Published in final edited form as:
J Immunol. 2014 February 15; 192(4): 1356–1360. doi:10.4049/jimmunol.1302835.

FYCO1 Recruitment to Dectin-1 Phagosomes is Accelerated by Light Chain 3 Protein and Regulates Phagosome Maturation and Reactive Oxygen Production

Jun Ma[*,†], **Courtney Becker**[*], **Christopher Reyes**[*], and **David M. Underhill**[*,§]

[*] F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048

[†]Biomedical Science and Translational Medicine Graduate Program, Cedars-Sinai Medical Center, Los Angeles, CA 90048

[§]Research Division of Immunology, Cedars-Sinai Medical Center, Los Angeles, CA 90048

Abstract

LC3-associated phagocytosis is a process in which LC3, a protein canonically involved in engulfing intracellular materials (autophagy), is recruited to traditional phagosomes during internalization of extracellular payloads. LC3 association with phagosomes has been implicated in regulating microbial killing, antigen processing, and phagosome maturation, however the mechanism by which LC3 influences these processes has not been clear. In this study, we report that FYCO1, a protein previously implicated in autophagosome trafficking, is recruited directly by LC3 to Dectin-1 phagosomes. During LC3-associated phagocytosis, FYCO1 recruitment facilitates maturation of early p40phox-positive phagosomes into late LAMP1-positive phagosomes. When FYCO1 is lacking, phagosomes stay p40phox-positive longer and produce more reactive oxygen.

Introduction

Autophagy is a cellular process for the clearance of cytosolic debris and damaged organelles. In immunological settings it has been implicated in clearance of cytosolic pathogens and regulation of inflammatory signaling (1-3). The process of autophagy involves the engulfment of targeted payloads into double membrane vesicles called autophagosomes that mature through fusion with lysosomes for degradation of their contents. The canonical autophagy pathway involves the lipidation and recruitment of Light Chain-3 protein (LC3) onto newly forming autophagosomes, and this recruitment is commonly used as a marker for the activation of autophagy (4,5).

Phagocytosis is the process by which cells engulf extracellular particles and form an intracellular phagosome that is bound by a single membrane. Similar to autophagosomes, phagosomes mature into highly degradative compartments. Recently, LC3 has been observed to be recruited to phagosomes formed during internalization of certain targets in a process that has been called LC3-associated phagocytosis (6,7). LC3 recruitment is specifically triggered by signaling through receptors including Toll-like receptors, Fc receptors, TIM4, and Dectin-1 (6-9). The consequences of LC3 recruitment to phagosomes are not entirely clear. Several studies have suggested that LC3 recruitment is important for killing bacterial and fungal pathogens, while others have focused on the potential for LC3 to

Address Correspondence to: David Underhill Phone: (310)423-7654 Fax: (310)423-0224 David.Underhill@csmc.edu.

affect phagosome maturation (6,7). We have previously shown that the fungal β-glucan receptor Dectin-1 triggers recruitment of LC3 to phagosomes and that this facilitates MHC-class II presentation of fungal-derived antigens (9). Despite recent advances in understanding LC3-associated phagocytosis, mechanisms by which LC3 could influence phagosome functions have not been determined.

FYVE and coiled–coil domain containing 1 (FYCO1) is a protein that has been recently implicated in autophagosomal trafficking (10). Pankiv et al. demonstrated that FYCO1 directly binds to LC3 on autophagosomes where it promotes the movement of the organelles to the plus ends of the microtubule transport system in HeLa cells (10). Human genetic studies have suggested a connection between variants of FYCO1 and congenital cataracts, and it has been proposed that alterations in vesicle trafficking may contribute to the pathology (11,12). In mouse macrophages, FYCO1 has been demonstrated to play a role in promoting lysosomal tubulation, a process of elongation of the lysosomal compartment (13).

