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Role of O-GlcNAcylation in the phagocytosis process of macrophages

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1. Abstract.

Phagocytosis is a critical immune mechanism for the elimination of foreign particles, yet the role of O-GlcNAcylation in this process remains poorly understood. Using J774A.1 macrophages, we investigated how the modulation of O-GlcNAcylation affects the phagocytosis process. OGA inhibition (TMG inhibitor) after 24 hours significantly increased zymosan uptake, as evidenced by a 41% higher mean fluorescence intensity by flow cytometry and a 65–95% increase in phagocytic index by fluorescence microscopy. Neither OGT or OGA inhibitor significantly increased Dectin-1 or CD11b surface expression, suggesting that the enhanced phagocytosis is not driven by receptor upregulation. To elucidate if this mechanism is dependent on phagolysosome maturation, we assessed key phagosome markers. OGA inhibition (TMG) increased LAMP1 expression while OSMI-1 decreased it. No changes were observed in Rab5, Rab7, or Rab11, furthermore, OGA inhibition improved LAMP1 formation around phagocytosed zymosan particles. These findings indicate that O-GlcNAcylation modulates macrophage phagocytic capacity and phagolysosome maturation, with increased O-GlcNAcylation promoting zymosan internalization potentially through LAMP1-dependent mechanisms.

2. Introduction.

Macrophages are cells widely distributed in tissues; in response to several stimuli, these cells initiate pro-inflammatory or anti-inflammatory responses [1]. Phagocytosis is the process by which macrophages internalize a wide variety of particles, including microorganisms, dead cells, and environmental debris [2]. This process occurs in different stages: (a) identification of the particle by opsonic and non-opsonic receptors, (b) activation of the internalization process, (c) formation of the phagosome, (d) maturation of the phagosome into a phagolysosome, and (e) resolution and recycling of the phagolysosome [3, 4].

Zymosan particles have been extensively used as a phagocytic stimulus. Dectin-1 and CR3 (CD11b/CD18) are the main receptors that promote the internalization of β-glucans, a process followed by the formation of the phagosome [5–9]. The early phagosome is a poorly degradative compartment, during this stage exhibits Rab5 and EEA1 (Early endosome antigen 1) on its surface. As the further transition into a late phagosome, several molecules such as Rab7, Flotilin, and LAMP1 (Lysosome-associated membrane glycoprotein 1) are recruited [10, 11]. The fusion of the phagosome with lysosomes gives rise to the phagolysosome, which is characterized by the acquisition of proteases, lysozymes, and V ATPases, a pH decrease, and the production of reactive oxygen species (ROS) [4, 12]. To maintain the phagosome during the maturation process, Rab11 is implicated in phagosome formation and recycling endosome [13, 14].

Cellular metabolism plays a relevant role in macrophage function, including polarization mechanisms, antigen presentation from pathogens, cytokine secretion, and the phagocytosis process [15]. O-GlcNAcylation is a reversible and dynamic post-translational that consists of the addition of a single monosaccharide N-acetylglucosamine (GlcNAc) to a serine or threonine residues of proteins [16]. This modification depends on glucose availability and metabolism through the hexosamine biosynthesis pathway, it is controlled by only two enzymes: the O-GlcNAc transferase (OGT) adds GlcNAc moeity to the serine and threonine in the proteins, and the O-GlcNAcase (OGA) performs the removal of this carbohydrate [17]. O-GlcNAcylation has been studied in macrophages; however, contradictory results have been obtained regarding its effect on the processes that regulate their function [18, 19]. O-GlcNAcylation has been linked to macrophage activation [15, 20]. The macrophage O-GlcNAc signaling has been identified as a homeostatic mechanism related to metabolism, inflammation, and antiviral activation [19, 21–23].

Furthermore, it has been suggested that O-GlcNAcylation takes part in the phagocytic process. In retinal cells, the knockout of OGT or its pharmacological inhibition blocks phagocytosis. Mechanistically, OGT interacts with Ezrin and promotes its localization to the cell cortex, thereby stimulating the membrane-cytoskeleton interaction needed for efficient phagocytosis [24]. In macrophages it was observed the participation O-GlcNAcylation of phagocytic index of bacteria [25]. However, OGT-deficient macrophages show an increased number of internalized particles, suggesting that the loss of O-GlcNAcylation increase phagocytosis [22].

The regulation of phagocytosis mechanisms by O-GlcNAcylation remains poorly understood [26]. To date, there is no information regarding the role of O-GlcNAcylation in endosomal maturation during phagocytosis in macrophages. Therefore, this project aims to identify the involvement of this process in phagocytosis and thus provide information that helps clarify possible alterations in endosomal trafficking present in neurodegenerative diseases, cancer, or some infections caused by intracellular pathogens.

3. Materials and methods.

Chemicals and Antibodies.

