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*J Immunol* 2014; 192:1356-1360; Prepublished online 17 January 2014; doi: 10.4049/jimmunol.1302835

http://www.jimmunol.org/content/192/4/1356

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/01/20/jimmunol.1302835.DCSupplemental

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*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Cutting Edge: FYCO1 Recruitment to Dectin-1 Phagosomes Is Accelerated by Light Chain 3 Protein and Regulates Phagosome Maturation and Reactive Oxygen Production

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L chain 3 (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’s association with phagosomes has been implicated in regulating microbial killing, Ag processing, and phagosome maturation; however, the mechanism by which LC3 influences these processes has not been clear. In this study, we report that FYVE and coiled–coil domain containing 1 (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+ phagosomes into late LAMP1+ phagosomes. When FYCO1 is lacking, phagosomes stay p40phox+ longer and produce more reactive oxygen. The Journal of Immunology, 2014, 192: 1356–1360.

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,” which mature through fusion with lysosomes for degradation of their contents. The canonical autophagy pathway involves the lipiddation and recruitment of L chain 3 (LC3) protein 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 was 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 TLRs, FcR, TIM4, and Dectin-1 (6–9). The consequences of LC3 recruitment to phagosomes are not entirely clear. Several studies suggested that LC3 recruitment is important for killing bacterial and fungal pathogens, whereas others focused on the potential for LC3 to affect phagosome maturation (6, 7). We showed previously 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 Ags (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 recently was implicated in autophagosomal trafficking. Pankiv et al. (10) 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. Human genetic studies suggested a connection between variants of FYCO1 and congenital cataracts, and it was proposed that alterations in vesicle trafficking may contribute to the pathology (11, 12). In mouse macrophages, FYCO1 was demonstrated to play a role in promoting lysosomal tubulation, a process of elongation of the lysosomal compartment (13).

Because there is evidence demonstrating that FYCO1 interacts with LC3 on autophagosomes and that it 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+ phagosomes into late LAMP1+ phagosomes. When FYCO1 is lacking, phagosomes stay p40phox+ 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 (GPs) were produced from zymosan particles, as previously described (15). These particles specifically activate Dectin-1 and do not engage TLRs. Other reagents include anti-LC3H (immunoblotting, MBL International clone 115; immunofluorescence, MBL International clone 153), anti–phospho-p40phox (immunoblotting and immunofluorescence; Cell Signaling), anti–phospho-p40phox (Cell Signaling), anti-p40phox (Millipore), anti–GADPH (Santa Cruz Biotechnology), anti-LAMP1 (Santa Cruz Biotechnology), and anti-GFP (Life Technology).

Cell culture

RAW264.7 cells expressing Dectin-1 (16) were cultured in RPMI 1640 medium (cellgro) with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Bone marrow–derived dendritic cells (BMDCs) were derived from primary C57BL/6 mouse bone marrow cultured in RPMI 1640 medium with 10 ng/ml mouse GM-CSF (PeproTech) for 7 d. Bone marrow–derived macrophages (BMMs) were derived from primary C57BL/6 mouse bone marrow cultured in RPMI 1640 medium with 10 ng/ml M-CSF (PeproTech) for 7 d and were stimulated with 25 μM mouse IFN-γ (PeproTech) overnight.

Viral transduction and short hairpin RNA knockdown

A cDNA coding for FYCO1 was isolated from mouse BMDC mRNA and cloned into the pMSCV-LMP retroviral vector (Open Biopolymer), 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 (Dec RAW), BMMs, and BMDCs, as previously described (16). Lentiviral vectors encoding short hairpin RNAs (shRNAs) targeting Map4k6 (LC3) and Fyco1 were purchased from Sigma (Mission shRNA TRCN0000184601, TRCN0000120800) and were expressed stably in Dec RAW, BMMs, and BMDCs. 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 the indicated primary Abs, followed by proximity ligation secondary Abs. 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/coverslip overnight. A total of 10 μg/ml GPs or indicated amounts of yeast were added and centrifuged at 500 × g to synchronize particle contact. After stimulation, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.1% saponin or ice-cold acetone. Cells were stained with the indicated primary Abs, followed by fluorescent-conjugated secondary Abs (Jackson ImmunoResearch). Cells were washed and mounted with ProLong Gold Antifade Reagent (Invitrogen). Images were collected with a Zeiss Axio Observer epifluorescence microscope system using a ×63 objective.

Immunoblotting

Cells were plated on 24-well plates overnight at 250,000 cells/well. A total of 25 μg/ml GPs 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 polyvinylidene difluoride membranes (Millipore), blocked for 60 min with 2% BSA, and stained overnight with the indicated primary Abs at 4°C. Blots were washed and stained with HRP-conjugated secondary Abs, and binding was detected by chemiluminescence (Thermo Scientific).

