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Corticosteroids Block Autophagy Protein Recruitment in Aspergillus fumigatus Phagosomes via Targeting Dectin-1/Syk Kinase Signaling

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Aspergillus fumigatus is the predominant airborne fungal pathogen in immunocompromised patients. Genetic defects in NADPH oxidase (chronic granulomatous disease [CGD]) and corticosteroid-induced immunosuppression lead to impaired killing of A. fumigatus and unique susceptibility to invasive aspergillosis via incompletely characterized mechanisms. Recent studies link TLR activation with phagosome maturation via the engagement of autophagy proteins. In this study, we found that infection of human monocytes with A. fumigatus spores triggered selective recruitment of the autophagy protein LC3 II in phagosomes upon fungal cell wall swelling. This response was induced by surface exposure of immunostimulatory β-glucans and was mediated by activation of the Dectin-1 receptor. LC3 II recruitment in A. fumigatus phagosomes required spleen tyrosine kinase (Syk) kinase–dependent production of reactive oxygen species and was nearly absent in monocytes of patients with CGD. This pathway was important for control of intracellular fungal growth, as silencing of Atg5 resulted in impaired phagosome maturation and killing of A. fumigatus. In vivo and ex vivo administration of corticosteroids blocked LC3 II recruitment in A. fumigatus phagosomes via rapid inhibition of phosphorylation of Src and Syk kinases and downstream production of reactive oxygen species. Our studies link Dectin-1/Syk kinase signaling with autophagy-dependent maturation of A. fumigatus phagosomes and uncover a potential mechanism for development of invasive aspergillosis in the setting of CGD and corticosteroid-induced immunosupression. The Journal of Immunology, 2013, 191: 000–000.
receptors in antifungal immunity (16–21). Dectin-1 recognizes β-glucan carbohydrates in the fungal cell walls and triggers intracellular signaling via a cytoplasmic ITAM-like motif via recruitment of spleen tyrosine kinase (Syk) and Raf-1 kinase (16, 22).

In contrast to the well-characterized role of pattern recognition receptors in activating signaling pathways for induction of cytokine release, their contribution in phagosome maturation is less well defined. Recently, the recruitment of proteins of the autophagy machinery, including LC3 II, Atg5, and Atg7, in phagosomes containing microbial ligands in response to TLR activation was found to be important for phagolysosomal fusion and pathogen elimination by murine macrophages (23). Although the signaling regulating autophagy protein recruitment in TLR-containing phagosomes has not been characterized, this response was shown to be dependent on NADPH-derived ROS production (24). At present, there is no clear evidence on whether and how innate sensing of A. fumigatus is linked to phagosome maturation and killing by professional phagocytes.

In this study, we found that A. fumigatus infection of human monocytes triggered a selective recruitment of LC3 II autophagy protein in phagosomes upon fungal cell wall swelling. This response was induced by surface exposure of immunostimulatory β-glucans, required activation of Dectin-1/Syk kinase/ROS signaling, and it was nearly absent in monocytes of patients with CGD. This pathway was important for fungal clearance because conditional inactivation of Arg5 resulted in attenuated phagolysosomal fusion and killing of A. fumigatus spores. Importantly, in vivo or ex vivo treatment of human monocytes with hydrocortisone blocked LC3 II recruitment in Aspergillus-containing phagosomes via rapid inhibition of phosphorylation of Syc and Syk kinases and subsequent blockade in ROS production. Overall, our studies link Dectin-1/Syk kinase signaling with autophagy-dependent maturation of fungal phagosomes and uncover a potential target for development of novel immunotherapies against invasive Aspergillus infections.

Materials and Methods

Reagents

Highly purified Escherichia coli LPS (catalog number 437627) was purchased from Calbiochem; Laminarin from Laminaria digitata (catalog number L9634), β-(1–3)-Glucan–specific mAb (catalog number 400-2) was from Biosupplies (Parkville, Australia). Blocking mAb for Dectin-1 (GE2) (catalog number ab82888; 10 µg/ml) was from Abcam. TLR2 (10 µg/ml), TLR4 (10 µg/ml), and appropriate isotype control Abs were from eBioscience. In some experiments, highly purified Bartonella LPS was used as a potent TLR4 inhibitor. A specific Syk kinase inhibitor (1 µM; catalog number 574711), piceatannol (40 µM; catalog number 527948), and raf-1 inhibitor (40 µM; catalog number 553808, GW5074) were from Calbiochem. Hydrocortisone (Lyco-cortin) was from Vianex. Anti-LC3 Ab used for immunofluorescence was from Nanotools (0231-100/LC3-5F10). FITC-conjugated Dectin-1 Ab (MCAA4661FT) was from AbD Serotec. Latex beads of 3-µm diameter were purchased from Sigma-Aldrich. Coating of latex beads with IgG or BSA was performed by overnight rotation incubation at 4°C with human IgG (1 mg/ml) or BSA (1 mg/ml) followed by three washes with PBS.

Isolation and stimulation of human primary cells from patients and controls

Healthy volunteers without any known infectious or inflammatory disorders donated blood as a control group for the assessment of LC3 II recruitment in fungal phagosomes. In addition, PBMCs were isolated from three patients with CGD harboring homozygous mutations in the NCF1 gene (p47-phox) in which the complete absence of ROS production has been demonstrated and three homozygous patients with the early stop codon mutation Fiz238X in Dectin-1 (Dectin-1<sup>−/−</sup>). After informed consent, blood was collected by venipuncture from these patients and volunteers into 10-ml EDTA tubes. Six consecutive patients with various rheumatologic diseases receiving treatment with a standard dose of corticosteroids (Table I) were recruited from the Rheumatology Department, University Hospital of Heraklion.

Monocytes from healthy controls and patients were isolated from PBMCs using magnetic bead separation with anti-CD14–coated beads (MACS, Miltenyi Biotec) according to the protocol supplemented by the manufacturer.