Thiamet G (TMG), O-GlcNAcase (OGA) inhibitor (SML0244), Zymosan A from Saccharomyces cerevisiae (Z4250), Lyticase from Arthrobacter luteus (L2524) were from Merck. OSMI-1, O-GlcNAc transferase (OGT) inhibitor (ab235455), Anti-O-Linked N-Acetylglucosamine antibody RL2 (ab2739) were from Abcam. Anti-CD11b PE (101208), anti-F4/80 APC (123116), and TruStain FcX ™ anti-mouse CD16/32 (101320) were from BioLegend. Anti-OGT (24083S), anti-Rab5a (46449), anti-Rab7 (9367), and anti-Rab11 (5589) were from Cell Signaling Technology. Anti-OGA (NBP2-32233) was from Novus Biologicals. Anti-LAMP1 (PA1-654A) was from Thermo Fisher Scientific. GhostDye 780 (13–0865-T100) was from Tonbo. Anti-Actin (GTX109639) was from GeneTex. HRP-conjugated anti-mouse and anti-rabbit were from Jackson ImmunoResearch Laboratories.

Cell culture.

J774A.1 murine macrophage cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 mg/mL gentamicin, 10 mM HEPES, 1 mM sodium pyruvate, 50 mM 2- Mercaptoethanol (2-ME), 10 mM Glucose, and incubated at 37ºC with 5% CO2. Pharmacological inhibition of macrophages was performed with OSMI-1 (25 µM) for OGT or TMG (1 µM) for OGA enzymes, incubation is noted in each experiment.

Preparation of FITC-stained zymosan particles.

Zymosan A particles were stained with FITC according to [27]. 10 mg of zymosan particles were washed with sterile water and suspended in 1 mL of 0.1M sodium carbonate buffer, pH 9, and mixed with FITC that had been previously dissolved in DMSO (0.5 mg/mL). The suspension was incubated in the dark for 12h at 4ºC in continuous agitation. After incubation, NH_4Cl was added to a final concentration of 50 mM for 2 hours at 4ºC to quench and stop the reaction. FITC-stained zymosan particles (Zymosan A-FITC) were washed with sterile water and suspended in PBS, pH 7.4.

Flow cytometry.

For the phagocytosis assay, concentration and time conditions are mentioned in each experiment. The culture medium was removed, and zymosan particles were added. After zymosan stimulation, 3 washes were performed with the same cold DPBS (138 mM NaCl, 8.1 mM Na2HP04, 2.7 mM KCl, 1.2 mM KH2P04) and 200 µL of Lyticase (Merck) was added for 15 minutes at 37ºC. The medium was removed and cold DPBS

was added for 10 min at 4ºC. Several washes were performed with the same cold DPBS to detach the cells and centrifugated for 5 min at 300 × g at 4 °C. Cells were resuspended with100 µL of DPBS and added 1 µL of Ghost-Dye780 and incubated for 20 minutes in the dark. Cells were resuspended with 200 µL of 1% paraformaldehyde (PFA) in DPBS and mixed at 4 °C

For immunostaining, cells were recovered and centrifuged for 5 min at 300 x g at 4 °C. After discarding the supernatant, cells were resuspended (2×10^5) in 200 µL of DPBS, washed again by centrifugation and the supernatant was discarded, cells were resuspended with100 µL of DPBS-1% BSA and added 0.25 µg of TruStain FcX and incubated for 10 min at 4 °C. The primary antibodies (F4/80, CD11b and Dectin-1) coupled to the fluorophore were added and incubated for 30 min at 4 °C in the dark. Then, cells were centrifuged and washed by centrifugation, discarded the supernatant, cells were resuspended and after added 1 µL of Ghost-Dye780® and incubated for 20 min in the dark. Finally, 200 µL of DPBS-1% BSA was added and washed twice by centrifugation. Cells were resuspended with 200 µL of 1%PFA in DPBS and mixed vigorously at 4 °C.

Phagocytic index was the average number of ingested zymosan particles per cell. Flow cytometry analysis was performed using a MacQuant flow cytometer with MACSQuantify Software 2.13, acquiring 25,000 events. The data was analyzed using FlowJo software version 10.6.2. MFI was median fluorescence intensity.

Western blotting.

Cells were lysed with lysis buffer (50 mM Tris–HCl (pH 8), 137 mM, NaCl, 10% (v/v) glycerol, 1% (v/v) Triton, 50 mM NaF, 1 mM Na3VO4, 1 mM PMSF, 1 mM cOmplete™ Mini Protease Inhibitor Cocktail (Roche), 10 µM TMG). Samples were incubated for 30 min at 4ºC under constant agitation, the resultant suspension was centrifuged at 25,000 × g for 30 min to eliminate cellular residues. Samples were separated by electrophoresis in a 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS-PAGE) using the Laemmli buffer system. After electrophoresis, the gel was equilibrated for 1h in 25 mM Tris, 193 mM glycine, 20% methanol, and transferred to nitrocellulose membranes (0.45-nm pore size) (Thermo Fisher) in a semidry blotting device (Bio-Rad), at 25 mV for 1h.

Nitrocellulose membranes were then washed for 10 min with TBS (50 mM Tris, 150 mM NaCl, pH 8), and the unoccupied binding sites were blocked using a blocking solution (TBS, 5% BSA, 0.1% Tween 20, 0.02% sodium azide) for 1h at room temperature. Then, membranes were incubated with primary antibodies (RL2, anti-OGT, OGA, Rab5a, Rab7, Rab11, LAMP1, and Actin) overnight with blocking solution at 4ºC. The

membranes were washed 3 times with washing solution (TBS, 0.1% Tween 20) for 10 min and incubated with secondary antibodies coupled with peroxidase in TBS-5% dry milk for 1h. Then, membranes were washed 3 times with washing solution and signals were detected by chemiluminescence using SuperSignal™ West Femto or West Pico (Thermo Fisher).