Since there is evidence both demonstrating that FYCO1 interacts with LC3 on autophagosomes and that FYCO1 may play a role in the trafficking of intracellular compartments, we explored whether FYCO1 might be recruited to phagosomes in an LC3-dependent manner to affect phagosome functions. In this study, we report that FYCO1 is a novel phagosomal protein that directly interacts with LC3 on Dectin-1-triggered phagosomes where it facilitates maturation of early p40phox-positive phagosomes into late LAMP1-positive phagosomes. When FYCO1 is lacking, phagosomes stay p40phox positive longer and produce more reactive oxygen.

Materials and Methods

Reagents

All reagents were from Sigma unless noted. *Saccharomyces cerevisiae* (14) was kindly provided by Dr. van Haan (Vu University, Amsterdam, The Netherlands). β-glucan particles (GP) were produced from zymosan particles as previously described (15). These particles specifically activate Dectin-1 and do not engage Toll-like receptors. Other reagents include anti-LC3II (immunoblotting, MBL International clone 115; immunofluorescence, MBL International clone 153), anti-phospho Syk (immunoblotting and immunofluorescence, Cell Signaling), anti-phospho p40phox (Cell Signaling), anti-p40phox (Millipore), anti-GAPDH (Santa Cruz), anti-LAMP1 (Santa Cruz), and anti-GFP (Life Technology).

Cell culture

RAW264.7 cells expressing Dectin-1 (16) were cultured in RPMI 1640 medium (CellGrow) with 10% FCS, 10% penicillin/streptomycin, and 10% L-glutamine supplemented. Bone marrow-derived dendritic cells (BMDC) were derived from primary C57BL/6 mouse bone marrow cultured in RPMI 1640 medium with 10 ng/ml mGM-CSF (PeproTech) for 7days. Bone marrow-derived macrophages (BMM) were derived from primary C57BL/6 mouse bone marrow cultured in RPMI 1640 medium with 10 ng/ml MCSF (PeproTech) for 7days and were stimulated with 25 u/ml mouse interferon-γ (PeproTech) overnight .

Viral transduction and shRNA knockdown

A cDNA coding for FYCO1 was isolated from mouse BMDC mRNA and cloned into the pMSCV-LMP retroviral vector (Open Biosystem) allowing for expression of a protein tagged at the C-terminus with eGFP. Retrovirus was produced and used to infect RAW264.7 cells expressing Dectin-1 (DecRaw), BMM, and BMDC as previously described (16). Lentiviral vectors encoding shRNAs targeting *Map1lc3b* (LC3) and *Fyco1* were purchased from Sigma (Mission shRNA TRCN0000184601, TRCN0000120800) and were expressed

stably in DecRaw, BMM, and BMDC. Knock down levels were assessed by quantitative PCR normalized to *EF1α* or by immunoblotting.

Proximity ligation assay

Proximity ligation *assay* (Sigma Duolink) was performed as described previously (17). Briefly, cells on coverslips were fixed, permeabilized and stained with indicated primary antibodies followed by proximity ligation secondary antibodies. Ligation and amplification were performed to detect proximity and cells were visualized by fluorescence microscopy.

Immunofluorescence microscopy

Cells were plated on coverslips at 50,000 cells per coverslip overnight. 10 μg/ml of GP or indicated amounts of yeast were added and centrifuged at 500X g to synchronize particle contact. After stimulations, cells were fixed in 4% paraformaldehyde for 30 minutes and permeabilized in 0.1% saponin or ice cold acetone. Cells were stained with the indicated primary antibodies followed by fluorophore-conjugated secondary antibodies (Jackson). Cells were washed and mounted with prolong gold antifade reagent (Invitrogen). Images were collected with a Zeiss Axio Observer epifluorescence microscope system.

Immunoblotting

Cells were plated on 24 well plates overnight at 250,000 cells per well. 25 μg/ml of GP or indicated amounts of yeast were added as above. After stimulation, cells were lysed in LDS sample buffer (Invitrogen), boiled and loaded onto SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes (Millipore), blocked for 60 minutes with 2% BSA and stained overnight with indicated primary antibodies at 4 °C. Blots were washed, stained with HRP-conjugated secondary antibodies, and binding was detected by chemiluminescence (Thermo Scientific).