The monocytes were resuspended in RPMI 1640 culture medium supplemented with 1% gentamicin, 1% L-glutamine, and 1% pyruvate. The cells were counted in a Bürker counting chamber, and their number was adjusted to 1 × 10⁶/ml. A total of 2 × 10⁵ monocytes per condition in a final volume of 200 µl were allowed to adhere to glass coverslips (Ø12 mm) for 1 h, after which they were exposed to A. fumigatus spores at a multiplicity of infection (MOI) of 3:1 at 37°C for 1 h. After stimulation, the coverslips were washed twice with PBS to remove medium, and nonphagocytosed spores and cells were fixed on the coverslips for 15 min in 4% paraformaldehyde (PFA). Subsequently, the coverslips were washed with PBS followed by a fixation in ice-cold methanol for 10 min in −20°C, after which coverslips were stored in PBS at 4°C until immunofluorescence staining was performed.

Microorganisms and culture conditions

The A. fumigatus strains AF293 (ATCC 46645) and the GFP-A. fumigatus strain (kind gift of K. A. Marr) were used in this study. All isolates were grown on YAG agar plates for 3 d at 37°C. Fungal spores in the presence of sterile 0.1% Tween 20 in PBS were harvested by gentle shaking. washed twice with PBS, filtered through a 40-µm pore size cell strainer (Falcon) to separate conidia from contaminating mycelial clumps, and subjected to a hemocytometer, and suspended at a concentration of 10⁷ spores/ml. Swollen spores of A. fumigatus were obtained following growth in liquid RPMI 1640 media for 4–6 h at 37°C. Typically, >90% of fungal spores were visibly swollen. The conidia were labeled with FITC as previously described (9). Briefly, freshly harvested conidia (5 × 10⁵/2 ml 50 mM Na carbonate buffer [pH 10.2]) were incubated with FITC at a final concentration of 0.1 mg/ml at 37°C for 1 h and washed by centrifugation three times in PBS at 0.1% Tween 20.

Enzymatic digestion of β-glucan in swollen spores of A. fumigatus was performed by using β-1–3-n-glucanase (Sigma-Aldrich). A. fumigatus spores were incubated overnight in a water bath with 100 U/ml β-glucanase at a temperature of 55°C and pH 5. Inactivation of enzyme was achieved by 10-min incubation at 100°C followed by three washes in PBS. Verification of β-glucan digestion was performed by immunostaining with a β-glucan mAb. Inactivation of fungi was done by heat exposure (30 min, 65°C) or exposure to 1% PFA (4°C, overnight) following by treatment with 0.1% Triton X-100 for 1 h and washed by centrifugation three times in PBS.

Immunofluorescence staining

For immunofluorescence imaging, cells were seeded in coverslips pretreated with polysylane, fixed with 4% PFA for 15 min in room temperature following by 10 min of fixation with ice-cold methanol at −20°C, washed twice with PBS, permeabilized by using 0.1% saponin (Sigma-Aldrich), blocked for 30 min in PBS plus 2% BSA, incubated for 1 h with a mouse mAb to CD63 (catalog number 558508, Invitrogen) and immunostaining with a mouse mAb to LC3 (1:50; Nanotools, Germany) followed by 10 µm TO-PRO-3 iodide (642/661; Invitrogen). After the washing steps, slides were mounted in Prolong Gold antifade media (Molecular Probes). Images were acquired using a laser-scanning spectral confocal microscope (TCS SP2; Leica Microsystems), LCS Lite software (Leica Microsystems), and a 40× Apochromat 1.25 NA oil objective using identical gain settings. A low-fluorescence immersion oil (11513859; Leica Microsystems) was used, and imaging was performed at room temperature. Unless otherwise stated, mean projections of image stacks were obtained using the LCS Lite software and processed with Adobe Photoshop CS2 (Adobe Systems).

Phagosome acidification was assessed by use of the acidic dye LysoTracker Red DND-99 according to the manufacturer’s instructions (Invitrogen) and immunostaining with a mouse mAb to CD63 (catalog number 556019; BD Pharmingen) in primary human monocytes and THP-1 cells. Immunofluorescence imaging of LysoTracker Red fluorescence in complete medium containing RPMI 1640 supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mM 2-ME, 4.5 g/l glucose, and 10% FCS.
spores (2 × 10^7 condition) were pelleted in propylene tubes, washed twice with PBS, blocked for 30 min in PBS plus 2% BSA, incubated for 1 h with a mouse mAb to linear-(1, 3)-β-glucan (BioSuppies; 1 μg/ml) at room temperature, washed twice in PBS plus 2% BSA, stained by a secondary Alexa Fluor 550 goat anti-mouse Ab ( Molecular Probes), and images were acquired by confocal microscopy.

**Immunoelectron LC3 microscopy in monocytes**

Immunoelectron microscopy was performed using mouse monoclonal LC3 Ab (NanoTools), applying the pre-embedding gold enhancement method as described previously (25). Primary human monocytes cultured on polylysine-pretreated coverslips were fixed with 4% PFA (Nacalai Tesque) for 15 min at room temperature. After washing with the same buffer three times for 5 min, the fixed cells were permeabilized using 0.25% saponin in PBS. The cells were washed with PBS, blocked by incubating for 30 min in PBS containing 0.1% saponin, 10% BSA, 10% normal goat serum, and then exposed overnight to 0.01 mg/ml anti-LC3 mouse mAb or 0.01 mg/ml rat serum in the blocking solution. After washing with PBS containing 0.005% saponin, the cells were incubated with colloidal gold (1.4-nm diameter; Nanoprobes)-conjugated goat anti-mouse IgG in the blocking solution for 2 h. The cells were then washed with PBS and fixed with 1% glutaraldehyde in PBS for 10 min. After washing with 50 mM glycine in PBS, 1% BSA in PBS, and finally with milliQ water (Millipore), gold labeling was intensified with a gold enhancement kit (GoldEnhance EM; Nanoprobes) for 3 min at room temperature according to the manufacturer’s instructions. After washing with distilled water, the cells were postfixed in 1% OsO₄ containing 1.5% potassium ferrocyanide in PBS for 60 min at room temperature and washed with distilled water. The cells were dehydrated in a series of graded ethanol solutions and embedded in epoxy resin. After the epoxy resin hardened, the plastic coverslip was removed from it. Ultrathin sections were cut horizontally to the cell layer and double stained with uranyl acetate and lead citrate. Samples were analyzed with an electron microscope. Serial sections were collected on pioloform-coated copper grids and samples analyzed in a Philips CM100 electron microscope (Philips, Eindhoven, The Netherlands).