Immunofluorescence.

J774A.1 macrophages were cultured onto LabTek Chamber 8 well slides (ThermoFisher). The phagocytosis assay with Zymosan A-FITC (20 particles:1 cell) was performed as previously reported [28]. Briefly, cells were gently washed with 100 µL of sterile PBS before adding media containing 100 U/ml of Lyticase to remove non-internalized particles. Cells were then incubated for 15 min at 37 °C with 5% CO_2. and subsequently washed with PBS. Afterwards, cells were fixed using 1% PFA in PBS for 15 min at 4 °C, followed by 3 washes with PBS. Cells were blocked with PBS-5% BSA for 1h at room temperature, followed by 3 washes with PBS, and incubated with the Anti-LAMP1 overnight at 4ºC. The following day, cells were washed 3 times with PBS and incubated with the secondary antibody for 1 h at room temperature followed by 3 washes with PBS. For nuclear staining, DAPI at a 1:2000 dilution in PBS was added and incubated for 15 min at 4 °C. Finally, 100 µL of Fluoromount-G (ThermoFisher) was added as mounting medium.

Images were examined under an inverted microscope (DM2000 model) equipped with a DFC/300FX camera (Leica, Cambridge, UK). Image analysis was performed using ImageJ software. Phagocytic index was calculated by the equal to the ratio of the total number of absorbed particles and the number of macrophages.

Statistical analysis.

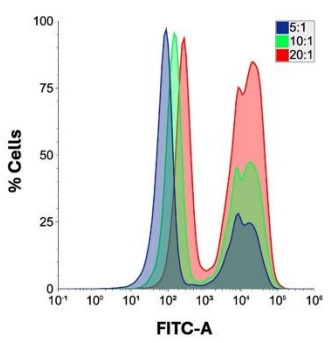
Quantitative data were expressed as mean ± standard deviation (SD). Statistical significance was determined using a one-way analysis of variance (ANOVA) or two-way ANOVA test using the GraphPad Prism 9. Values were considered statistically significant at $P < 0.05$. All experiments were independently repeated 3 times.

4. Results

Macrophages phagocyte zymosan particles

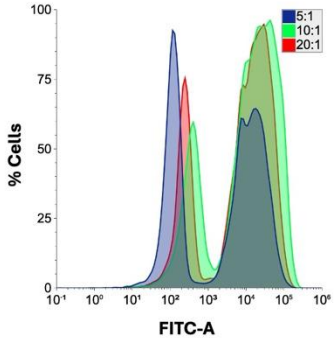
J774.A1 macrophages were stimulated with zymosan at different times and concentrations. It was observed that after 2 hours stimulation with a relation of 20 particles per cell was the optimal relation condition (Fig 1).

30 min



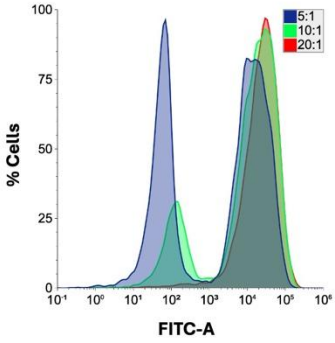
PARTICLES	%
5:1	33.9
10:1	50.8
20:1	61.1