Phagocytosis & Reactive Oxygen assays

GP were biochemically conjugated to Alexa-647 fluorophore (Invitrogen). 25 μg/ml of conjugated GP was centrifuged onto cells as above, and cells were allowed to interact with particles for 15 minutes. Cells were washed, fixed in 4% paraformaldehyde, and fluorescence was assessed by flow cytometry. Reactive oxygen production was detected by luminol-enhanced chemiluminescence as previously described (16).

Statistical analysis

Statistical significance was determined by a two sided student t test with an α level of .05.

Results and Discussion

FYCO1 is recruited to Dectin-1 phagosomes

In order to identify proteins that might influence phagosome maturation during LC3-associated phagocytosis, we examined whether FYCO1 associates with phagosomes. We stably expressed GFP-tagged FYCO1 in RAW264.7 mouse macrophages expressing Dectin-1. When fed β-glucan particles for 20 minutes, these cells readily bound and phagocytosed the particles via Dectin-1, and we observed accumulation of FYCO1-GFP on the compartments (Fig. 1A). We observed similar results when we performed the same experiment in primary mouse macrophages and dendritic cells expressing FYCO1-GFP (Fig. 1A). To determine whether this effect was specific to pure β-glucan particles, we fed macrophages either live or heat-killed *S. cerevisiae* yeast and observed similar recruitment of FYCO1-GFP to phagosomes (Fig. 1B). To define more carefully the kinetics of FYCO1 recruitment to phagosomes, we fed β-glucan particles to macrophages and followed in real-

time the localization of FYCO1-GFP (Fig. 1C, and Supplementary Video 1). Initially, newly-formed phagosomes did not contain FYCO1 (time = 0 min.). FYCO1 began to accumulate within 5 minutes and became maximal within 15 minutes after phagosome formation.

LC3 recruitment to phagosomes downstream of Dectin-1 signaling is dependent on Syk activation and production of reactive oxygen species by the NADPH phagocyte oxidase (8,9). We therefore examined whether these signals are similarly important for stimulating recruitment of FYCO1 to phagosomes. Inhibition of Syk with the inhibitor piceatannol and inhibition of the NADPH oxidase with diphenyliodonium both caused a delay in FYCO1 recruitment to phagosomes (Fig. 1D, E) suggesting that these signals promote accelerated recruitment of the protein.

LC3 directly promotes the recruitment of FYCO1 to Dectin-1 phagosomes

To specifically examine the role of LC3 in directing FYCO1 recruitment to phagosomes, we first examined whether the two proteins localize to the same organelles. We observed colocalization of FYCO1-GFP with endogenous LC3 on phagosomes containing β-glucan particles (Fig. 2A). The proximity ligation assay is a method for directly visualizing protein-protein interactions (17). Using this approach we observed that the two proteins directly interact with each other on phagosomes (Fig. 2B) and that this interaction is suppressed when the NADPH oxidase is inhibited with diphenyliodonium and LC3 recruitment to phagosomes is blocked.

To directly test whether LC3 recruitment to phagosomes promotes subsequent recruitment of FYCO1, we generated macrophages expressing shRNA targeting *Map1lc3b* (the gene for LC3) in which LC3 expression was suppressed (Fig. 2C). While suppression of LC3 expression did not affect the ability of the cells to bind and internalize β-glucan particles (Fig. 2D), FYCO1 recruitment was delayed (Fig. 2E). Together, the data suggest that LC3 directly interacts with FYCO1 on phagosomes and accelerates its recruitment to phagosomes.

FYCO1-deficiency results in enhanced reactive oxygen production by the phagocyte oxidase

In order to understand the functional consequences of the interaction of FYCO1 with phagosomes, we generated macrophages and dendritic cells in which *Fyco1* expression was suppressed by shRNA (Fig. 3A). Suppression of *Fyco1* had no effect on phagocytosis of β-glucan particles indicating that binding and initial internalization were normal (Fig. 3B). However, production of reactive oxygen species by the NADPH phagocyte oxidase that assembles on newly-formed β-glucan particle-containing phagosomes was enhanced and prolonged in both macrophages (Fig. 3C) and dendritic cells (Fig. 3D) after *Fyco1* suppression. Similar results were obtained when the cells were infected with live yeast (Fig. 3E) or heat-killed yeast (Fig. 3F). These data suggest that FYCO1 plays an unexpected role in limiting ROS production on phagosomes.

