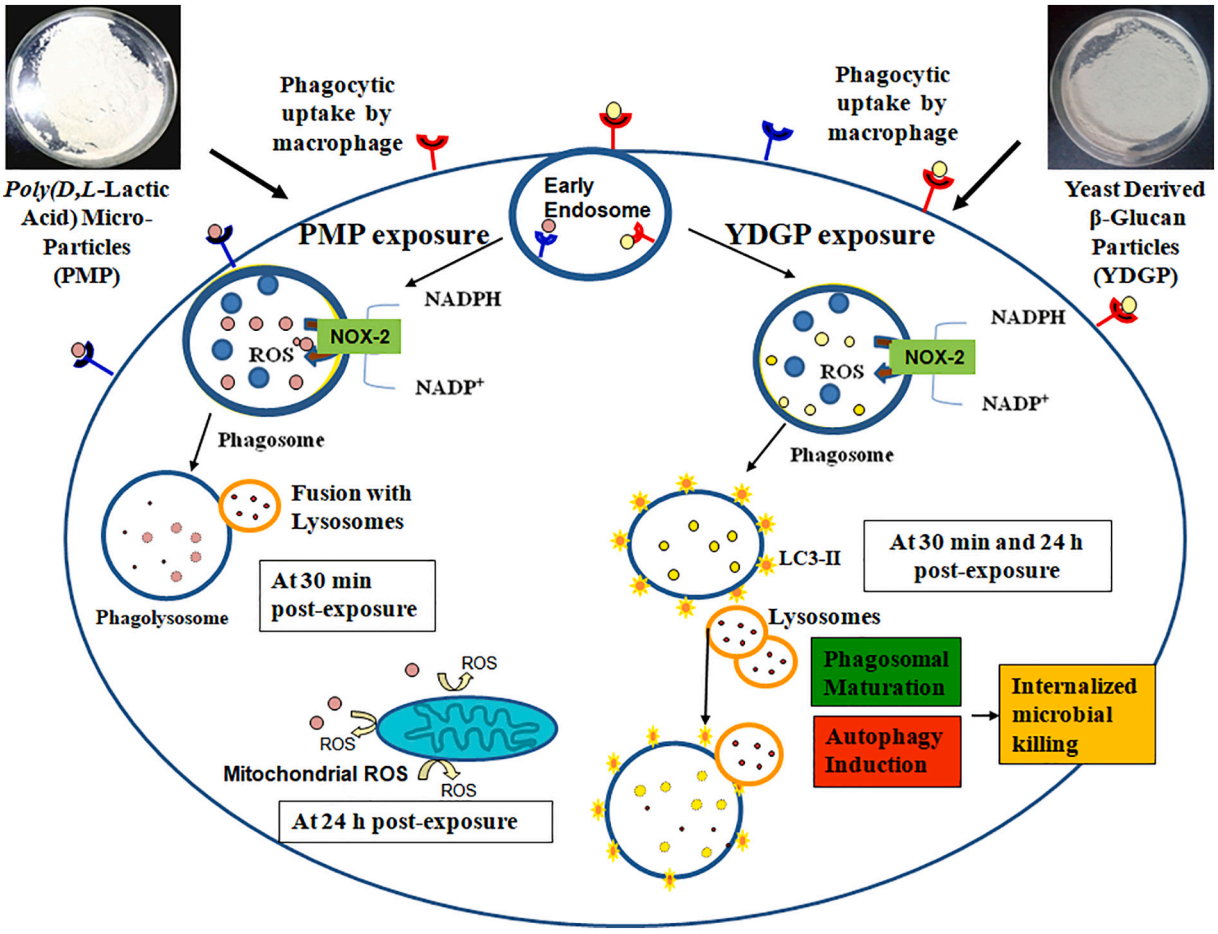

Fig. 6. Schematic representation of the role of NADPH oxidase-2 in regulating phagosomal maturation and autophagy induction by YDGP. YDGP (○) and PMP (●) both induce NOX-2 mediated ROS (●) generation at early (10 min–30 min) and delayed (24 h) time points after exposure. YDGP administration to macrophage induces AVO accumulation and phagosomal maturation, leads to autophagy induction at both early and delayed time points. Further, YDGP administration also results in decreased survival of intracellular microbes (*M. tb.* and *S. typhi*). PMP activate NOX-2 and mitochondrial ROS at early and delayed time(s) respectively, and do not induce autophagy at either of the time points.

(equivalent amount of) free RB drug, at a much lower therapeutic dose [55]. Thus, the autophagy-induction and anti-bacterial activity of YDGP is expected to have a synergistic impact with the chemotherapeutic cargo, thereby contributing further to the efficacy of these MPs, against intracellular infections (Fig. 6).

5. Conclusion

While both YDGP and PMP are biocompatible particles, used widely as drug delivery systems, and also induce significant ROS generation within macrophage, this study demonstrates that particulate β-glucans (YDGP), but not PMP, induce phagosomal maturation and autophagy within macrophage, at early and delayed exposure in a NOX-2 dependent manner. Our data suggests that YDGP are able to trigger signaling for two kinds of autophagy, at two different time points in a NOX-2 dependent manner. This study enhances our current understanding on the 'crosstalk of NOX-2 mediated signaling and activation of autophagy by particulate β-glucans' with respect to duration of stimulation, and is thereby expected to be further helpful in improving their use as a delivery system, but also their use as a delivery system. In addition, the antibacterial protection provided by particulate glucans (but not by PLA MPs with similar dimensions) against intracellular *M. tb.* and *S. typhi*, promises new therapeutic implications for these particles. These results reveal the potential of particulate glucans (YDGP) and support harnessing these as immunotherapeutics in addition to drug delivery systems, that can be exploited in the development of 'value-added' autophagy mediated therapeutics, as an adjunct to existing treatments against various diseases including intracellular infections.

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CRediT authorship contribution statement

Nida Fatima: Contriving the Concept and Explanation, Preparing-Original Draft, Writing, Reviewing and Editing.
Tarun Upadhayay: Formatting, Examining and Editing.
Firoz Ahmad: Examination.
Md Arshad: Investigation.
Mohammad Amjad Kamal: Reviewing and Editing.
Deepak Sharma: Providing Resources.
Rolee Sharma: Contriving the Concept and Explanation, Resources, Supervision, Writing, Reviewing and Editing, Funding procurement.

Declaration of competing interest

None.

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