60 min



PARTICLES	%
5:1	59.5
10:1	74.5
20:1	79.0

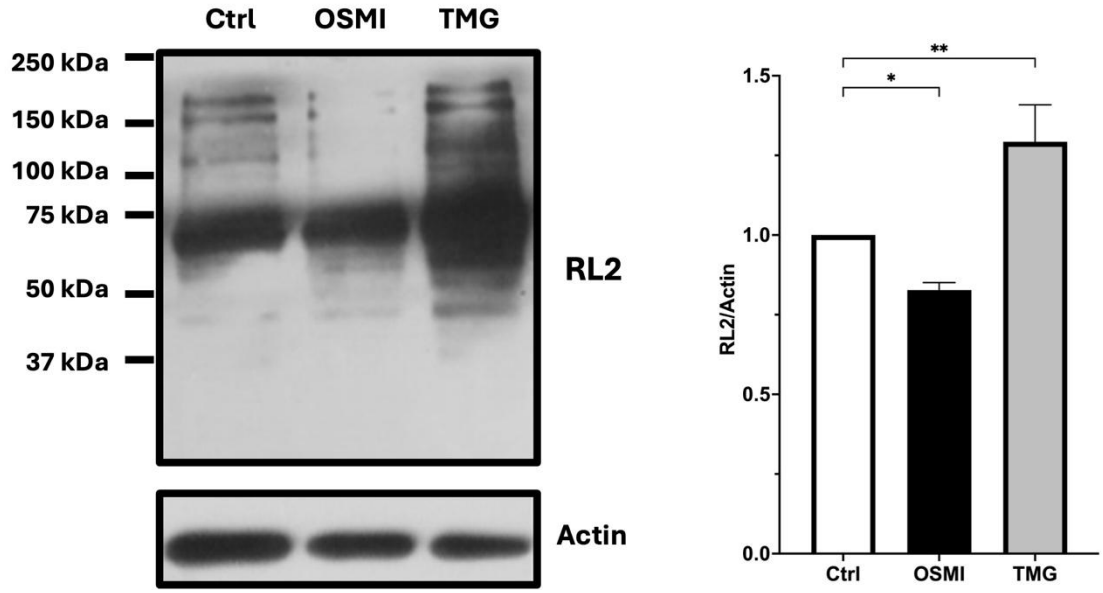
120 min



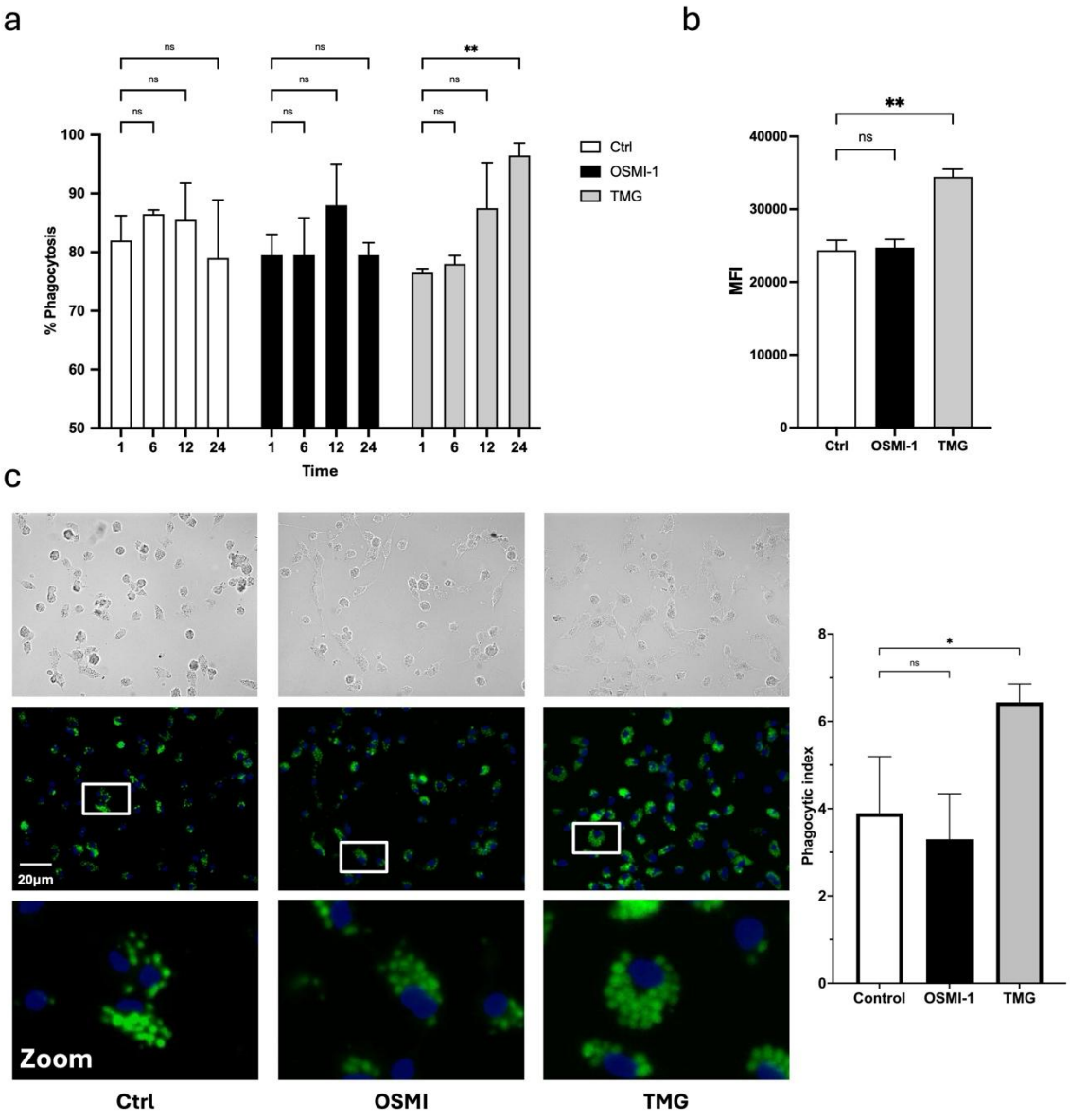
PARTICLES	%
5:1	57.9
10:1	79.6
20:1	91.6

Increased O-GlcNAcylation promotes zymosan phagocytosis.

The O-GlcNAcylation profile in J774.A1 macrophages was established by treating cells with 25 µM OSMI-1 (OGT inhibitor) or 10 µM TMG (OGA inhibitor) for 24 hours. These conditions were validated by Western blot using an anti-O-GlcNAc antibody (RL2) (Fig. S1).



A time-course inhibition assay using TMG and OSMI-1 was performed, and phagocytosis was evaluated by flow cytometry. Our results showed that OGA inhibition increased zymosan particle uptake after 24 hours (Fig. 2A). At this time point, the mean fluorescence intensity was 41% higher in TMG-treated cells compared to OSMI-1 and control groups (Fig. 2B).