**Western blot analysis**

Primary human monocytes were stimulated with A. fumigatus conidia for the indicated time points at an MOI of 10:1. Where appropriate, cells were preincubated with DMSO or the indicated concentrations of inhibitors for 30 min prior to stimulation. Cells were washed once in PBS prior to lysis in 1% Nonidet P-40 containing RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 1 mM Na₂VO₃, and 1 mM PMSF plus a mixture of protease inhibitors [Roche Molecular Biochemicals]). Cell lysis was performed on ice for 20 min, and samples were centrifuged. After protein estimation of supernatants, addition of SDS sample buffer, and boiling, samples were separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes. Western blotting was performed according to the instructions of the manufacturer using the following primary Abs: rabbit anti-LC3 (NB100-2222; Novus), mouse anti-actin (MAB 1501; Millipore), mouse anti-tubulin (T5168; Sigma-Aldrich), rabbit anti-Syk (sc-1077), rabbit anti-Phospho-SyK (Tyr557/559; 2710; Cell Signaling Technology), rabbit anti-Phospho Src (Tyr416; 1221; Cell Signaling Technology), and goat anti-ATG5 (sc-8666; Santa Cruz Biotechnology). Secondary Abs used in Western blotting were purchased from Cell Signaling Technology (anti-rabbit HRP and anti-goat HRP) as well as Millipore (anti-mouse HRP). The blots were developed using chemiluminescence (ECL; Thermo Scientific).

**Measurement of ROS production in human monocytes**

ROS measurements were performed by means of a DCFH assay (26). Stock solution of DCFH-DA was dissolved in DMSO to a final concentration of 100 mM. Human monocytes (2 × 10^5) were plated on 96-well round-bottom plates, incubated at 37°C for the indicated time (2 h) with or without hydrocortisone, and accordingly stimulated for 1 h with A. fumigatus spores in the presence of DCFH-DA added to a final concentration of 10 μM. After 30 min of exposure, the content of the wells were transferred to vials and the fluorescence of the cells from each well measured by flow cytometry. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Killing of A. fumigatus spores by THP-1 cells**

THP-1 cells were plated onto 12-well plates and allowed to differentiate to macrophages in the presence of PMA (100 μg/ml). Cells that were adherent after 48 h were used in phagocytosis and killing experiments. To measure macrophage killing of conidia, PMA was removed by adding fresh medium, and THP-1 cells were allowed to ingest A. fumigatus conidia at an MOI of 1:10 for 1 h at 37°C. Medium containing nonadherent, nonphagocytosed conidia was removed, and wells were washed three times using warm PBS. Macrophages were then allowed to kill conidia for 2 and 6 h before intracellular conidia were harvested. Macrophages were removed by scraping, placed in propylene tubes, snap frozen with the use of liquid nitrogen, and rapidly thawed at 37°C to lyse the THP-1 cells and harvest conidia. The process of cellular lysis was performed twice and confirmed by light microscopy. Lysates left overnight at 4°C in RPMI 1640. The percentage of killing (number of nongerminated spores per 100 counted conidia) in the culture well after 6–8 h of incubation at 37°C was assessed under a microscope. Control wells containing only A. fumigatus conidia showed that the percentage of germination of the conidia used was always >90%.

**Silencing of Atg5 expression by specific short interfering RNA**

Short interfering RNA (siRNA) targeting was used to knockdown Atg5 expression in human THP-1 monocytes. A human monocyte nucleofector kit (Amaxa, Gaithenberd, MD) and Nucleofector device (Amaxa) were used for delivering siRNA into monocytes by following the instructions provided by the manufacturer. In brief, 1.5 × 10^6 THP-1 cells were suspended in 100 μl human monocyte nucleofector solution (Amaxa) and transfected with siRNA at a final concentration of 100 nM using the V-001 program. Transfected cells were immediately diluted with prewarmed growth media and cultured in 12-well plates for 24 h. THP-1 cells were allowed to differentiate for an additional 48 h in the presence of PMA (25 μg/ml) and then used for experiments. The following siRNA pool of oligonucleotide sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): ATGS RNA interference (RNAi; sc-41445) and control RNAi (C RNAi) oligonucleotide sequences (sc-37003). Specific gene knockdowns were assessed by immunoblotting.

**Statistical analysis**

The data were expressed as means ± SE. Statistical significance of differences was determined by Student t test and Bonferroni t test (p < 0.05 was considered statistically significant). Analysis was done in GraphPad Prism software (version V; GraphPad). All experiments were performed at least in triplicate and replicated at least twice.

