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Dectin-1-triggered Recruitment of Light Chain 3 Protein to Phagosomes Facilitates Major Histocompatibility Complex Class II Presentation of Fungal-derived Antigens*

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Background: Dectin-1 is a phagocytic receptor important for host immune responses to fungal infections.
Results: Dectin-1 signaling directs LC3 to phagosomes leading to increased MHC class II recruitment and increased presentation of fungal-derived antigens.
Conclusion: Dectin-1 signaling enhances the efficiency of MHC class II presentation of fungal-derived antigens.
Significance: Dectin-1 signaling can influence how phagosomal contents are handled.

Dectin-1 is a pattern recognition receptor that is important for innate immune responses against fungi in humans and mice. Dectin-1 binds to β-glucans in fungal cell walls and triggers phagocytosis, production of reactive oxygen by the NADPH oxidase, and inflammatory cytokine production which all contribute to host immune responses against fungi. Although the autophagy pathway was originally characterized for its role in the formation of double-membrane compartments engulfing cytosolic organelles and debris, recent studies have suggested that components of the autophagy pathway may also participate in traditional phagocytosis. In this study, we show that Dectin-1 signaling in macrophages and bone marrow-derived dendritic cells triggers formation of LC3II, a major component of the autophagy machinery. Further, Dectin-1 directs the recruitment of LC3II to phagosomes, and this requires Syk, activation of reactive oxygen production by the NADPH oxidase, and ATG5. Using LC3-deficient dendritic cells we show that whereas LC3 recruitment to phagosomes is not important for triggering phagocytosis, killing or Dectin-1-mediated inflammatory cytokine production, it facilitates recruitment of MHC class II molecules to phagosomes and promotes presentation of fungal-derived antigens to CD4 T cells.

Dectin-1 is an innate immune receptor that recognizes β-glucan in fungal cell walls (1, 2). Mice and humans lacking Dectin-1 are more susceptible to a wide variety of fungal infections. Dectin-1 is a C-type lectin receptor that is primarily expressed by myeloid cells such as macrophages, dendritic cells, and neutrophils. Upon ligation, Dectin-1 triggers phagocytosis, activates reactive oxygen production by the NADPH oxidase, and stimulates production of inflammatory cytokines and chemokines. In addition to its role in innate antimicrobial

responses to fungi, Dectin-1 also influences adaptive immune responses through stimulating production of IL-1β, IL-6, and IL-23 which play important roles polarizing Th17 T cells (3, 4).

Autophagy is a cellular process in which cytosolic material is engulfed in double membrane vesicles called autophagosomes, degraded, and recycled for energy. The signaling pathway that triggers autophagy is highly conserved and has been widely studied in organisms ranging from yeast to mammals (5, 6). Two essential components of the autophagy pathway that are important for this paper are autophagy-related protein (ATG)[2] 5 and light chain (LC)3. ATG5 is an upstream signaling component that is part of a complex that directs lipidation of cytosolic LC3I to form membrane-bound LC3II which decorates newly forming autophagosomes.

In innate immunity, autophagy has been shown to be important for host defense against intracellular bacteria including group A *Streptococcus* (7) and *Mycobacterium tuberculosis* (8). In addition to orchestrating engulfment of intracellular organisms, it is becoming increasingly clear that components of the autophagy pathway regulate aspects of host defense not directly related to autophagy. For example, ATG5 complexes have been reported to directly bind and negatively regulate RIG-I and IPS-1, signaling proteins important for defense against viruses (9). Also, ATG5 has been implicated in recruiting interferon-inducible GTPases to *Toxoplasma gondii* parasitophorous vacuoles that are important for destruction of this membrane (10, 11).

Recent findings have begun to suggest that proteins that play a role in autophagy (double membrane engulfment of intracellular material) may also play significant roles in phagocytosis (single membrane engulfment of extracellular material). These studies showed that TLR2, Fcγ receptors, and TIM4 can stimulate recruitment of LC3 to phagosomes (12–14). For example, Sanjuan *et al.* showed that LC3 can be recruited to macrophage

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[2] The abbreviations used are: ATG, autophagy-related protein; BMDC, bone marrow-derived dendritic cells; DPI, diphenyleneiodonium; LC, light chain; m.o.i., multiplicity of infection; Ova, ovalbumin; ROS, reactive oxygen species; Syk, spleen tyrosine kinase; TLR, Toll-like receptor.

phagosomes containing beads coated with the TLR2 ligand, PAM$_3$CSK$_4$ (12). Similarly, Huang *et al.* showed that Fcγ receptor stimulation triggers recruitment of LC3 to phagosomes and further showed that in neutrophils this signal requires reactive oxygen (ROS) production by the phagocyte NADPH oxidase (13). The importance of LC3 recruitment to these phagosomes is not yet clear. Sanjuan *et al.* showed that macrophages lacking the upstream signaling component ATG7 are less efficient at killing yeast. Also, Lee *et al.* showed that ATG5-deficient dendritic cells have a reduced ability to process antigens delivered on beads coated with LPS (15). However, the specific role of LC3 recruitment to phagosomes has not been directly assessed.

It is not yet known whether signaling through Dectin-1 can trigger recruitment of LC3 to phagosomes or how such recruitment would be important for Dectin-1-mediated host responses. In this study, we show that Dectin-1 signaling triggers recruitment of LC3 to phagosomes. LC3 recruitment requires activation of Syk, reactive oxygen production by the NADPH oxidase, and the presence of ATG5. Further, using antigen-presenting cells lacking LC3 we directly show that LC3 promotes efficient MHC class II antigen presentation through facilitating recruitment of MHC class II molecules to Dectin-1 phagosomes. These data demonstrate that in addition to the previously recognized roles for Dectin-1 in host defense, the receptor also directs intracellular processing and presentation of fungal antigens through a mechanism facilitated by a component of the autophagy pathway.