FYCO1 expression regulates the duration of Dectin-1 signaling and phagosome maturation

Since Dectin-1 signaling directly activates the NADPH phagocyte oxidase, we hypothesized that FYCO1 expression influences the duration of Dectin-1 activation. To directly examine the effects of FYCO1 on Dectin-1 signaling, we measured phosphorylation of Syk, a kinase activated by Dectin-1 and required for NADPH phagocyte oxidase activation. Syk became activated rapidly upon Dectin-1 engagement and then became deactivated over the course of 60 minutes as measured both by immunoblotting (Fig. 4A) and by immunofluorescence

microscopy (Fig. 4B). Upon suppression of FYCO1expression, Syk activation was noticeably prolonged.

Further, to directly assess the activation of the NADPH oxidase complex on phagosomes, we measured the phosphorylation of the cytosolic p40 subunit of the NADPH oxidase and its recruitment to phagosomal membranes. Suppression of FYCO1 expression notably prolonged activation of p40phox as measured by immunoblotting (Fig 4A) and by immunofluorescence microscopy (Fig. 4B). These data are consistent with the observed enhanced and prolonged production of reactive oxygen species noted above.

We and others have observed that LC3 recruitment to phagosomes accelerates their association with later compartments (6), and we therefore hypothesized that FYCO1 recruitment is responsible for accelerated phagosome maturation. Indeed, we found that FYCO1 always colocalized with mature phagosomes, as indicted by the presence of LAMP1, a late endosomal and lysosomal marker, while it did not colocalize with p40phox on phagosomes (Fig. 4C). Even though we have demonstrated that FYCO1 colocalized with LC3, we observed that phagosomes became LC3-positive first, then became LC3-FYCO1-LAMP1-positive transiently, and then finally remained FYCO1-LAMP1-positive (Supplemental Fig. 1A). Further, suppression of FYCO1 expression by shRNA resulted in significant inhibition in the acquisition of LAMP1 by Dectin-1 phagosomes (Fig. 4D). The data suggest that FYCO1 plays a role in maturing early p40phox-positive phagosomes into late LAMP1-positive phagosomes. When FYCO1 is lacking, phagosomes stay p40phox-positive longer and produce more reactive oxygen.

In this study we have identified FYCO1 as a novel phagosome-associated protein. The data demonstrate that FYCO1 is important for regulating the rate of phagosome maturation. LC3 on phagosomes directly interacts with FYCO1, suggesting that a key function of LC3 when localized to phagosomes is to recruit FYCO1-associated LAMP1 positive compartments thus accelerating maturation. This explains why previous studies have observed a delay in phagosome maturation in macrophages deficient in the ability to recruit LC3 (6). Considering these data, we conclude that when a phagocyte engulfs a ROS-inducing pathogen, it activates LC3-associated phagocytosis. This leads to accelerated phagosome maturation via FYCO1-LAMP1 recruitment, and this maturation curtails ROS production (Supplemental Fig. 1B).

Rosas et al. have previously noted that Dectin-1 signaling is turned off upon internalization of the receptor (18). Our data suggest further that it is not only internalization, but also the maturation of the phagosome that facilitates turning off Dectin-1 signaling. As a result, in the absence of FYCO1 when phagosome maturation is inhibited, Dectin-1 signaling remains active longer.

Mrakovic et al. have previously observed that FYCO1 is associated with lysosomes in macrophages and is involved in the tubulation of this compartment (13). Additionally Pankiv et al. have demonstrated that FYCO1 is involved in the trafficking of autophagosomes (10). Our study adds an innate immune dimension to the role of FYCO1, implicating it in regulating phagosome maturation and production of reactive oxygen, processes important for handling extracellular pathogens. Future studies should investigate the role of FYCO1 in the antimicrobial activity of myeloid phagocytes and investigate the mechanisms by which FYCO1 influences maturation of phagosomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants AI071116 & GM085796 to DMU.