**Results**

*The autophagy protein LC3 II is selectively recruited in A. fumigatus phagosomes upon fungal cell wall swelling*

To evaluate whether autophagy proteins participate in immune responses against *A. fumigatus*, we monitored the kinetics of LC3 II recruitment to phagosomes of primary human monocytes infected with live spores of GFp- or FITC-labeled *A. fumigatus* by immunostaining with the use of an LC3-specific Ab. In contrast to the previously reported rapid LC3*⁺* phagosome formation, within minutes of the uptake of beads coated with TLR ligands (24), we noticed a delayed LC3 II recruitment in *A. fumigatus*-containing phagosomes that was pronounced only after 2 h of infection (Fig. 1A). Next, we asked whether the formation of LC3*⁺* phagosomes is elicited by fungal molecules that are either released or exposed during intracellular fungal cell wall swelling of *A. fumigatus* spores (9). Thus, we infected human monocytes with PFA-killed resting spores of *A. fumigatus* and assessed LC3 II recruitment. Surprisingly, we noticed minimal LC3 II recruitment in phagosomes even at late (4 h) time points of infection of human monocytes with PFA-killed resting spores of
A. fumigatus (Fig. 1B). Similarly, although monocyte infection with live A. fumigatus spores triggered high levels of LC3 II protein expression, there was no evidence of significant LC3 II protein expression in monocytes infected with PFA-killed A. fumigatus resting spores (Fig. 1C), by Western blot analysis. In contrast to PFA-killed resting spores of A. fumigatus, PFA-killed swollen spores triggered robust LC3 III phagosome formation (Fig. 1D, 1E) and pronounced LC3 II protein expression (Fig. 1F) by Western blot analysis. Collectively, these data reveal that LC3 II protein recruitment in A. fumigatus phagosomes is not dependent on release of soluble factors and occurs upon fungal cell wall swelling.

In agreement with previous studies that reported lack of classic double membrane autophagosome formation in LC3 + phagosomes containing TLR ligands (23), we found that A. fumigatus swollen spores were contained within single membrane phagosomes, which was also suggested by the presence of gold-conjugated Ab against LC3 only in the outer part of the phagosome membrane in immunoelectron microscopy studies (Fig. 1G).

b-glucan surface exposure during fungal cell wall swelling triggers LC3 II recruitment in A. fumigatus–containing phagosomes

Recent studies demonstrated that resting A. fumigatus spores are immunologically inert because of concealing of immunostimulatory molecular patterns by a surface layer of hydrophobin (27). Importantly, swelling of A. fumigatus spores leads to surface exposure of the immunostimulatory fungal polysaccharide β-1-3-β-
glucan (β-glucan) and induction of robust inflammatory responses (28). Therefore, we assessed whether stage-specific surface exposure of β-glucan in swollen spores of *A. fumigatus* accounts for selective LC3 II protein recruitment in *A. fumigatus* phagosomes. Accordingly, we performed enzymatic digestion of β-glucan in PFA-swollen spores of *A. fumigatus* by using a β-1-3-α-glucanase and assessed the effect on LC3 II protein recruitment in fungal phagosomes. Efficient digestion of β-glucan layer in *A. fumigatus* swollen spores was confirmed by immunofluorescence microscopy with the use of a β-glucan–specific Ab. We found that enzymatic digestion of β-glucan resulted in significant reduction in LC3*. A. fumigatus* phagosome formation (Fig. 2A, 2B) following infection of human monocytes with swollen *A. fumigatus* spores. Furthermore, laminarin, a nonimmunostimulatory soluble β-glucan that acts as competitive inhibitor of β-glucan receptors (28), almost completely abolished LC3*. A. fumigatus* phagosome formation (Fig. 2A, 2B) and LC3 II protein induction in human monocytes stimulated with swollen *A. fumigatus* spores (Fig. 2C). Notably, laminarin treatment had no effect in LC3 II protein conversion in human monocytes stimulated with IgG-coated latex beads (Fig. 2D).

The cell wall of *A. fumigatus* also contains galactomannan moieties (29), and previous studies have implicated mannose- or mannan-specific receptors, including dendritic cell–specific ICAM-3–grabbing nonintegrin and the long pentraxin PTX3, in the recognition of *A. fumigatus* (30, 31). To address the possible role of a mannose- or mannan-specific receptor in LC3*. A. fumigatus* phagosome formation by swollen spores of *A. fumigatus*, we pretreated human monocytes with *Saccharomyces cerevisiae*–derived mann (31) prior to their addition to swollen spores and observed no effect on LC3 II recruitment by immunofluorescence imaging or LC3 II expression by Western blot analysis, in contrast to the effect of laminarin (Supplemental Fig. 1).

To confirm the ability of β-glucan to trigger LC3* phagosome formation, we stimulated human monocytes with different forms of purified insoluble β-glucan, including curdlan and yeast-derived WGP of ~3 μm size. Stimulation of human monocytes with curdlan particles elicited robust autophagosome formation that was blocked by pretreatment with laminarin (Fig. 2E, 2F); in contrast, laminarin had no measurable effect in autophagy induction by LPS in human monocytes (Fig. 2F). In addition, stimulation of human monocytes with fluorescein dichlorotriazine–labeled WGP resulted in a high degree of LC3*. *A. fumigatus* phagosome formation, comparable to that induced by stimulation with IgG-coated latex beads (Fig. 2G). Similarly, we noticed high levels of LC3 II conversion following stimulation of human monocytes with WGP, a response completely inhibited by laminarin (Fig. 2H). Collectively, these studies demonstrate that β-glucan surface exposure in *A. fumigatus* fungal cell wall activates the recruitment of the autophagy protein LC3 II in fungal phagosomes.

**LC3 II recruitment in *A. fumigatus* phagosomes depends on Dectin-1 signaling and is mediated by Syk kinase**

Sensing of β-glucan by human myeloid cells predominantly occurs via engagement of the C-type lectin receptor Dectin-1 (16, 17). Human patients with the homozygous early stop-codon mutation Tyr238X in Dectin-1 display lack of surface receptor expression, defective cytokine release, and hypersusceptibility to mucocutaneous fungal infections (20). We tested whether Dectin-1 receptor is involved in β-glucan–induced LC3* phagosome formation by infecting monocytes of three patients having homozygous Dectin-1 Tyr238X mutation (Dectin-1<sup>−/−</sup>) with PFA-killed resting and swollen spores of *A. fumigatus*. We found that monocytes of Dectin-1<sup>−/−</sup> patients had significant reduction in formation of LC3* phagosomes following infection with swollen spores of *A. fumigatus* when compared with monocytes of Dectin-1<sup>+/−</sup> controls (Fig. 3A).