EXPERIMENTAL PROCEDURES

Reagents—Unless noted, all reagents were from Sigma. *Saccharomyces cerevisiae*-expressing ovalbumin under a galactose-inducible promoter (16) was kindly provided by Dr. van Haan (VU University, Amsterdam, The Netherlands). *Candida albicans* was from the American Type Culture Collection (ATCC 90028). Other reagents include lipopolysaccharide (Invivogen), β-glucan particles (Wellmune WGP; Biothera) (17), anti-LC3II antibody (immunoblotting, MBL International clone 115; immunofluorescence, MBL International clone 153), anti-ATG5 antibody (ABCam clone AB54033), anti-GAPDH antibody (Santa Cruz clone 6C5), phycoerythrin-conjugated anti-CD69 (Jackson Laboratory), streptavidin beads (Dynabeads; Invitrogen), Ova-peptide SIINFEKL (Anaspec), Ova-peptide 323–339 (Anaspec), and anti-MHCII antibody (eBioscience clone M5/114.15.2).

ShRNA Knockdown—ATG5 shRNA (sense, ACCAGATA-ACTTTCTTCATATT; antisense, AATATGAAGAAAGT-TATCTGGG) (12) was cloned into the pMSCV-LMP vector (Open Biosystem) and expressed stably in RAW264.7 cells expressing streptavidin-binding peptide-tagged Dectin-1 (18).

Cell Culture—RAW246.7 Cells expressing streptavidin-binding peptide-tagged Dectin-1 were cultured in RPMI 1640 medium (CellGrow) with 10% FCS, 10% penicillin/streptomycin, and 10% L-glutamine. Bone marrow-derived dendritic cells (BMDC) were derived from primary mouse bone marrow cultured in RPMI 1640 medium as described above together with 10 ng/ml mGM-CSF (PeproTech) for 7 days. Wild type BMDC were derived from C57BL/6 mice (Jackson Laboratory), Dectin-1$^{-/-}$ BMDC were from CLEC7-deficient mice (19) (gift from

G. D. Brown, University of Aberdeen). For generation of Syk$^{-/-}$ BMDC, fetal liver cells from these mice were used to generate bone marrow chimeras as described previously (20). NOX2$^{-/-}$ BMDC were from p91^{phox}-deficient mice (Jackson Laboratory). These mice were all backcrossed at least eight generations on a C57BL/6 background. LC3β$^{-/-}$ BMDC were from LC3β-deficient mice (Jackson Laboratory) which were on a B6;129P2 mixed background and were compared with wild type cells from littermate controls.

LC3 Immunoblotting—Stimuli were added to the cells and centrifuged at $500 \times g$ to synchronize particle contact. Inhibitors were added 10 min before stimulation and remained in culture for the duration of the experiments. To control for specificity in streptavidin-binding peptide-tagged Dectin-1 cross-linking experiments, 9.7 mM biotin was added to the streptavidin beads to block binding sites before they were added to cells. Whole cell lysates were made in LDS sample buffer (Invitrogen), boiled, and loaded onto 12% SDS-polyacrylamide gels. Proteins were transferred to PVDF microporous membranes (Millipore), blocked overnight with 4% BSA at 4 °C, and stained with the indicated antibodies. Binding was detected by chemiluminescence (Thermo Scientific).

Immunofluorescence Microscopy—Cells on coverslips were fixed in 4% paraformaldehyde in PBS and permeabilized with either acetone or 0.2% Triton/PBS. Primary antibodies and secondary antibodies were incubated with coverslips for 1 h each. Coverslips were mounted on microscope glass slides with Prolong Gold antifade reagent (Invitrogen). Images were collected with a Leica TCS SP spectral confocal microscope.

Antigen Presentation—*S. cerevisiae*-expressing ovalbumin was generated by inducing expression with galactose as described previously (16). Yeast were added to the BMDC and centrifuged at $500 \times g$ to synchronize contact. Control peptide antigens were added as indicated. After 8 h, cells were washed, and new medium was added. OTII T cells were sorted from spleens of OTII mice (Jackson Laboratory) using CD4$^+$ negative selection (Miltenyi) and AutoMacs Pro (Miltenyi). The OTI hybridoma cell line B3Z was cultured as above in medium supplemented with β-mercaptoethanol. T cells were added to washed BMDC (10:1), and supernatants were collected after 36 h. IL-2 and IL-17 levels were measured by ELISA (Biolegend).
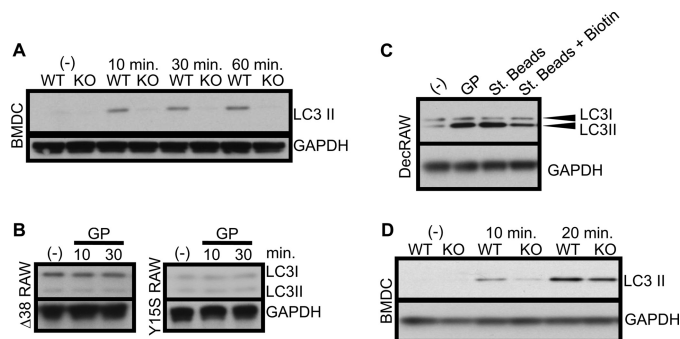
RESULTS