References

1. Jounai N, Takeshita F, Kobiyama K, Sawano A, Miyawaki A, Xin KQ, Ishii KJ, Kawai T, Akira S, Suzuki K, Okuda K. The Atg5 Atg12 conjugate associates with innate antiviral immune responses. Proc Natl Acad Sci U S A. 2007; 104:14050–14055. [PubMed: 17709747]
2. Kumar D, Nath L, Kamal MA, Varshney A, Jain A, Singh S, Rao KV. Genome-wide analysis of the host intracellular network that regulates survival of Mycobacterium tuberculosis. Cell. 2010; 140:731–743. [PubMed: 20211141]
3. Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, Kamimoto T, Nara A, Funao J, Nakata M, Tsuda K, Hamada S, Yoshimori T. Autophagy defends cells against invading group A Streptococcus. Science. 2004; 306:1037–1040. [PubMed: 15528445]
4. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008; 451:1069–1075. [PubMed: 18305538]
5. Shahnazari S, Brumell JH. Mechanisms and consequences of bacterial targeting by the autophagy pathway. Curr Opin Microbiol. 2010; 14:68–75. [PubMed: 21112809]
6. Sanjuan MA, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, Komatsu M, Tanaka K, Cleveland JL, Withoff S, Green DR. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature. 2007; 450:1253–1257. [PubMed: 18097414]
7. Martinez J, Almendinger J, Oberst A, Ness R, Dillon CP, Fitzgerald P, Hengartner MO, Green DR. Microtubule-associated protein 1 light chain 3 alpha (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. Proc Natl Acad Sci U S A. 2011; 108:17396–17401. [PubMed: 21969579]
8. Huang J, Canadien V, Lam GY, Steinberg BE, Dinauer MC, Magalhaes MA, Glogauer M, Grinstein S, Brumell JH. Activation of antibacterial autophagy by NADPH oxidases. Proc Natl Acad Sci U S A. 2009; 106:6226–6231. [PubMed: 19339495]
9. Ma J, Becker C, Lowell CA, Underhill DM. Dectin-1-triggered recruitment of light chain 3 protein to phagosomes facilitates major histocompatibility complex class II presentation of fungal-derived antigens. J Biol Chem. 2012; 287:34149–34156. [PubMed: 22902620]
10. Pankiv S, Alemu EA, Brech A, Bruun JA, Lamark T, Overvatn A, Bjorkoy G, Johansen T. FYCO1 is a Rab7 effector that binds to LC3 and PI3P to mediate microtubule plus end-directed vesicle transport. J Cell Biol. 2010; 188:253–269. [PubMed: 20100911]
11. Chen J, Ma Z, Jiao X, Fariss R, Kantorow WL, Kantorow M, Pras E, Frydman M, Riazuddin S, Riazuddin SA, Hejtmancik JF. Mutations in FYCO1 cause autosomal-recessive congenital cataracts. Am J Hum Genet. 2011; 88:827–838. [PubMed: 21636066]
12. Aldahmesh MA, Khan AO, Mohamed JY, Hijazi H, Al-Owain M, Alswaid A, Alkuraya FS. Genomic analysis of pediatric cataract in Saudi Arabia reveals novel candidate disease genes. Genet Med. 2012; 14:955–962. [PubMed: 22935719]
13. Mrakovic A, Kay JG, Furuya W, Brumell JH, Botelho RJ. Rab7 and Arl8 GTPases are necessary for lysosome tubulation in macrophages. Traffic. 2012; 13:1667–1679. [PubMed: 22909026]
14. van Leeuwen F, Gafken PR, Gottschling DE. Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell. 2002; 109:745–756. [PubMed: 12086673]
15. Goodridge HS, Shimada T, Wolf AJ, Hsu YM, Becker CA, Lin X, Underhill DM. Differential use of CARD9 by dectin-1 in macrophages and dendritic cells. J Immunol. 2009; 182:1146–1154. [PubMed: 19124758]
16. Underhill DM, Rossnagle E, Lowell CA, Simmons RM. Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. Blood. 2005; 106:2543–2550. [PubMed: 15956283]
17. Misawa T, Takahama M, Kozaki T, Lee H, Zou J, Saitoh T, Akira S. Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. Nat Immunol. 2013; 14:454–460. [PubMed: 23502856]