Phagocytosis and reactive oxygen assay

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

Statistical analysis

Statistical significance was determined by a two-sided Student t test with an α level of 0.05. An asterisk denotes a statistically significant difference.

Results and Discussion

FYCO1 is recruited to Dectin-1 phagosomes

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 GPs for 20 min, 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 GPs, 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 GPs to macrophages and followed, in real-time, the localization of FYCO1-GFP (Fig. 1C, Supplemental Video 1). Initially, newly formed phagosomes did not contain FYCO1 (time = 0 min). FYCO1 began to
accumulate within 5 min and became maximal within 15 min after phagosome formation.

LC3 recruitment to phagosomes downstream of Dectin-1 signaling is dependent on Syk activation and production of reactive oxygen species (ROS) by the NADPH phagocyte oxidase (8, 9). Therefore, we 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 (DPI) both caused a delay in FYCO1 recruitment to phagosomes (Fig. 1D, 1E), 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 GPs (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 DPI 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). Although suppression of LC3 expression did not affect the ability of the cells to bind and internalize GPs (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

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 GPs, indicating that binding and initial internalization were normal (Fig. 3B). However, production of reactive oxygen production by the phagocyte oxidase was increased in FYCO1-deficient cells (Fig. 3C), and this increase was enhanced when yeast were ingested (Fig. 3D). Together, these data suggest that FYCO1 is required for the efficient production of reactive oxygen species on Dectin-1 phagosomes.
ROS by the NADPH phagocyte oxidase that assembles on newly formed GP-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**

Because 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 min, as measured both by immunoblotting (Fig. 4A) and by immunofluorescence microscopy (Fig. 4B). Upon suppression of FycO1 expression, 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 ROS noted above.

We and others investigators observed that LC3 recruitment to phagosomes accelerates their association with later compartments (6); therefore, we 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, whereas it did not colocalize with p40phox on phagosomes (Fig. 4C). Although we demonstrated that FycO1 colocalized with LC3, we observed that phagosomes became LC3+ first, then became LC3–FycO1–LAMP1+ transiently, and finally remained LC3–FycO1–LAMP1+ (Supplemental Fig. 1A). Further, suppression of FycO1 expression by shRNA resulted in significant inhibition of the acquisition of LAMP1 by Dectin-1 phagosomes (Fig. 4D). The data suggest that FycO1 plays a role in maturing early p40phox+ phagosomes into late LAMP1+ phagosomes. When FycO1 is lacking, phagosomes stay p40phox+ longer and produce more reactive oxygen.

In this study, we 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+ compartments, thus accelerating maturation. This explains why previous studies 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. (18) noted that Dectin-1 signaling is turned off upon internalization of the receptor. Our data suggest further that 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. (13) observed that FycO1 is associated with lysosomes in macrophages and is involved in the tubulation of this compartment. Additionally, Pankiv et al. (10) demonstrated that FycO1 is involved in the trafficking of autophagosomes. Our study adds an innate immune dimension to the role of FycO1, implicating it in the regulation of 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.

### Disclosures

The authors have no financial conflicts of interest.

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**FIGURE 4.** FycO1 expression regulates the duration of Dectin-1 signaling and phagosome maturation. (A) RAW264.7 macrophages or BMMs, with or without FycO1 knocked down, were stimulated with GPs 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 GPs for the indicated times. Phospho-Syk and phospho-p40phox levels were detected by immunoblotting (Fig. 4A) and by immunofluorescence microscopy (Fig. 4B). These data are consistent with the observed enhanced and prolonged production of ROS noted above.

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References


Supplementary Figure 1. (A) GFP-tagged FYCO1-expressing RAW264.7 macrophages were stimulated with GP for the indicated times. P40phox, LAMP1 colocalization with GFP-tagged FYCO1 was assessed by immunofluorescence microscopy. Representative images from each time point are shown. (B) A proposed model of phagosome maturation during normal phagocytosis of a reactive oxygen-inducing pathogen, phagocytosis in the absence of LC3, and phagocytosis in the absence of FYCO1.
Supplementary Video Legend

Supplementary Video 1. RAW264.7 macrophages expressing GFP-tagged FYCO1 were plated on chambered coverglass (Lab Tek) at 50,000 cells per chamber overnight. Cells were fed 10 μg/ml of β-glucan particles, and GFP localization was followed in real time by fluorescence microscopy. Images were collected at 15 second intervals and are shown at 5 frames per second.