Fungi and β-Glucan Particles Stimulate LC3 Lipidation in Macrophages and Dendritic Cells—To determine whether fungi can trigger LC3 lipidation, we stimulated RAW264.7 cells (a mouse macrophage cell line) expressing Dectin-1 (18) with heat-killed *S. cerevisiae* or *C. albicans*. We assessed endogenous LC3II (the lipidated form of LC3) levels by immunoblotting cell lysates. We observed rapid formation of LC3II within 10 min of stimulation (Fig. 1*A*). We observed similar formation of LC3II in primary mouse BMDC stimulated with heat-killed yeasts (Fig. 1*B*). Also, we observed similar LC3II formation when cells were infected with live yeasts (Fig. 1*C*). To determine whether fungal cell wall β-glucan is sufficient to trigger LC3 lipidation, we stimulated cells with β-glucan particles. β-Glucan particles strongly triggered LC3II formation in macrophages and den-
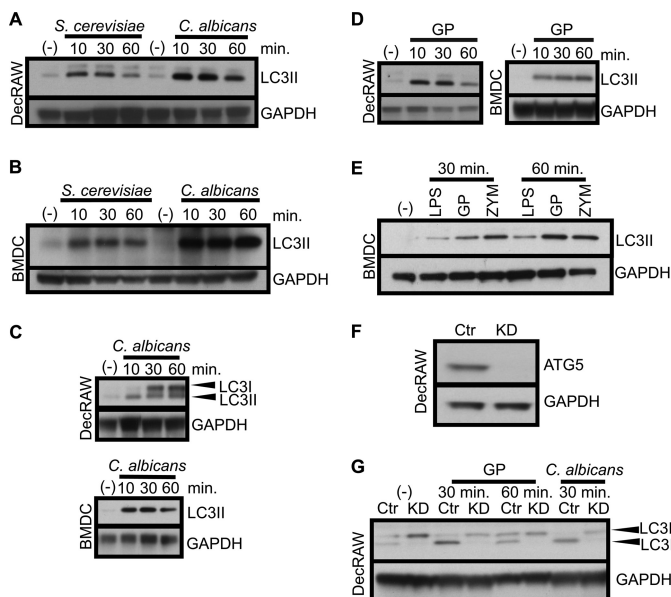
FIGURE 1. **Fungi and β-glucan particles stimulate LC3 lipidation in macrophages and dendritic cells.** *A* and *B*, RAW 264.7 cells stably expressing Dectin-1 (*DecRAW*) or BMDC as indicated were stimulated with heat-killed *S. cerevisiae* (m.o.i. 10:1) or heat-killed *C. albicans* (m.o.i. 10:1) for the indicated times. Cell lysates were prepared, and levels of LC3II protein were determined by immunoblotting. Levels of GAPDH in the same lysates were determined by immunoblotting as loading controls. *C*, DecRAW or BMDC were infected with live *C. albicans* (m.o.i. 10:1) for the indicated times. LC3II and GAPDH protein levels were determined by immunoblotting. *D* and *E*, DecRAW or BMDC were stimulated with β-glucan particles (*GP*, 100 μg/ml), LPS (100 nM), or zymosan (*ZYM*, 100 μg/ml), for the indicated times. LC3II and GAPDH protein levels were determined by immunoblotting. *F*, ATG5 and GAPDH levels were determined by immunoblotting of lysates from cells stably expressing a control vector (*Ctr*) or a shRNA targeting ATG5 (*KD*). *G*, control and ATG5 knockdown cells were stimulated with β-glucan particles (100 μg/ml) or heat-killed *C. albicans* (m.o.i. 10:1) for the indicated times. Cell lysates were prepared, and LC3II and GAPDH protein levels were determined by immunoblotting.

FIGURE 2. **Dectin-1 signaling is necessary and sufficient to trigger LC3II formation.** *A*, wild type (*WT*) or Dectin-1-deficient BMDC (*KO*) were stimulated with 100 μg/ml β-glucan particles for the indicated times. Cell lysates were prepared, and LC3II protein levels were determined by immunoblotting. GAPDH levels of the same lysates were determined by immunoblotting as loading controls. *B*, RAW264.7 cells stably expressing Dectin-1 with the cytoplasmic domain deleted (*Δ38RAW*) or Dectin-1 with a point mutation at tyrosine 15 (*Y15SRAW*) were stimulated with 100 μg/ml β-glucan particles for the indicated times. Cell lysates were prepared, and LC3II protein levels were determined by immunoblotting. GAPDH levels of the same lysates were determined by immunoblotting as loading controls. *C*, RAW267.4 cells stably expressing a streptavidin-binding peptide epitope-tagged Dectin-1 were stimulated for 10 min with 100 μg/ml β-glucan particles (*GP*), 100 μg/ml streptavidin-coated beads (*St. Beads*), or streptavidin-coated beads blocked with biotin. LC3II and GAPDH protein levels were determined by immunoblotting. *D*, wild type or Dectin-1-deficient BMDC (*KO*) were stimulated with heat-killed *C. albicans* for the indicated times. LC3II protein levels were determined by immunoblotting. GAPDH levels were determined by immunoblotting as loading controls.

dritic cells (Fig. 1*D*). To explore how this level of LC3II induction compares with inducers of LC3II published previously, we compared β-glucan particles with lipopolysaccharide (LPS) and zymosan. β-Glucan particle-triggered LC3II formation was higher than that induced by LPS and was comparable with zymosan (Fig. 1*E*). Consistent with previous studies on the mechanism of LC3 lipidation, formation of LC3II in response to β-glucan particles and fungi required ATG5 (Fig. 1, *F* and *G*).

Dectin-1 Signaling Is Necessary and Sufficient to Trigger LC3II Formation—Because β-glucan particles are recognized by Dectin-1, we examined whether Dectin-1 is necessary for stimulating LC3II formation. We stimulated wild type and Dectin-1-deficient BMDC with β-glucan particles and assessed formation of LC3II by immunoblotting (Fig. 2*A*). LC3II formation was completely ablated in cells lacking Dectin-1. To investigate further the role of Dectin-1 we used RAW264.7 cells expressing signaling-deficient mutants of Dectin-1 with either the cytoplasmic domain deleted (Δ38RAW) or with tyrosine 15 mutated (Y15SRAW) (21). The cells were stimulated with β-glucan, and LC3II formation was assessed by immunoblotting (Fig. 2*B*). Cells expressing signaling-deficient forms of Dectin-1 failed to stimulate LC3II formation, suggesting that Dectin-1 signaling is necessary for β-glucan-stimulated formation of LC3II.

We next assessed whether Dectin-1 cross-linking is sufficient to trigger LC3II formation using an approach we have previously employed to specifically cluster Dectin-1 receptors on a particle (18). We stimulated RAW267.4 cells stably expressing a streptavidin-binding peptide epitope-tagged Dectin-1 with streptavidin-coated beads. The beads specifically bind to the receptor via the epitope tag and triggered LC3II formation to levels similar to that observed using β-glucan particles (Fig. 2*C*). LC3II formation induced by the streptavidin-coated beads was substantially blocked in the presence of biotin, which competes against the epitope tag for binding to the beads. Previous studies have shown that TLR2 signaling can trigger LC3II formation (12), and whole yeast activate both Dectin-1 and TLR2 (22, 23). To assess the net contribution of Dectin-1 in triggering LC3II formation in response to whole yeast, we stimulated wild type and Dectin-1-deficient BMDC with heat-killed yeast. LC3II formation was reduced in Dectin-1-deficient cells (Fig. 2*D*). Together, these data demonstrate that Dectin-1 signaling is necessary and sufficient to trigger LC3II formation in response to β-glucan and that it contributes to LC3II formation in response to whole yeast.