18. Rosas M, Liddiard K, Kimberg M, Faro-Trindade I, McDonald JU, Williams DL, Brown GD, Taylor PR. The induction of inflammation by dectin-1 in vivo is dependent on myeloid cell programming and the progression of phagocytosis. J Immunol. 2008; 181:3549–3557. [PubMed: 18714028]

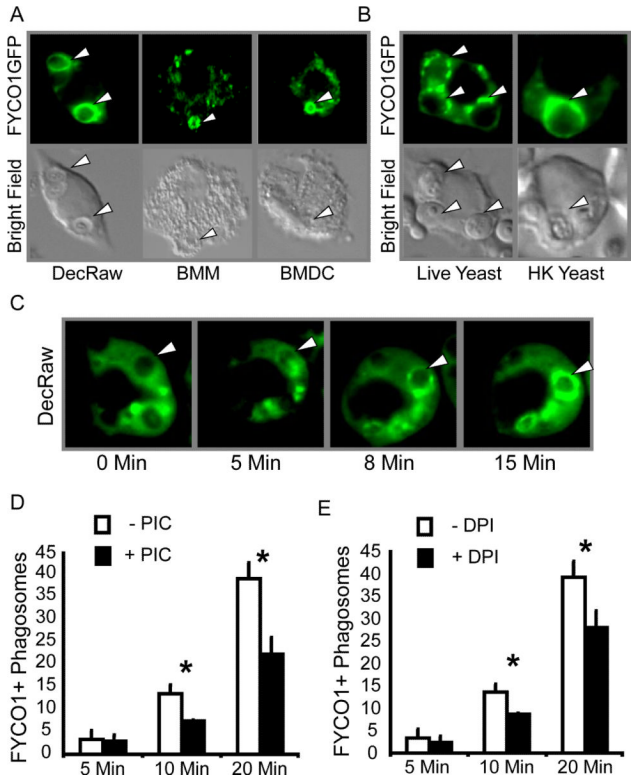

Figure 1.

FYCO1 is recruited to Dectin-1 phagosomes in a Dectin-1 signaling dependent manner. (**A-B**) RAW264.7 macrophages stably expressing Dectin-1 (DecRaw), interferon-γ-primed bone marrow-derived macrophages (BMM) and bone marrow-derived dendritic cells (BMDC) stably expressing GFP-tagged FYCO1 were stimulated with 10 μg/ml of β-glucan particles (A) or DecRaw cells were infected with live or heat-killed yeast at MOI of 5 (B) for 20 minutes. Cells were fixed, mounted, and localization of GFP-tagged FYCO1 was observed by fluorescence microscopy. Arrowheads indicate phagosomes. (**C**) RAW264.7 macrophages stably expressing GFP-tagged FYCO1 were stimulated with 10 μg/ml of GP, and GFP-localization was followed in real time by fluorescence microscopy. Arrowhead indicates the maturation of a single phagosome over 15 minutes. (**D-E**) RAW264.7 macrophages were pretreated with or without 25 μM piceatannol (PIC) (D) or 25 μM diphenyliodonium (DPI) (E) 10 minutes before stimulating with β-glucan particles. Cells were fixed at the indicated times post-stimulation, and localization of GFP-tagged FYCO1 was observed by fluorescence microscopy. GFP-positive phagosomes were counted, and numbers were normalized to per 100 cells counted.