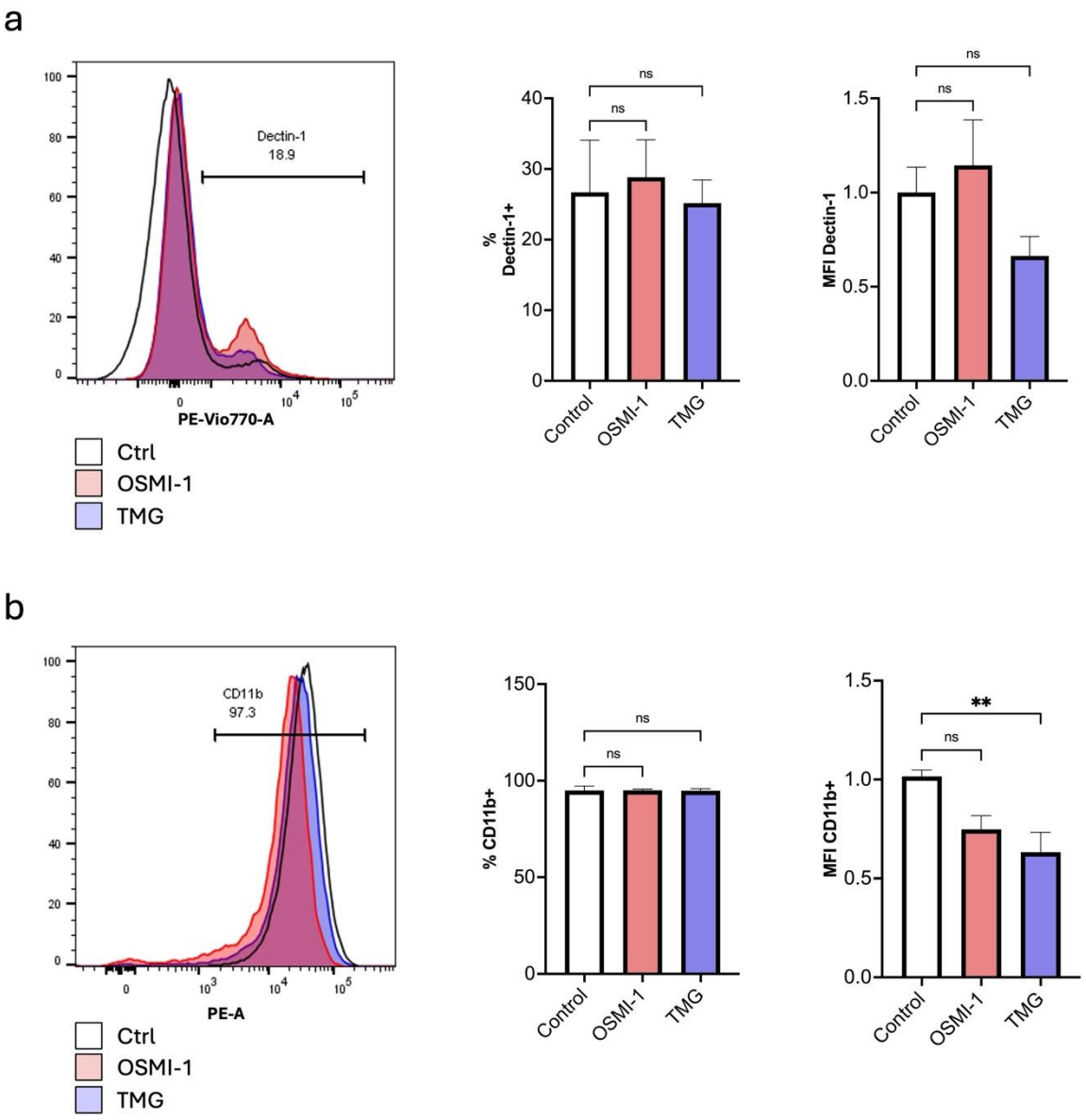


205

206 Fluorescence microscopy revealed that, under control conditions, the phagocytic
207 index was 3.8 particles per cell. OSMI-1 treatment showed a slight, non-significant
208 decrease of phagocytosis (3.2 particles per cell). In contrast, TMG treatment resulted
209 in a 65% and 95% increase in zymosan phagocytosis compared to control and OSMI-1,
210 respectively (Fig. 2C).