Dectin-1 Triggers Recruitment of LC3II to Phagosomes—Previous studies have shown that TLR signaling can lead to recruitment of LC3II to phagosomes containing TLR ligands (12). We therefore examined whether Dectin-1 signaling is also capable of directing LC3II recruitment to phagosomes. We stimulated macrophages and dendritic cells with β-glucan particles and evaluated LC3II recruitment to phagosomes by immunofluorescence microscopy (Fig. 3*A*). We observed strong recruitment of LC3II to phagosomes. LC3II was recruited rapidly during internalization where it co-localized with F-actin (Fig. 3*A*), after which LC3II was slowly depleted from the phagosomes
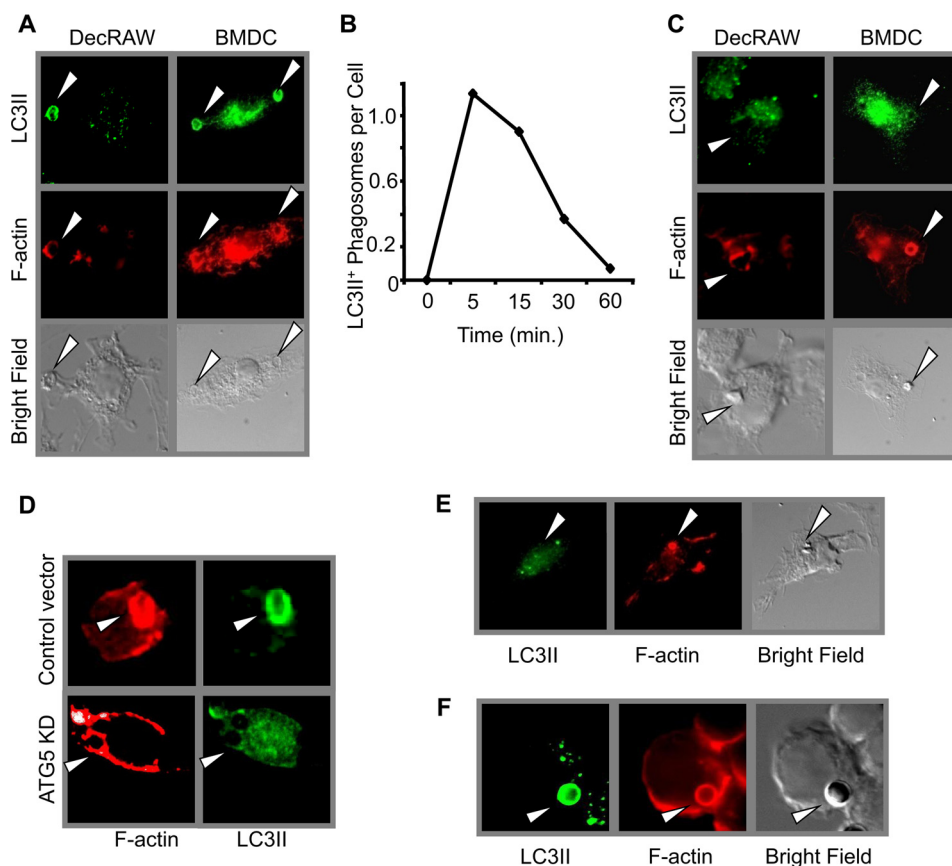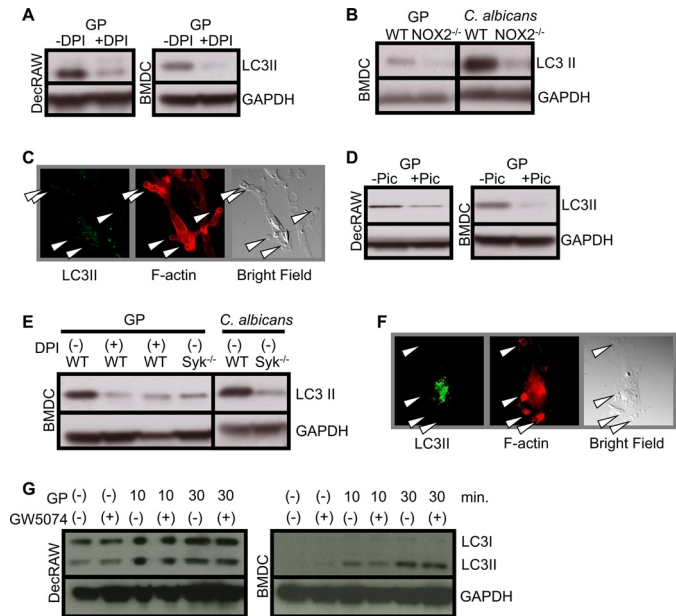
FIGURE 3. **Dectin-1 triggers recruitment of LC3II to phagosomes.** *A*, RAW264.7 cells stably expressing Dectin-1 (*DecRAW*) or BMDC were pulsed with β-glucan particles (25 μg/ml) and chased for 15 min. Cells were stained for LC3II (*green*) and F-actin (*red*) and analyzed by immunofluorescence microscopy. *B*, BMDC were stimulated with β-glucan particles, chased for the indicated times, and stained as in *A*. LC3II$^+$ phagosomes per number of cells (*n* >90) were counted. *C*, cells were imaged as in *A* except they were pulsed with 3-μm latex beads. *D*, cells stably expressing a control vector or an shRNA targeting ATG5 (*ATG5 KD*) were pulsed for 15 min with β-glucan particles (25 μg/ml). Cells were fixed, permeabilized, and stained for LC3II and F-actin. *E*, Dectin-1-deficient BMDC were pulsed with β-glucan particles (25 μg/ml) for 15 min. Cells were stained as indicated and analyzed by immunofluorescence microscopy. *F*, RAW267.4 cells stably expressing a streptavidin-binding peptide epitope-tagged Dectin-1 were stimulated for 15 min with streptavidin-coated beads (100 μg/ml) and imaged as in *E*.

over a period of 60 min (Fig. 3*B*). LC3II was not recruited to phagosomes containing latex beads (Fig. 3*C*). Recruitment of LC3II to phagosomes containing β-glucan particles required ATG5 (Fig. 3*D*). To confirm in two ways that Dectin-1 signaling specifically triggers recruitment of LC3II to phagosomes, we first fed β-glucan particles to Dectin-1-deficient BMDC. Although these cells bind and internalize β-glucan particles poorly, we could still find some particles that were internalized. These phagosomes did not recruit LC3II (Fig. 3*E*), demonstrating that Dectin-1 signaling is necessary for LC3II recruitment to phagosomes containing β-glucan particles. Second, we fed streptavidin-coated beads to RAW267.4 cells expressing the streptavidin-binding peptide epitope-tagged Dectin-1 to directly cluster and activate Dectin-1. Phagosomes containing these beads strongly recruited LC3II (Fig. 3*F*). Together these data demonstrate that Dectin-1 signaling is necessary and sufficient to trigger recruitment of LC3II to phagosomal membranes.