In addition, blocking of Dectin-1 receptor in monocytes from healthy individuals with the use of a specific Ab resulted in significant reduction in LC3* phagosome formation following infection with swollen spores of *A. fumigatus* (Fig. 3B). Because TLR2 and TLR4 receptors are the main TLRs involved in sensing of *A. fumigatus* (2, 14, 15), we tested whether they also regulate autophagy protein recruitment in the phagosome. There was no evidence of significant reduction in LC3 II recruitment in phagosomes containing swollen spores of *A. fumigatus* following blockade of either TLR2 receptor using TLR2-specific Ab or TLR4 receptor using either TLR4-specific Ab (Fig. 3B) or Bartonella Quintana LPS, a specific TLR4 inhibitor. Because β-glucan has been reported to activate complement receptor 3 in human phagocytes (17), we blocked this receptor by using competitive inhibition with N-acetyl-α-glucosamine (32, 33) and assessed the effect in LC3* *A. fumigatus* phagosome formation. We did not find significant reduction in LC3 II recruitment and LC3 II protein conversion in human monocytes pre-exposed to N-acetyl-α-glucosamine and subsequently infected with swollen spores of *A. fumigatus* (Supplemental Fig. 1). These studies suggest that LC3 II recruitment in *A. fumigatus* phagosomes depends mainly on activation of the Dectin-1 receptor.

Coupling of Syk kinase with Dectin-1 and other c-type lectin receptors activates multiple downstream pathways (16, 17, 34). However, the role of Syk kinase in phagosome maturation has not been earlier evaluated. In agreement with a stage-specific pattern of β-glucan exposure in the cell wall surface of *A. fumigatus*, we found selective activation of Syk kinase following monocyte infection with swollen and not with resting spores of *A. fumigatus* (Fig. 3C). Importantly, treatment of human monocytes with two different Syk kinase inhibitors almost completely abolished LC3 II recruitment in phagosomes containing swollen *A. fumigatus* spores and blocked LC3 II protein conversion by Western blot analysis (Fig. 3D, 3E).

Similarly, treatment with Syk kinase inhibitor blocked LC3 II recruitment in phagosomes containing purified β-glucan particles (WGP; Supplemental Fig. 2). Of interest, Syk kinase inhibitors also blocked LC3 II recruitment in phagosomes containing IgG-coated latex beads (Supplemental Fig. 2), implying that Syk kinase controls LC3* phagosome formation upon activation of a broad range of pattern recognition receptors that contain ITAM motifs.

Raf-1 kinase has been implicated in Dectin-1 signaling via a Syk-independent alternative noncanonical pathway of activation of NF-κB (22). Thus, we tested whether signaling mediated by raf-1 kinase is involved in LC3 recruitment in *A. fumigatus* phagosomes. Blocking of raf-1 kinase by use of a specific raf-1 inhibitor did not cause significant reduction in LC3* phagosome formation (Fig. 3E, 3F) and LC3 II protein expression (Fig. 3G) in human monocytes stimulated with swollen *Aspergillus* spores. Collectively, these studies demonstrate that Dectin-1/Syk kinase signaling regulates the formation of LC3* *A. fumigatus* phagosomes.

**Syk kinase–dependent ROS production regulates formation of LC3* Aspergillus-containing phagosomes**

Recent studies implicate NOX-2–dependent ROS production in regulation of LC3 II recruitment in phagosomes of murine macrophages containing TLR and FcγR ligands (24). Because Syk kinase regulates ROS production in response to β-glucan (16, 17, 34, 35), we tested whether Syk-mediated LC3 II recruitment in *A. fumigatus*–containing phagosomes was dependent on production of ROS. We initially confirmed that similar to murine macrophages (35), treatment with Syk kinase inhibitor in primary human monocytes resulted in complete inhibition of ROS production in human monocytes stimulated with swollen *A. fumigatus* spores (Fig. 4A).
Importantly, patients with GCD have mutations in various components of NADPH oxidase and unique susceptibility to invasive A. fumigatus infection via incompletely characterized mechanisms (1, 2, 11). Thus, we tested whether abolished ROS production in monocytes of CGD patients results in defective LC3 II recruitment in A. fumigatus–containing phagosomes. When compared with monocytes of control healthy individuals, monocytes of three CGD patients displayed almost complete abolishment of LC3+ phagosome formation following infection with A. fumigatus (Fig. 4B, and 4C). In addition, we noticed decreased LC3 II protein expression in lysates of monocytes from CGD patients infected with A. fumigatus in comparison with lysates of monocytes from healthy control patients infected with the fungus (Fig. 4D). Therefore, NADPH-derived ROS production regulates LC3 II recruitment in A. fumigatus–containing phagosomes, and this pathway is defective in patients with CGD.

Silencing of Atg5 in human macrophages results in attenuated phagosome maturation and killing of A. fumigatus. Recent studies demonstrated that silencing or knockdown of autophagy related genes Atg5 and Atg7 in murine macrophages resulted in impaired LC3 II protein expression in lysates of monocytes from CGD patients infected with A. fumigatus in comparison with lysates of monocytes from healthy control patients infected with the fungus (Fig. 4D). Therefore, NADPH-derived ROS production regulates LC3 II recruitment in A. fumigatus–containing phagosomes, and this pathway is defective in patients with CGD.