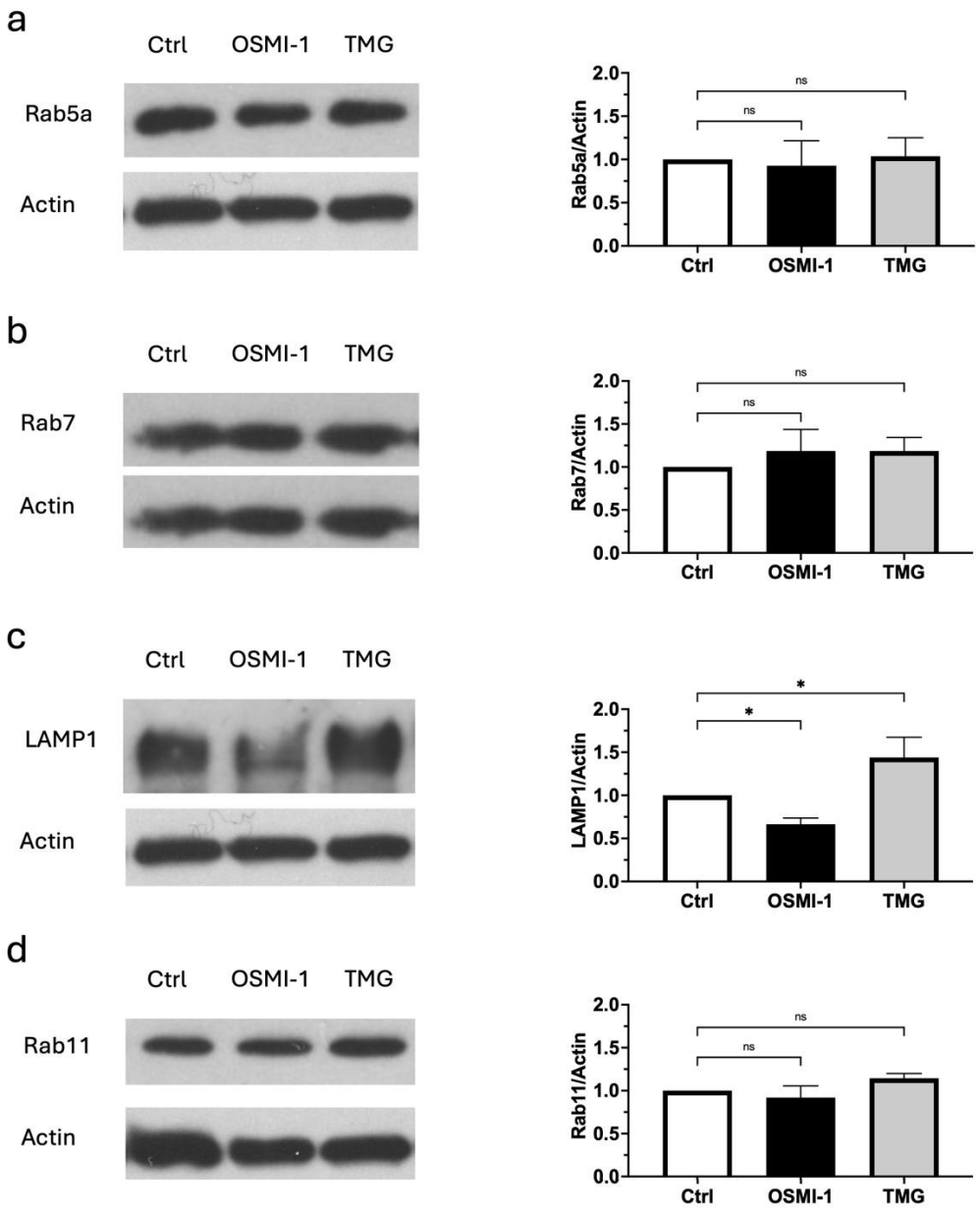
Inhibition O-GlcNAcylation decreases expression of zymosan receptors.

The expression of receptors that recognize zymosan was analyzed by flow cytometry (Fig. 3). Neither OSMI-1 nor TMG treatment significantly affected the percentage of Dectin-1$^+$ cells; however, TMG treatment resulted in a non-significant 34% decrease in mean fluorescence intensity (MFI) compared to control (Fig. 3A). Similarly, no significant changes were observed in the percentage of CD11b$^+$ cells following OSMI-1 or TMG treatment. In contrast, a significant 37% decrease in CD11b MFI was observed under TMG treatment (Fig. 3B).



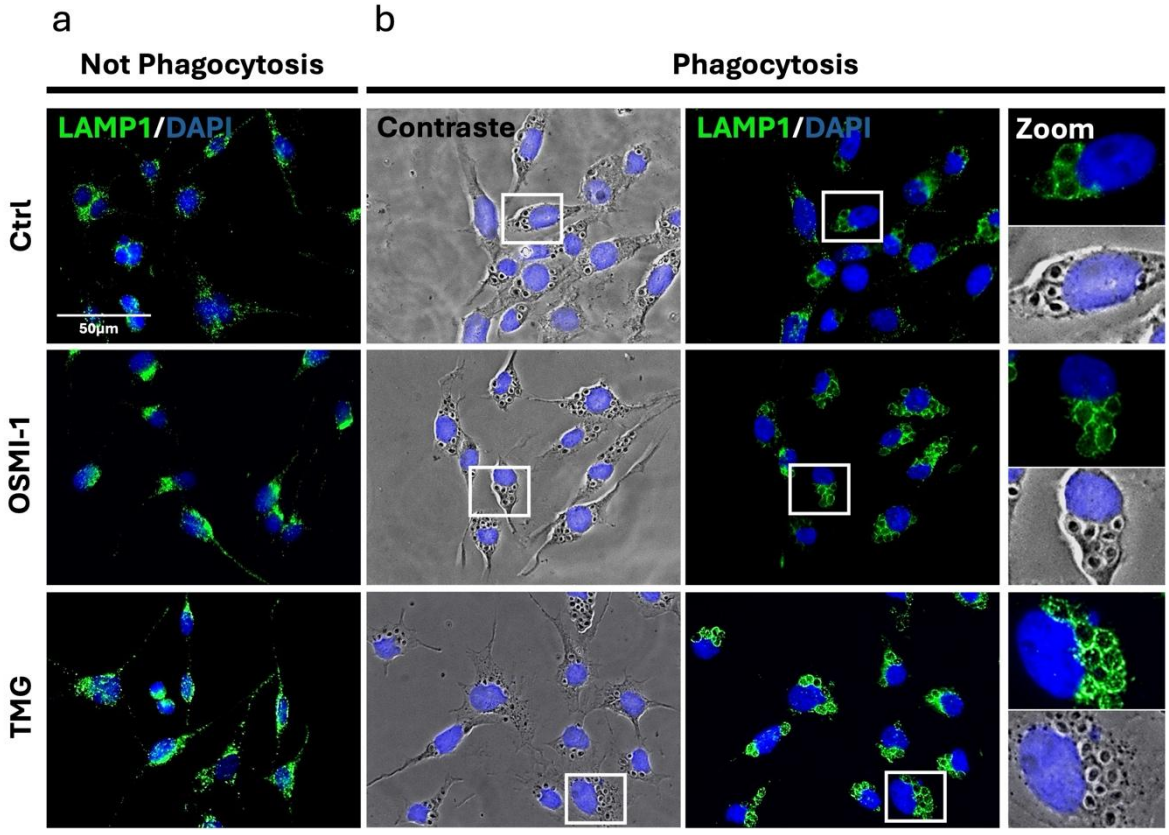
Increased O-GlcNAcylation increases LAMP1 protein expression.