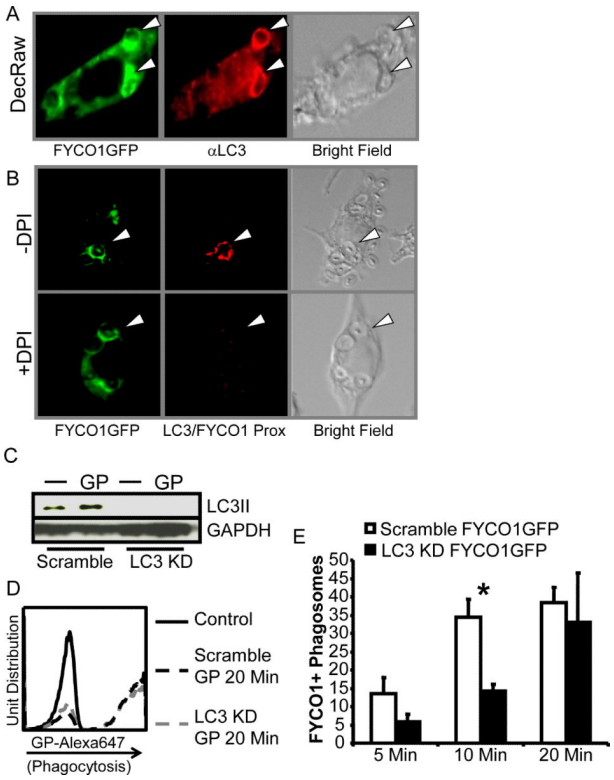

Figure 2.

LC3 directly promotes the recruitment of FYCO1 to Dectin-1 phagosomes. (**A**) RAW264.7 macrophages stably expressing GFP-tagged FYCO1 were stimulated with 10 μg/ml of β-glucan particles for 20 minutes. Cells were fixed, permeabilized and stained for endogenous LC3. Arrowheads indicate phagosomes with FYCO1 and LC3 colocalization. (**B**) RAW264.7 macrophages were stimulated as in (A) with or without pretreatment with 25 μM DPI. Proximity ligation assay was performed to detect the interaction of endogenous LC3 with FYCO1-GFP on phagosomes. Arrowheads indicate GFP-tagged FYCO1-positive phagosomes. (**C**) RAW264.7 macrophages stably expressing GFP-tagged FYCO1 and expressing either a scramble control shRNA or an LC3-targeting shRNA were stimulated with β-glucan particles (GP) for 20 minutes and expression of lipidated LC3II was assessed by immunoblotting. GAPDH levels were determined as a loading control. (**D**) GFP-tagged FYCO1-expressing macrophages with or without LC3 knocked down were fed Alexa647-labeled GP for 20 minutes, and particle internalization was determined by flow cytometry. Scramble shRNA-expressing cells not fed particles were used as a control. (**E**) GFP-tagged FYCO1-expressing macrophages with or without LC3 knocked down were fed GP for the indicated times. Cells were fixed and GFP accumulation was assessed by fluorescence microscopy. GFP+ phagosomes were counted and were normalized to per 100 cells counted.

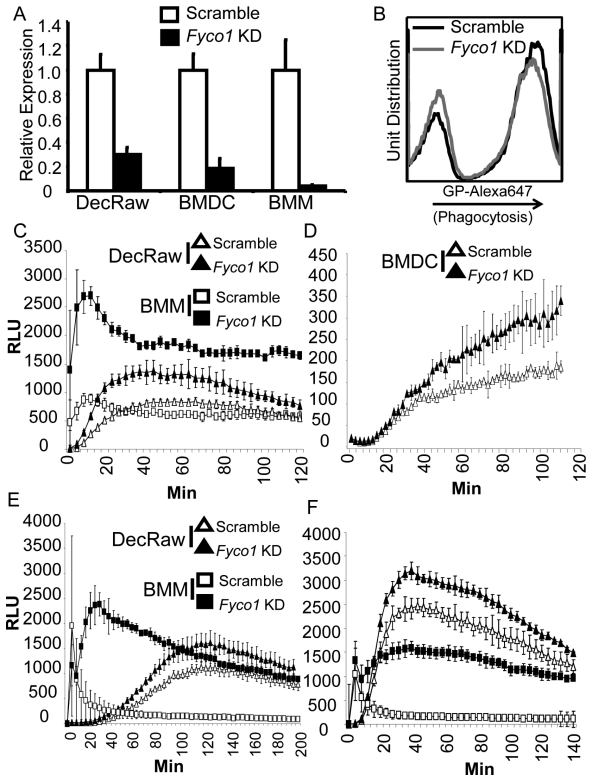

Figure 3.