Dectin-1-triggered Recruitment of LC3II Requires Signaling to Syk and the NADPH Oxidase—We next explored which signaling mechanisms activated by Dectin-1 are required for signaling to LC3II. Dectin-1 signaling activates ROS production by the NADPH oxidase, and this response requires Syk (21).
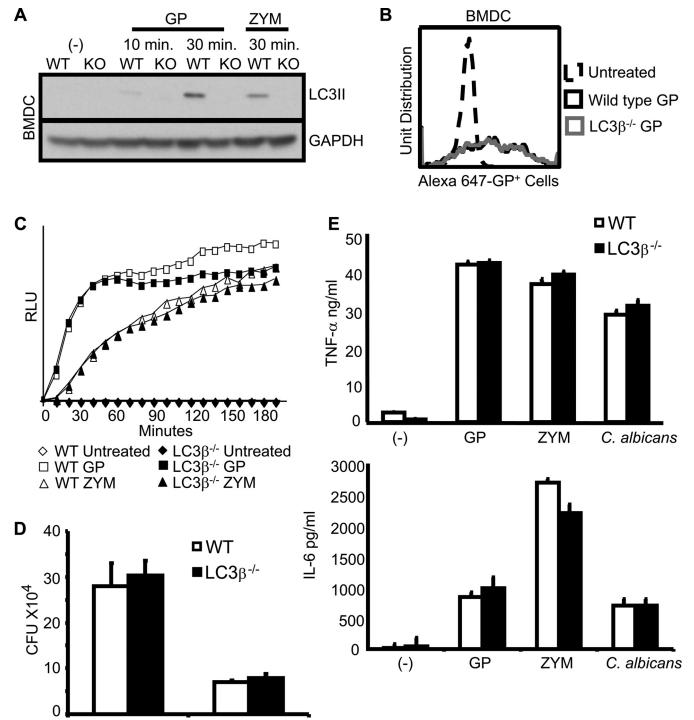
Huang *et al.* have previously shown that Fcγ receptors can trigger LC3II recruitment via activation of ROS production (13). We therefore examined whether NADPH oxidase-mediated ROS production is required for Dectin-1-triggered LC3II formation. Inhibition of ROS production with the inhibitor diphenyleneiodonium (DPI) blocked formation of LC3II in response to β-glucan particles in macrophages and dendritic cells (Fig. 4*A*). Consistent with this observation, BMDC from gp91^{phox}-deficient mice stimulated with β-glucan particles or heat-killed yeast also showed reduced LC3II formation compared with wild type cells (Fig. 4*B*). Inhibition of ROS production did not block phagocytosis, but did block recruitment of LC3II to phagosomes containing β-glucan particles (Fig. 4*C*). Dectin-1 signaling requires tyrosine phosphorylation of a "HemITAM" motif in its cytoplasmic tail that recruits Syk kinase. We therefore examined whether LC3II formation requires activation of Syk. We first inhibited Syk with the specific inhibitor piceatannol which blocked β-glucan particle-induced LC3II formation in macrophages and dendritic cells (Fig. 4*D*). Next, we stimulated wild type and Syk-deficient BMDC with β-glucan particles or heat-killed yeast (Fig. 4*E*). LC3II formation in response to both stimuli was reduced in Syk-deficient cells similar to ROS inhibition by DPI. Consistent with previous reports (21,

FIGURE 4. **Dectin-1-triggered recruitment of LC3II requires signaling to Syk and NADPH oxidase.** *A*, DecRAW or BMDC were stimulated for 30 min with β-glucan particles (*GP*) (100 μg/ml) with or without DPI (25 μM). Cell lysates were prepared and LC3II, and GAPDH protein levels were determined by immunoblotting. *B*, wild type (*WT*) or gp91^{phox}-deficient (*NOX2$^{-/-}$*) BMDC were stimulated for 30 min with GP (100 μg/ml) or heat-killed *C. albicans* (m.o.i. 10:1). LC3II and GAPDH protein levels were determined by immunoblotting. *C*, BMDC were pulsed for 15 min with GP (25 μg/ml) in the presence of DPI (25 μM), stained, and analyzed by immunofluorescence microscopy. *D*, DecRAW or BMDC were stimulated as in *A* with or without piceatannol (*Pic*, 25 μM). LC3II and GAPDH protein levels were determined by immunoblotting. *E*, wild type or Syk-deficient BMDC were stimulated as in *B* with or without DPI (25 μM). LC3II and GAPDH protein levels were determined by immunoblotting. *F*, BMDC were pulsed with GP (25 μg/ml) in the presence of 25 μM piceatannol for 15 min, stained, and analyzed by immunofluorescence microscopy. *G*, RAW264.7 cells stably expressing Dectin-1 (*DecRAW*) or BMDC were stimulated with GP (100 μg/ml) for the indicated times with or without the addition of 1.0 μM Raf-1 inhibitor GW5074. Cell lysates were prepared, and levels of LC3II and GAPDH were determined by immunoblotting.

FIGURE 5. **LC3β-deficiency does not alter many Dectin-1-triggered responses.** *A*, wild type (*WT*) or LC3β-deficient BMDC (*KO*) were stimulated with β-glucan particles (*GP*, 100 μg/ml) or zymosan (*ZYM*, 100 μg/ml), for the indicated times. Cell lysates were prepared, and LC3II and GAPDH protein levels were determined by immunoblotting. *B*, BMDC were pulsed with Alexa Fluor 647-labeled β-glucan particles (25 μg/ml) for 10 min. Cells were washed, fixed, and Alexa Fluor 647-positive cells were measured by flow cytometry. *C*, wild type or LC3β-deficient BMDC were pulsed with β-glucan particles or zymosan, and reactive oxygen production was measured by luminol-enhanced chemiluminescence. *RLU*, relative light units. *D*, wild type or LC3β-deficient BMDC were infected with *S. cerevisiae* (m.o.i. 10:1), and cells were washed and lysed in water after the indicated periods. Lysates were plated, and surviving *S. cerevisiae* colony-forming units (*CFU*) were counted. *E*, wild type or LC3β-deficient BMDC were stimulated overnight with β-glucan particles (100 μg/ml), zymosan (100 μg/ml), or heat-killed *C. albicans* (m.o.i. 10:1). Supernatant TNF-α and IL-6 levels were measured by ELISA. *Error bars*, S.D.