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fusion of zymosan-containing phagosomes with lysosomes (23) and defective killing of *S. cerevisiae* (23) and *Candida albicans* (36). To evaluate the role of autophagy in human macrophage effector function against *A. fumigatus*, we performed silencing of *Atg5* in THP-1–differentiated macrophages (Fig. 5A), a human cell line previously shown to efficiently internalize and kill *A. fumigatus* (37). Silencing of *Atg5* in THP-1 macrophages resulted in significant reduction of the percentage of *A. fumigatus* spores within acidified lysosomes, as evidenced by LysoTracker staining (Fig. 5B, 5C).

We next assessed the effect of *Atg5* silencing in killing of *A. fumigatus* by THP-1 macrophages. Previous studies demonstrated that elimination of *A. fumigatus* occurs following an initial 2-h lag phase and reaches maximum levels at ~6 h of infection (9, 10, 37). In agreement with previous studies (35), we found that THP-1 cells prevented germination of ~60% of *A. fumigatus* spores at 6 h of infection, whereas there was little evidence of inhibition of *A. fumigatus* growth at earlier (2-h) time points of infection (Fig. 5E). Silencing of *Atg5* in THP-1 human macrophages had no significant effect on the uptake of fungal spores (Fig. 5D), but
resulted in attenuated killing of *A. fumigatus* (Fig. 5E). Collectively, these studies demonstrate that autophagy proteins regulate phagosome maturation and intracellular killing of *A. fumigatus*. Corticosteroids block LC3 II recruitment in *A. fumigatus*-containing phagosomes via inhibiting Src and Syk kinase–dependent ROS production. Seminal studies in the 1970s demonstrated that corticosteroids block the fusion of lysosomes with *Aspergillus*-containing phagosomes in murine macrophages, leading to impaired killing of *A. fumigatus* (12, 13); however, a mechanistic explanation of the immunosuppressive action of corticosteroids on fungal phagosomes is lacking.

Because we found that components of autophagy regulate maturation of *A. fumigatus* phagosomes, we evaluated whether corticosteroids target this pathway. Therefore, we assessed LC3 III phagosome formation in monocytes of patients with rheumatologic diseases before and 2 h after i.v. administration of corticosteroids (Table I). Notably, we found a significant reduction in LC3 III *A. fumigatus*-containing phagosomes following corticosteroid treatment in monocytes of all patients tested (Fig. 6A, 6C). In addition, ex vivo administration of corticosteroids resulted in significant reduction in recruitment of LC3 II protein in *A. fumigatus* phagosomes when compared with control untreated monocytes (Fig. 6B). We next assessed whether Dectin-1/Syk kinase signaling regulating antifungal autophagy responses is also targeted by corticosteroids. Of interest, we found no difference in the uptake of *A. fumigatus* spores and Dectin-1 receptor expression following administration of corticosteroids (Supplemental Fig. 3). Because corticosteroids block TCR signaling by inhibiting phosphorylation of ITAM motifs of TCR mediated by tyrosine kinases (38, 39), we reasoned that they might as well inhibit phosphorylation of Src and Syk tyrosine kinases in human monocytes. Importantly, we found that hydrocortisone administration caused a rapid block in phosphorylation of Src and Syk kinases within 5 and 10 min of *A. fumigatus* infection, respectively (Fig. 6D, 6E).
Corticosteroids inhibit ROS production in murine macrophages following infection with A. fumigatus (10). Because we found that ROS production in response to A. fumigatus infection in human monocytes is dependent on Syk kinase signaling, we reasoned that corticosteroid-mediated blockade in Syk kinase activation would result in defective ROS production. Indeed, human monocytes treated with corticosteroids displayed a significant reduction in the levels of ROS production following infection with A. fumigatus (Fig. 6E). These studies demonstrate that corticosteroids target autophagy protein recruitment in A. fumigatus phagosomes via inhibiting Src/Syk-dependent ROS production and provide a potential mechanism for their direct immunosup-
pressive properties in phagosome maturation and killing of Aspergillus spp.

Discussion

In the present work, we shed light in the signaling regulating A. fumigatus phagosome maturation and uncover a potential mechanism for development of invasive fungal disease in patients with CGD and corticosteroid-induced immunosuppression. In particular, we found that activation of Dectin-1/Syk kinase/ROS signaling upon exposure of β-glucan in A. fumigatus spores triggers the recruitment of autophagy protein LC3 II in fungal phagosomes, a response that is abolished in monocytes of patients with CGD. Furthermore, by silencing Atg5 in human phagocytes, we demonstrate that autophagy protein assembly is important for maturation of A. fumigatus phagosomes and fungal clearance. Very important from a clinical point of view, we also discovered that corticosteroids target the pathway of LC3+ A. fumigatus phagosome formation by causing an early block in phosphorylation of Src and Syk kinase and downstream production of ROS.

Autophagy is a lysosomal degradation pathway that, among other immune-related actions, mediates clearance of intracellular pathogens via their engulfment upon escape to the cytosol (40). Little is known about the role of autophagy pathway in immunity against extracellular pathogens, including fungi. Recent studies implicating autophagy proteins in regulation of maturation of phagosomes containing TLR ligands prompted us to study the physiologic relevance of this pathway in immunity against A. fumigatus (23, 24). Our initial experiments identified that fungal cell wall swelling is the trigger for LC3 II recruitment in A. fumigatus phagosomes. Of interest, these studies provide a mechanistic explanation of previous observations by electron microscopy on the intracellular lifecycle of A. fumigatus, sug-

![Image](http://www.jimmunol.org/Downloadedfrom)
sugesting that fungal cell wall swelling is a prerequisite for efficient phagosome maturation and killing of *A. fumigatus* by murine macrophages (9).