The expression of proteins involved in phagosome maturation was determined by Western blot, as shown in Fig. 4. OSMI-1 treatment resulted in a significant 44% decrease in LAMP1 levels (Fig. 4C), whereas TMG treatment led to a significant 34% increase in LAMP1 expression (Fig. 4C). No significant changes were observed in Rab5a (Fig. 4A), Rab7 (Fig. 4B), or Rab11 (Fig. 4D) protein levels under either inhibition condition.



Increased O-GlcNAcylation recruits LAMP1 to the phagosome.

Further analysis by microscopy were performed, LAMP1 is constitutively expressed in Ctrl, OSMI-1, TMG treatment (Fig. 5A) in accordance with the western blot experiments (Fig. 4C). After 2 hours of zymosan phagocytosis, in all conditions was observed that phagosome formation and LAMP1 recruitment/localization occur regardless the presence of OSMI-1 or TMG (Fig 5B). However, TMG showed the most robust, well-defined LAMP1 formation around phagocytosed zymosan particles, whereas OSMI-1 showed fainter and sparser LAMP1 structures, consistent with globally reduced LAMP1 protein expression (Fig. 4C).



5. Discussion.

Phagocytosis is a vital mechanism for the elimination of foreign particles and the maintenance of cellular homeostasis [2]. Integrating metabolic aspects with the phagocytic process is essential for a comprehensive understanding of this mechanism [25]. O-GlcNAcylation serves as a key integrator of carbohydrates, amino acids, nucleic acids, and lipids metabolic pathways [29]. O-GlcNAcylation has been linked to the macrophage activation; however, there are few reports related to phagocytosis process [15, 20].

Our results showed that inhibition of OGA with TMG for 24 hours increase O-GlcNAcylation levels in J774A.1 macrophages and promotes an enhanced zymosan phagocytosis compared to the control group. On the contrary, decreasing O-GlcNAcylation with OSMI-1 showed a slight reduction of phagocytosis. In retinal cells, the knockout of OGT or its pharmacological inhibition blocks phagocytosis of latex beads [24]. In macrophages, the inhibition of OGT with OSMI-1 reduced the phagocytic index of bacteria [25]. However, in bone marrow derived macrophages (BMDM) from KO OGT mice internalized 75% more zymosan particles compared to WT BMDMs [22]. These results indicate that O-GlcNAcylation participates in phagocytic process.

Particle recognition is a critical step in phagocytosis. To determine whether the observed increase in phagocytosis was associated with receptor expression, we evaluated the levels of key zymosan receptors. Our results showed that treatment with OSMI-1 or TMG did not alter Dectin-1 expression, whereas CD11b expression decreased following OGA inhibition (TMG). Previous studies have reported that O-GlcNAcylation is involved in the regulation of PRR expression and localization in macrophages. For instance, peritoneal macrophages from OGT knockout mice showed slightly decrease of CD11b levels [21]. In immortalized bone marrow-derived macrophage (iBMDM), TMG augmented CD206 protein level [30]. Additionally, in J774A.1 macrophages, TMG has been shown to promote CD36 re-localization to early endosomes [31]. Therefore, our results indicate that the increase in phagocytic capacity is not mediated by changes in Dectin-1 or CD11b expression at the cell membrane. Further studies are required to determine whether other receptors, such as Scavenger receptors [32] contribute to the recognition of zymosan particles in this context.

Next, we analyzed several endosomal proteins that participate in phagolysosome maturation. Our results showed that OGA inhibition increased LAMP1 expression and promoted the formation of well-defined LAMP1 structures around zymosan particles. These findings indicate that increased O-GlcNAcylation enhances phagocytosis by

improving phagolysosome formation efficiency. Consistently, in human colon cells, OGT inhibition has been shown to disrupt Rab7 localization on lysosomes and late endosomes [33], suggesting that O-GlcNAcylation may also influence the subcellular distribution of key endolysosomal trafficking proteins.

Furthermore, it has been identified that both mTOR and TFEB participate in LAMP1 expression and localization, and that these proteins could be regulated directly or indirectly by O-GlcNAcylation [34–37]. Together, these results suggest a potential O-GlcNAc–mTOR/TFEB regulatory axis in phagolysosome maturation; however, further studies are required to more precisely elucidate the role of O-GlcNAcylation in this process during phagocytosis.