24), Dectin-1-mediated phagocytosis was not blocked by the Syk inhibitor or by Syk deficiency, and phagosomes formed normally (data not shown). However, LC3II was not recruited to these phagosomes (Fig. 4F). Together these data show that Dectin-1-triggered formation of LC3II and its recruitment to phagosomes requires signaling to the NADPH oxidase and Syk. A recent study by Gringhuis *et al.* suggested that Raf-1 signaling plays a role parallel to Syk in Dectin-1 activation of inflammation (25). We investigated the Raf-1 role in Dectin-1-triggered LC3II formation by stimulating macrophages and BMDC with β-glucan with or without the Raf-1 inhibitor GW5074 and assessed LC3II by immunoblotting (Fig. 4G). Levels of LC3II formation were not affected by Raf-1 inhibition, suggesting that the Raf-1 pathway does not play a role.

Dectin-1-triggered Recruitment of LC3II Enhances Efficiency of Presentation of Fungal Antigens—Even though LC3II has been a reliable marker for autophagy and the formation of autophagosomes, its function and role in traditional phagocytosis are not clear. We therefore examined whether LC3II recruitment to phagosomes is important for downstream responses to Dectin-1 stimulation using wild type and LC3β-deficient BMDC (Fig. 5A). Dectin-1 triggers phagocytosis, reactive oxygen production, killing, and cytokine secretion in

response to β-glucan particles, and none of these responses was altered in LC3β-deficient cells (Fig. 5, *B–E*). This suggests that although LC3II is recruited to Dectin-1 phagosomes, it is not necessary for these well studied Dectin-1-induced responses.

Phagocytosis is important for acquisition, degradation, and presentation of extracellular antigens. We therefore explored whether Dectin-1-triggered recruitment of LC3II to phagosomes affected processing and presentation of fungal antigens. We pulsed wild type or LC3β-deficient BMDC with yeast expressing ovalbumin (16) and measured their ability to activate OTII T cells by measuring IL-2 production, CD69 up-regulation, and IL-17 production. OTII T cell activation was significantly reduced when antigen was presented by LC3β-deficient BMDC compared with wild type cells (Fig. 6, *A–C*). The LC3β-deficient cells did not have an inherent defect in antigen presentation because presentation of soluble ovalbumin peptide was not affected. The defect in antigen presentation was specific to MHCII because cross-presentation of fungal antigens on MHCI was not affected (Fig. 6D).

Because MHCII presentation was reduced in LC3β-deficient BMDC, we examined whether LC3 is important for recruit-
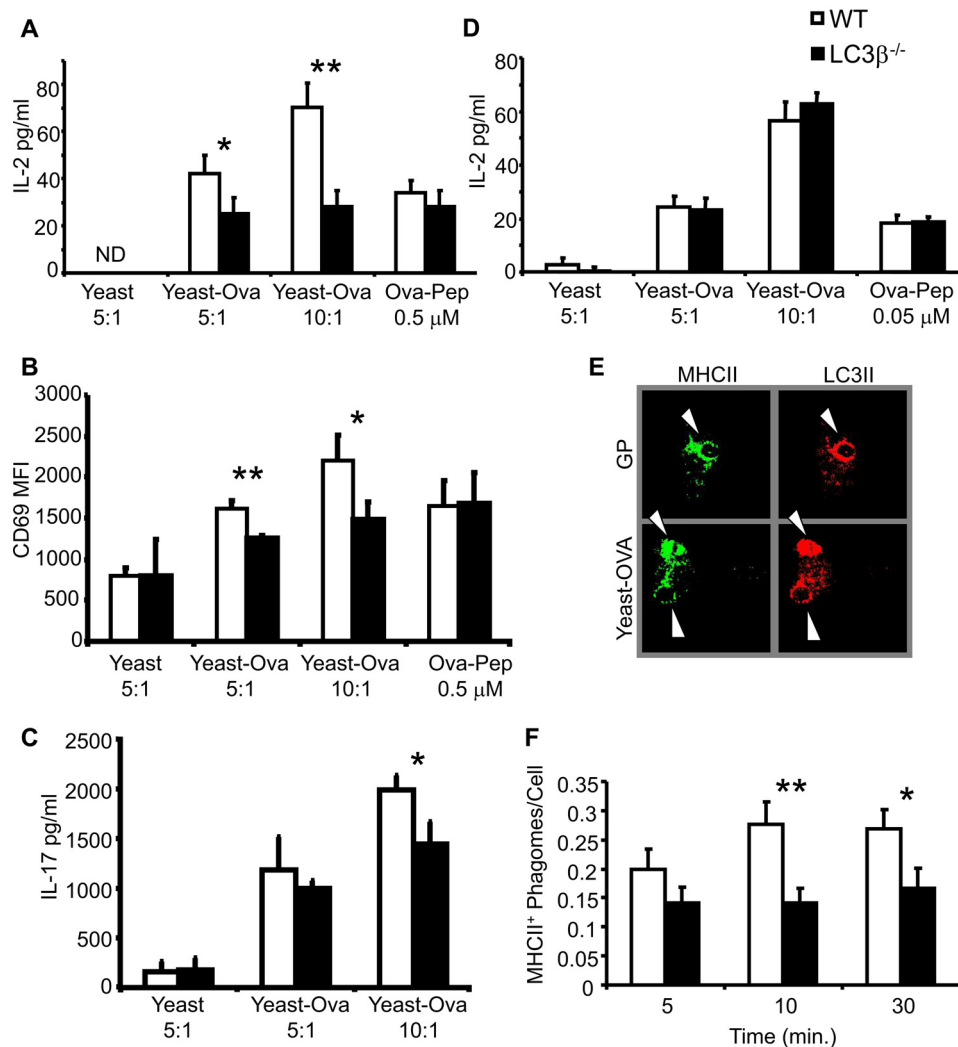
FIGURE 6. **Dectin-1-triggered recruitment of LC3II enhances efficiency of presentation of fungal antigens.** *A–C*, wild type or LC3β-deficient BMDC were pulsed with heat-killed *S. cerevisiae* (*Yeast*) or heat-killed *S. cerevisiae* expressing ovalbumin (*Yeast-Ova*) at the indicated m.o.i. or soluble Ova-peptide 323–339 (0.5 μM) for 8 h. Cells were washed, and CD4+ T cells from OTII mice were added to the BMDC (10:1). Supernatant IL-2 and IL-17 protein levels were assessed after 36 h by ELISA. Surface expression of CD69 was determined by flow cytometry and depicted as mean fluorescence intensity (*MFI*). *D*, wild type or LC3β-deficient BMDC were pulsed with heat-killed *S. cerevisiae* or heat-killed *S. cerevisiae* expressing ovalbumin at the indicated m.o.i. or soluble Ova-peptide SIINFEKL (0.05 μM) for 8 h. Cells were washed and fixed, and B3Z T cells (OTI CD8+ T cell hybridoma) were added to the BMDC (10:1). Supernatant IL-2 protein levels were assessed after 24 h by ELISA. *E*, BMDC were pulsed for 15 min with β-glucan particles (25 μg) or heat-killed *S. cerevisiae*-expressing ovalbumin (10:1). Cells well fixed, permeabilized, and stained with anti-MHCII (*green*) and anti-LC3II (*red*), and analyzed by immunofluorescence microscopy. *F*, wild type or LC3β-deficient BMDC were pulsed with β-glucan particles (25 μg) for the indicated times. Cells were stained as in *E*, and MHCII+ phagosomes were counted and normalized to the number of cells counted (*n* >90). *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