FYCO1 on Dectin-1 phagosomes regulates reactive oxygen production. (**A**) FYCO1 expression was knocked down in RAW264.7 macrophages, BMM and BMDC by shRNA expression. Knockdown was confirmed by measuring mRNA levels by quantitative PCR. (**B**) RAW264.7 macrophages with our without FYCO1 knocked down were fed Alexa647-labeled GP for 20 minutes. Phagocytosis was assessed by flow cytometry. (**C-D**) RAW264.7 macrophages, BMM (C) or dendritic cells (D) expressing control (Scramble) or *Fyco1*-targeting shRNAs were fed GP, and reactive oxygen production was detected by luminol-enhanced chemiluminescence and expressed in relative light units (RLU). (**E-F**) RAW264.7 macrophages or BMM expressing control or *Fyco1*-targeting shRNAs were fed live yeast at an MOI of 5 (E) or heat-killed yeast at an MOI of 5 (F). Reactive oxygen production was detected as above.

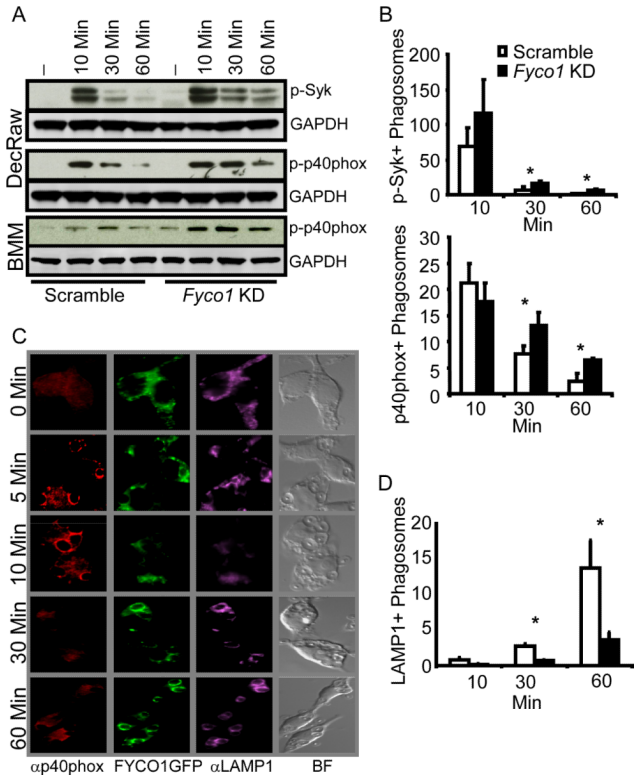

Figure 4.

FYCO1 expression regulates the duration of Dectin-1 signaling and phagosome maturation. (**A**) RAW264.7 macrophages or BMM with or without FYCO1 knocked down were stimulated with GP for the indicated times. Phospho-Syk levels and phospho-p40phox levels were detected by immunoblotting. GAPDH levels were measured as a loading control. (**B**) RAW264.7 macrophages with or without FYCO1 knocked down were stimulated with GP for the indicated times. Phospho-Syk-positive or p40phox-positive phagosomes were detected by immunofluorescence microscopy. Positive phagosomes were normalized to per 100 cells counted. (**C**) GFP-tagged FYCO1-expressing RAW264.7 macrophages were stimulated with GP for the indicated times. p40phox and LAMP1 colocalization with GFP-tagged FYCO1 was assessed by immunofluorescence microscopy. Representative images from each time point are shown. (**D**) BMM with or without FYCO1 knocked down were stimulated with GP for the indicated times. LAMP1-positive phagosomes were observed by immunofluorescence microscopy. Positive phagosomes were normalized to per 100 cells counted.