Because immunostimulatory β-glucans are selectively exposed at the surface of the fungal cell wall surface upon swelling of *A. fumigatus* spores (28), we tested whether this could be the trigger for LC3 II recruitment in fungal phagosomes. By using different assays, including β-glucan enzymatic digestion, competitive inhibition with laminarin, and stimulation with purified β-glucans particles, we found that LC3+ *A. fumigatus* phagosome formation was dependent on cell wall β-glucans. Previous studies in the murine RAW macrophage cell line using zymosan, a crude fungal cell wall extract rich in β-glucans, reported robust LC3+ phagosome formation around zymosan particles mediated by TLR2 engagement (23, 24). However, because RAW macrophages express low levels of the β-glucan–sensing receptor Dectin-1 (41) and because zymosan is a mixture of β-glucan and TLR ligands, it was difficult to dissect the contribution of β-glucan sensing in LC3 II recruitment. Overall, our study identified β-glucan as the key molecule activating recruitment of autophagy proteins in fungal phagosomes.

In a following set of experiments, we tested whether LC3+ *Aspergillus* phagosome formation was defective in monocytes of patients with the homozygous early stop-codon mutation Tyr238X in Dectin-1 (Dectin-1<sup>−/−</sup>). Indeed, we found a significant reduction in recruitment of LC3 protein in monocyes of Dectin-1<sup>−/−</sup> patients when compared with control Dectin-1<sup>+/+</sup> monocytes infected with *A. fumigatus*. Similarly, blocking Dectin-1 receptor in monocyes of healthy individuals with the use of a specific Ab resulted in significant reduction in LC3* A. fumigatus* phagosomes, whereas blocking TLR2 and TLR4 did not affect LC3 recruitment. Our findings corroborate a recent study reporting that in murine dendritic cells Dectin-1 activation was required for LC3 II recruitment in *C. albicans* phagosomes (42). Importantly, Dectin-1<sup>−/−</sup> patients are not at risk for invasive aspergillosis in the absence of additional immunosuppression (20, 21). In our studies, we noticed residual LC3 II recruitment in *A. fumigatus* phagosomes of Dectin-1<sup>−/−</sup> patients, which is suggestive of redundancy in upstream innate receptors implicated in antifungal autophagy responses. Although blocking of other known fungal pattern recognition receptors, including mannose, mannan receptors, and complement receptor 3, had no significant effect on LC3* A. fumigatus* phagosome formation, we cannot exclude that cooperative activation of other c-type lectin receptors (e.g., Dectin-2, Mingle) may play an important role in LC3 II recruitment in fungal phagosomes.

In addition, we assessed the role of Syk kinase in LC3 II recruitment in *A. fumigatus* phagosomes. Pharmacologic inhibition of Syk kinase almost completely abolished LC3 protein recruitment in *Aspergillus* phagosomes. Notably, inhibition of rap-1 kinase that also activates an alternative signaling pathway downstream of Dectin-1 had no impact on LC3<sup>+</sup> phagosome formation. Because Syk kinase is downstream of many different signaling receptors (34), our finding could have broad spectrum implications on regulation of autophagy responses following sensing of endogenous or pathogen-related ligands. Importantly, a recent study in Syk<sup>−/−</sup> bone marrow chimeric mice found an indispensable role of Syk kinase in intracellular killing of *A. fumigatus* by neutrophils and alveolar macrophages (43). An important role of Dectin-1/Syk kinase signaling in acidification of phagosomes containing β-glucan–coated particles has also recently been reported (44). In agreement, we found that inhibition of Syk kinase impaired acidification, as evidenced by defective CD63 protein recruitment (45) in *A. fumigatus* phagosomes upon infection of primary monocytes and THP-1–differentiated macrophages (Supplemental Fig. 4).

NADPH oxidase–derived ROS production was recently shown to regulate recruitment of autophagy proteins in phagosomes of murine macrophages containing TLR or FcγR ligands (24). In agreement with previous studies in murine and human phagocytes demonstrating that ROS production in response to zymosan is dependent on activation of Syk kinase (35), we found that ROS production was selectively induced in response to swollen spores of *A. fumigatus* in a Syk-dependent fashion. Studies in monocytes of CGD patients also revealed a block in LC3* A. fumigatus* phagosome formation, confirming that NADPH-derived ROS also regulate recruitment of autophagy proteins in fungal phagosomes. Because patients with CGD have increased susceptibility to invasive aspergillosis (1, 2, 11), and macrophages of mice with mutations in NADPH oxidase display defective phagolysosomal fusion and killing following the uptake of *A. fumigatus* spores (10), our studies suggest that defective autophagy protein recruitment could play an important role for development of invasive fungal infections in CGD.

Previous studies in murine macrophages demonstrated an important role of Atg7 and Atg5 proteins in phagosome maturation and clearance of yeast, including *S. cerevisiae* and *C. albicans* (23, 37). We also found that silencing of Atg5 in human THP-1 macrophages did not affect the uptake of fungal spores, but resulted in impaired maturation of *A. fumigatus* phagosomes and attenuated killing of the fungus. In humans, there are no previous studies to suggest a link between defective autophagy protein function and invasive fungal disease. Because full disruption of Atg5 have not been described, it has been difficult to assess the direct in vivo role of autophagy in *Aspergillus* immunity. Future studies in conditional Atg5 knockout mice should define the in vivo role of autophagy in *A. fumigatus* host defense and allow studying this pathway in neutrophils and other immune cell types with important role in antifungal immunity. An important future direction of research is represented by genetic association studies of polymorphisms in autophagy genes with susceptibility to fungal infection, studies that could validate the present in vitro data in a clinical setting.