ment of MHCII molecules to phagosomes containing β-glucan particles by immunofluorescence microscopy. We observed that LC3II and MHCII co-localized on phagosomes containing either β-glucan particles or whole yeast (Fig. 6*E*). We further observed that in LC3β-deficient BMDC, phagosomes containing β-glucan particles are significantly less likely to acquire MHCII molecules (Fig. 6*F*). Together the data suggest that Dectin-1-triggered recruitment of LC3II to phagosomes enhances recruitment of MHCII molecules and promotes presentation of fungal-derived antigens.

DISCUSSION

In this study, we showed that Dectin-1 activation specifically recruits LC3 to phagosomes containing Dectin-1 ligands. Using a variety of inhibitors and knock-out mice, we showed that LC3 recruitment requires activation of Syk, reactive oxygen produc-

tion by the NADPH oxidase, and the presence of ATG5. Further, we directly showed by using antigen-presenting cells lacking LC3 that LC3 recruitment to phagosomes facilitates subsequent recruitment of MHC class II molecules and efficient MHC class II antigen presentation. These data demonstrate that in addition to the previously recognized roles for Dectin-1 in host defense, the receptor also directs intracellular processing and presentation of fungal antigens through a mechanism utilizing components of the autophagy machinery.

Previous studies have shown that LC3 was recruited to phagosomes containing TLR, Fcγ receptor, and TIM4 ligands (12–14). Huang *et al.* showed that Fcγ receptor-triggered recruitment of LC3 to phagosomes in neutrophils and macrophages is dependent on reactive oxygen production by the NADPH oxidase, but the functional significance of this recruitment is unclear (13). Although not identical, Fcγ receptors and Dec-

tin-1 signaling mechanisms are highly similar, and our study shows that Dectin-1-triggered LC3 recruitment to phagosomes is also dependent on activation of the NADPH oxidase and further shows that Syk is required. We demonstrate that ATG5, an upstream component of the autophagy pathway, is required, although it is not clear how much of the classical autophagy pathway is involved. Green and co-workers showed that TIM4-triggered LC3 recruitment to phagosomes containing dead cells and that this requires ATG5, ATG7, and Beclin-1. However, it does not require the upstream autophagy signaling protein ULK1 (14). This suggests that even though some upstream mediators for LC3 recruitment to phagosomes and for canonical autophagy are the same, some are different. Furthermore, upstream signaling molecules of autophagy have been shown to be involved in processes other than directing LC3II formation (for example, ATG5 complexes bind and negatively regulate RIG-I and IPS-1) (9). Thus, using phagocytes deficient in upstream autophagy signaling proteins may not be sufficient to examine the specific role of LC3 recruitment to phagosomes. None of the prior studies reporting LC3 recruitment to other types of phagosomes has yet specifically examined the effect of LC3 deficiency on phagocytosis. Using LC3β-deficient dendritic cells, we have now shown a specific role for LC3 recruitment to Dectin-1-triggered phagosomes in regulating the efficiency of processing and presenting fungal-derived antigens. The mechanisms by which LC3 influences the properties of maturing phagosomes are not yet known. Future studies will have to establish how LC3 influences MHCII recruitment. One possibly significant interaction is the reported association of LC3 with FYCO1, a RAB7 effector protein that also regulates microtubule transport (26). It is possible that altered peripheral transport of LC3-tagged phagosomes influences fusion with MHCII-positive compartments.

Dectin-1 recruits and activates the NADPH oxidase to phagosomes to facilitate killing of fungi. It recruits signaling adaptor molecules such as CARD9 to trigger production of cytokines that influence local inflammatory responses and polarization of T cell responses. This study now shows that Dectin-1 also recruits LC3 to phagosomes to influence the efficiency of presentation of fungal-derived antigens. Pattern recognition receptors coordinate immune responses to be tailored appropriately to the types of threats that they recognize. Further understanding of the mechanisms employed by these receptors will help us develop better vaccines and therapies for infectious diseases.