6. Conclusions.

This report demonstrates that O-GlcNAcylation plays a regulatory role in the phagocytosis of zymosan particles in macrophages. Increase of O-GlcNAcylation significantly enhanced zymosan internalization. Our results suggest that O-GlcNAcylation modulates phagocytosis through Dectin-1 and CR3 receptor-independent mechanisms, the observed changes in LAMP1 expression indicate that O-GlcNAcylation may influence phagolysosome maturation, potentially facilitating particle degradation. These findings open new perspectives for understanding how the hexosamine biosynthetic pathway shape's phagocytic function. Moreover, the modulation of O-GlcNAcylation could represent a novel strategy for developing treatments for several pathological contexts where impaired phagocytosis contributes to disease progression such as neurodegenerative diseases, cancer, or some infections caused by intracellular pathogens.

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8. Authors contributions

Conceptualization: [Vanessa Palma-Lojero, Javier Beltrán-Couoh, Johana Torres-Barrón, Alí Pereya, Wilton Gomez Henao, Eleazar Ramírez-Hernandez, Edgar Zenteno, José Luis Sánchez-Salgado]; Methodology: [Vanessa Palma-Lojero, Javier Beltrán-Couoh, Johana Torres-Barrón, Alí Pereya, Wilton Gomez Henao, Edgar Zenteno, José Luis Sánchez-Salgado]; Formal analysis and investigation: [Vanessa Palma-Lojero, Javier Beltrán-Couoh, Johana Torres-Barrón, Alí Pereya, Wilton Gomez Henao, Eleazar Ramírez-Hernandez, Edgar Zenteno, José Luis Sánchez-Salgado]; Writing - original draft preparation: [Vanessa Palma-Lojero, Johana Torres-Barrón, Alí Pereya, Wilton Gomez Henao, Edgar Zenteno, José Luis Sánchez-Salgado]; Writing - review and editing: [Vanessa Palma-Lojero, Johana Torres-Barrón, Alí Pereya, Wilton Gomez Henao, Edgar Zenteno, José Luis Sánchez-Salgado]; Funding acquisition: [Eleazar Ramírez-Hernandez, Edgar Zenteno, José Luis Sánchez-Salgado]; Resources: [Eleazar Ramírez-Hernandez, Edgar Zenteno, José Luis Sánchez-Salgado]; Supervision: [Edgar Zenteno, José Luis Sánchez-Salgado]

The authors have no relevant financial or non-financial interests to disclose.

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Figure captions

Fig. 1 J774.A1 Macrophages phagocyte Zymosan particles. Representative histogram overlay with diferent particles proportion by cell

Fig 2 Increased O-GlcNAcylation promotes Zymosan particles phagocytosis. *(a)* Percentage of J774.A1 macrophages that phagocyte Zymosan-FITC paticles treated with inhibitor of OGT (OSMI-1) and OGA (TMG). *(b)* MFI of J774.A1 macrophages ingesting Zymosan-FITC particles treated with inhibitor of OGT (OSMI-1) and OGA (TMG) for 24 hours. *(c)* Representative fluoresence microscopy of of J774.A1 macrophages ingesting Zymosan-FITC particles treated with inhibitor of OGT (OSMI-1) and OGA (TMG) for 24 hours. MFI: Median Fluoresence Intensity. Results are the means of three independent experiments. *$p < 0.05$, ** $p < 0.01$

Fig. 3 Inhibition O-GlcNAcylation decreases expression of Zymosan receptors. *(a)* Dectin-1 and *(b)* CD11b expression and MFI in J774.A1 macrophages treated with inhibitor of OGT (OSMI-1) and OGA (TMG) for 24 hours. Left panel, representative histogram overlay with Ctrl, OSMI-1, and TMG. Center panel, Porcentage of protein expression. Right panel, MFI: Mean Fluoresence Intensity. Results are the means of three independent experiments. ns: no significance; ** $p < 0.01$

Fig. 4 Increased O-GlcNAcylation increases LAMP1 protein expression. Protein expression level of *(a)* Rab5a, *(b)* Rab7, *(c)* LAMP1, and *(d)* Rab11 in total cell lysates from J774.A1 macrophages treated with inhibitor of OGT (OSMI-1) and OGA (TMG) for 24 hours. Left panel, representative Western blot is shown. Right panel, densitometric analysis of the blots. Results are the means of three independent experiments. ns: no significance; * $p < 0.05$

Fig. 5 Increased O-GlcNAcylation recruits LAMP1 to the phagosome. *(a)* J774.A1 macrophages treated with inhibitor of OGT (OSMI-1) and OGA (TMG) for 24 hours with out phagocytosis. *(b)* J774.A1 macrophages ingesting Zymosan-FITC particles treated with inhibitor of OGT (OSMI-1) and OGA (TMG) for 24 hours

Supplementary Information

SI1 Inhibition of OGT and OGA modulates O-GlcNAcylation profile. J774.A1 macrophages treated with inhibitor of OGT (OSMI-1) and OGA (TMG) for 24 hours. Proteins were extracted and analyzed by Western blot using an anti–O-GlcNAc Ab (RL2) and anti-actin for caculate relative epxression. Each blot is representative of three independent experiments. *$p < 0.05$, ** $p < 0.01$

Supplementary Files

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