Finally, we assessed whether corticosteroids, the major risk factor for development of invasive aspergillosis, target autophagy protein recruitment in *A. fumigatus* phagosomes. Surprisingly, we found that administration of a relatively low dose of corticosteroids blocked LC3 recruitment in *A. fumigatus* phagosomes within 2 h of exposure. Because of the rapid inhibition of LC3* A. fumigatus* phagosome formation by hydrocortisone, we reasoned that this effect is mediated by nongenomic action of corticosteroids on Dectin-1/Syk kinase signaling. Notably, corticosteroids had no effect on *A. fumigatus* uptake and expression of Dectin-1 receptor. Because corticosteroids have been shown to block tyrosine kinase phosphorylation within minutes of exposure in T cells (39, 40) and B cells (47), we focused on their effects in phosphorylation of Src and Syk kinases in monocytes. Notably, we found that hydrocortisone almost completely inhibited phosphorylation of both Src and Syk kinases within min of exposure. Because Syk kinase regulates ROS production in response to *A. fumigatus* infection, and corticosteroids have been shown to block ROS in macrophages during fungal infection (10), we tested whether hydrocortisone blocked ROS production in monocytes infected with *A. fumigatus*. Indeed, hydrocortisone caused a significant reduction in ROS production following infection with *A. fumigatus*. Of interest, recent studies on T cells demonstrate that glucocorticoids induce macroautophagy prior to the induction of apoptosis, because of their ability to inhibit Src kinases and downstream inositol 1,4,5-triphosphate–mediated calcium signaling (48). We also found evidence of increased
macropautophagy in monocytes pretreated with corticosteroids, an effect that precluded the assessment of blockade in LC3β phagosome formation by Western blot analysis. Thus, our studies reveal a selective property of corticosteroids to inhibit LC3 II recruitment in fungal phagosomes, which is regarded as a specialized form of autophagy. Collectively, our studies demonstrate an important physiologic role of autophagy pathway in restriction of intracellular growth of A. fumigatus within human phagocytes. Furthermore, our findings on defective antifungal autophagy as a result of impaired Dectin-1/Syk kinase/ROS signaling could provide a mechanistic explanation for the defective phagocyte function in two distinct groups of patients with increased susceptibility for invasive aspergillosis. Future studies are warranted to explore the therapeutic potential of autophagy induction in these patients and better define the in vivo role of autophagy in antifungal immunity.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Effect of N-acetyl-D-glucosamine (NADG) or yeast mannan pre exposure in LC3 II recruitment in A. fumigatus phagosomes. A. Primary human monocytes (2 × 10^5 cells/condition) isolated from healthy individuals were infected with FITC-labeled A. fumigatus swollen spores with or without mannan (500 µg/ml) or NADG (500 µg/ml) at a MOI 5:1 for 1h. Cells were fixed, permeabilized, and stained for LC3 II. The percentages of LC3+ A. fumigatus-containing phagosomes (LC3+ Aspergillus; n > 150 per group) were quantified and data are presented as mean ± S.E.M. of 2 independent experiments. B. Primary human monocytes (2 × 10^6 cells/condition) were stimulated with A. fumigatus swollen spores alone or in the presence of increasing concentrations of laminarin, or (D) IgG coated 3mm latex beads alone or in the presence of increasing concentrations of laminarin for 1h. B. Primary human monocytes (2 × 10^6 cells/condition) were stimulated as in A. Cell lysates were prepared and levels of LC3 II protein were determined by immunoblotting.

Supplementary Figure 2. Effect of Syk and raf-1 inhibitor in LC3 II phagosome recruitment in human monocytes stimulated with purified b-glucan particles (WGP) or IgG coated latex beads. Primary human monocytes (2 × 10^5 cells/condition) isolated from healthy individuals were stimulated with IgG coated beads with or without pretreatment with a specific Syk inhibitor (574711, Calbiochem, 1 µM) or yeast derived whole glucan particles (WGP) both at MOI 5:1 for 1h. Cells were fixed, permeabilized, stained for LC3 II with the use of an Alexa555 secondary antibody (red) and TOPRO-3 (blue, nuclear staining) and analyzed by immunofluorescence confocal microscopy. The percentages of LC3+ A. fumigatus-containing phagosomes (LC3+ Aspergillus; n > 150
Supplementary Figure 3. Effect of hydrocortisone on Dectin-1 expression and the uptake of FITC-labeled *A. fumigatus* spores by human monocytes. A. Primary human monocytes (2 × 10^5 cells/condition) from healthy individuals (n = 2) were stimulated before (0h) and after (2h) *ex vivo* exposure to hydrocortisone (20 µg/ml), infected with FITC-labeled swollen spores of *A. fumigatus* for 1h, washed three times with PBS to removed unbound spores, and the degree of internalization of FITC-labeled *A. fumigatus* spores with monocytes was immediately assessed by FACS analysis. B. Primary human monocytes stimulated as in A were stained with the use of a FITC-conjugated Dectin-1 monoclonal antibody or appropriate isotype control antibody and levels of Dectin-1 surface expression were analyzed by FACS.

Supplementary Figure 4. Syk kinase signaling regulates acidification of *A. fumigatus* phagosomes. Primary human monocytes (2 × 10^5 cells/condition) isolated from healthy individuals and THP-1 PMA-differentiated macrophages (10^5 cells/condition) were stimulated with FITC-labeled resting or swollen spores of *A. fumigatus* with or without 30 min pretreatment with Chloroquine (Sigma, 100 µM) or a specific Syk inhibitor (574711, Calbiochem, 1 µM) at MOI 5: 1 for 1h. Cells were fixed, permeabilized, stained for CD63 with the use of an Alexa^555^ secondary antibody (red) and TOPRO-3 (blue, nuclear staining) and analyzed by immunofluorescence confocal microscopy. The percentages of CD63^+ *A. fumigatus*-containing phagosomes (> 150 per group) were quantified and data are presented as mean + S.E.M. of 3 independent experiments. *, P < 0.0001, paired Student’s *t* test.
Supplementary Figure 2
Supplementary Figure 3

Hydrocortisone 0h

Hydrocortisone 2h

CD14+ monocytes

FITC-A. fumigatus

CD14+ monocytes

FITC-Dectin-1
Supplementary Figure 4

CD14⁺ Monocytes

THP-1 cells