REFERENCES

1. Drummond, R. A., and Brown, G. D. (2011) The role of Dectin-1 in the host defense against fungal infections. *Curr. Opin. Microbiol.* **14,** 392–399
2. Goodridge, H. S., Wolf, A. J., and Underhill, D. M. (2009) β-Glucan recognition by the innate immune system. *Immunol. Rev.* **230,** 38–50
3. Gringhuis, S. I., Wevers, B. A., Kaptein, T. M., van Capel, T. M., Theelen, B., Boekhout, T., de Jong, E. C., and Geijtenbeek, T. B. (2011) Selective c-*rel* activation via Malt1 controls anti-fungal Th17 immunity by dectin-1 and dectin-2. *PLoS Pathog.* **7,** e1001259
4. Vautier, S., Sousa Mda, G., and Brown, G. D. (2010) C-type lectins, fungi and Th17 responses. *Cytokine Growth Factor Rev.* **21,** 405–412
5. Mizushima, N., Levine, B., Cuervo, A. M., and Klionsky, D. J. (2008) Autophagy fights disease through cellular self-digestion. *Nature* **451,** 1069–1075
6. Shahnazari, S., and Brumell, J. H. (2011) Mechanisms and consequences of bacterial targeting by the autophagy pathway. *Curr. Opin. Microbiol.* **14,** 68–75
7. Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., Hamada, S., and Yoshimori, T. (2004) Autophagy defends cells against invading group A *Streptococcus. Science* **306,** 1037–1040
8. Kumar, D., Nath, L., Kamal, M. A., Varshney, A., Jain, A., Singh, S., and Rao, K. V. (2010) Genome-wide analysis of the host intracellular network that regulates survival of *Mycobacterium tuberculosis. Cell* **140,** 731–743
9. Jounai, N., Takeshita, F., Kobiyama, K., Sawano, A., Miyawaki, A., Xin, K. Q., Ishii, K. J., Kawai, T., Akira, S., Suzuki, K., and Okuda, K. (2007) The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 14050–14055
10. Zhao, Z., Fux, B., Goodwin, M., Dunay, I. R., Strong, D., Miller, B. C., Cadwell, K., Delgado, M. A., Ponpuak, M., Green, K. G., Schmidt, R. E., Mizushima, N., Deretic, V., Sibley, L. D., and Virgin, H. W. (2008) Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. *Cell Host Microbe* **4,** 458–469
11. Khaminets, A., Hunn, J. P., Könen-Waisman, S., Zhao, Y. O., Preukschat, D., Coers, J., Boyle, J. P., Ong, Y. C., Boothroyd, J. C., Reichmann, G., and Howard, J. C. (2010) Coordinated loading of IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole. *Cell. Microbiol.* **12,** 939–961
12. Sanjuan, M. A., Dillon, C. P., Tait, S. W., Moshiach, S., Dorsey, F., Connell, S., Komatsu, M., Tanaka, K., Cleveland, J. L., Withoff, S., and Green, D. R. (2007) Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* **450,** 1253–1257
13. Huang, J., Canadien, V., Lam, G. Y., Steinberg, B. E., Dinauer, M. C., Magalhaes, M. A., Glogauer, M., Grinstein, S., and Brumell, J. H. (2009) Activation of antibacterial autophagy by NADPH oxidases. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 6226–6231
14. Martinez, J., Almendinger, J., Oberst, A., Ness, R., Dillon, C. P., Fitzgerald, P., Hengartner, M. O., and Green, D. R. (2011) Microtubule-associated protein 1 light chain 3α (LC3)-associated phagocytosis is required for the efficient clearance of dead cells. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 17396–17401
15. Lee, H. K., Mattei, L. M., Steinberg, B. E., Alberts, P., Lee, Y. H., Chervonsky, A., Mizushima, N., Grinstein, S., and Iwasaki, A. (2010) *In vivo* requirement for Atg5 in antigen presentation by dendritic cells. *Immunity* **32,** 227–239
16. van Leeuwen, F., Gafken, P. R., and Gottschling, D. E. (2002) Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* **109,** 745–756
17. Goodridge, H. S., Reyes, C. N., Becker, C. A., Katsumoto, T. R., Ma, J., Wolf, A. J., Bose, N., Chan, A. S., Magee, A. S., Danielson, M. E., Weiss, A., Vasilakos, J. P., and Underhill, D. M. (2011) Activation of the innate immune receptor Dectin-1 upon formation of a "phagocytic synapse." *Nature* **472,** 471–475
18. Goodridge, H. S., Shimada, T., Wolf, A. J., Hsu, Y. M., Becker, C. A., Lin, X., and Underhill, D. M. (2009) Differential use of CARD9 by dectin-1 in macrophages and dendritic cells. *J. Immunol.* **182,** 1146–1154
19. Taylor, P. R., Tsoni, S. V., Willment, J. A., Dennehy, K. M., Rosas, M., Findon, H., Haynes, K., Steele, C., Botto, M., Gordon, S., and Brown, G. D. (2007) Dectin-1 is required for β-glucan recognition and control of fungal infection. *Nat. Immunol.* **8,** 31–38
20. Mócsai, A., Zhou, M., Meng, F., Tybulewicz, V. L., and Lowell, C. A. (2002) Syk is required for integrin signaling in neutrophils. *Immunity* **16,** 547–558
21. Underhill, D. M., Rossnagle, E., Lowell, C. A., and Simmons, R. M. (2005) Dectin-1 activates Syk tyrosine kinase in a dynamic subset of macrophages for reactive oxygen production. *Blood* **106,** 2543–2550
22. Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003) Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* **197,** 1107–1117
23. Brown, G. D., Taylor, P. R., Reid, D. M., Willment, J. A., Williams, D. L., Martinez-Pomares, L., Wong, S. Y., and Gordon, S. (2002) Dectin-1 is a major β-glucan receptor on macrophages. *J. Exp. Med.* **196,** 407–412

24. Herre, J., Marshall, A. S., Caron, E., Edwards, A. D., Williams, D. L., Schweighoffer, E., Tybulewicz, V., Reis e Sousa, C., Gordon, S., and Brown, G. D. (2004) Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* **104,** 4038–4045

25. Gringhuis, S. I., den Dunnen, J., Litjens, M., van der Vlist, M., Wevers, B., Bruijns, S. C., and Geijtenbeek, T. B. (2009) Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-κB activation through Raf-1 and Syk. *Nat. Immunol.* **10,** 203–213

26. Pankiv, S., Alemu, E. A., Brech, A., Bruun, J. A., Lamark, T., Overvatn, A., Bjørkøy, G., and Johansen, T. (2010) FYCO1 is a Rab7 effector that binds to LC3 and PI3P to mediate microtubule plus end-directed vesicle transport. *J. Cell Biol.* **188,** 253–269