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Corticosteroids block autophagy protein recruitment in *Aspergillus fumigatus* phagosomes via targeting Dectin-1/syk kinase signaling

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Abstract

Aspergillus fumigatus is the predominant airborne fungal pathogen in immunocompromised patients. Genetic defects in NADPH oxidase (chronic granulomatous disease; CGD) and corticosteroid-induced immunosupression lead to impaired killing of *A. fumigatus* and unique susceptibility to invasive aspergillosis via incompletely characterized mechanisms. Recent studies link Toll-like receptor activation with phagosome maturation via the engagement of autophagy proteins. Herein, 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 syk kinase-dependent production of reactive oxygen species (ROS) 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 syk kinase phosphorylation and downstream production of ROS. Our studies link Dectin-1/syk kinase signaling with maturation of *A. fumigatus* phagosomes and uncover a mechanism for development of invasive fungal disease.

Keywords

Autophagy; *Aspergillus fumigatus*; Dectin-1; β-glucan; syk; corticosteroids

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Contributions: E.K., M.S.G., T.A., and G.C. analyzed data and performed experiments. G.C. conceived and supervised the study, designed and performed experiments and wrote the manuscript. G.S., P.S., D.B. D.P.K., M.G.N., and F.L.V. analyzed the data. M.G.N. and F.L.V. contributed reagents.

INTRODUCTION

Aspergillus fumigatus, a ubiquitous saprophytic mold, is a leading cause of morbidity and mortality in immunocompromised patients[1]. Acquired quantitative and qualitative innate immune defects, typically encountered in hematological malignancy patients with severe chemotherapy-induced neutropenia and recipients of transplants following treatment with high doses of corticosteroids, are major predisposing factors for development of invasive aspergillosis[1–3]. *A. fumigatus* is currently regarded as an emerging fungal pathogen in a broad range of non-neutropenic hosts who receive prolonged courses of corticosteroid therapy[4], including patients with autoimmune and inflammatory diseases, and prolonged stay in intensive care units[1,4–6]. Moreover, patients with chronic granulomatous disease (CGD), a rare primary immunodeficiency characterized by genetic defects in NADPH oxidase complex, are uniquely susceptible for development of invasive aspergillosis[1,2].

Although risk factors for development of invasive aspergillosis are well characterized, the immunopathogenesis of this frequently lethal opportunistic mycosis is incompletely understood. In immunocompetent individuals, professional phagocytes, including resident alveolar macrophages, circulating monocytes, and neutrophils, efficiently eliminate *A. fumigatus* spores, which are inhaled in a daily basis, to prevent germination of spores to hyphae and development of invasive fungal disease[1,2,7,8]. *A. fumigatus* spores are degraded within acidified lysosomal compartments of human phagocytes via the complex process of phagolysosomal fusion[9,10]. Genetic defects in NADPH oxidase-derived ROS generation and corticosteroid therapy are associated with impaired maturation of *A. fumigatus* phagosomes and attenuated fungal killing, via incompletely characterized mechanisms[11–13].

The past few years have witnessed major advances in understanding innate sensing of fungi. Initial studies demonstrated that *A. fumigatus* preferentially activates Toll like receptors (TLR)-2 and TLR-4, and results in NF-κB mediated immune responses[14,15]. Recent evidence suggests an emerging role for Dectin-1 and other C-type lectin 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.

Herein, 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 and required activation of Dectin-1 receptor. LC3 II recruitment in *Aspergillus* phagosomes was independent of raf-1 kinase signaling, but required syk kinase-mediated ROS production and it was nearly absent in monocytes of patients with CGD. This pathway was important for fungal clearance because conditional inactivation of A*tg5* resulted in attenuated phagolysosomal fusion and killing of *A. fumigatus* spores. Importantly, *in-vivo* administration of corticosteroids in patients with rheumatologic diseases or *ex-vivo*

treatment of human monocytes with hydrocortisone blocked LC3 II recruitment in *Aspergillus*-containing phagosomes via rapid inhibition of phosphorylation of syk kinase and downstream ROS production. Overall, our studies link Dectin-1/syk kinase signaling with phagosome maturation and uncover a potential target for development of novel immunotherapies against invasive fungal infection.

MATERIALS AND METHODS

Reagents

Highly purified *Escherichia coli* LPS (cat#, 437627) was purchased from Calbiochem, Laminarin from *Laminaria digitata* (cat#, L9634), β-1,3-D-glucanase from *Aspergillus niger* (cat#, 49101), and 2',7'-Dichlorofluorescin diacetate (DCFH-DA; cat#, D6883) were all obtained from Sigma-Aldrich. Purified particulate β-glucan (curdlan) was from Waiko (Tokyo, Japan). Yeast whole glucan particles (WGP) were from Biothera. For immunofluorescence imaging studies, WGP was labeled with fluorescein dichlorotriazine (DTAF; Molecular Porbes-Invitrogen). b-(1–3)-glucan-specific monoclonal antibody (cat#, 400-2) was from Biosupplies (Parkville, Australia). Blocking monoclonal antibody for Dectin-1 [GE2] (cat#, ab82888; 10 μg/ml) was from Abcam. TLR-2 (10 μg/ml), and TLR-4 (10 μg/ml) and appropriate isotype control antibodies were from eBioscience. In some experiments highly purified Bartonella LPS was used as a potent TLR-4 inhibitor. A specific syk kinase inhibitor (1μM) (cat#, 574711), piceatannol (40 μM) (cat#, 527948), and raf-1 inhibitor (40 μM) (cat#, 553008) were from Calbiochem. Hydrocortisone (Lyo-cortin) was from Vianex S.A.. Anti- LC3 antibody used for immunofluorescence was from Nanotools (0231-100/LC3-5F10). FITC conjugated Dectin-1 antibody (MCAA4661FT) was from AbD Serotec. Latex beads of 3μm diameter were purchased from Sigma. Coating of latex beads with IgG or BSA was performed by overnight rotating incubation at 4 °C with human IgG (1 mg/ml) or BSA (1 mg/ml) followed by 3 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, peripheral blood mononuclear cells (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 3 homozygous patients with the early stop-codon mutation *Tyr238X* in Dectin-1 (Dectin $1^{-/-}$). 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 1) were recruited from the Rheumatology Department, University Hospital of Heraklion (Greece).

Monocytes from healthy controls, and patients were isolated from PBMCs using magnetic bead separation with anti-CD14 coated beads (MACS miltenyi Germany) according to the protocol supplemented by the manufacturer. The monocytes were resuspended in RPMI culture medium supplemented with gentamicin 1%, L-glutamine 1%, and pyruvate 1%. The cells were counted in a Bürker counting chamber, and their number was adjusted to 1×10^6/ml. 2×10^5 monocytes per condition in a final volume of 200μL were allowed to adhere to glass coverslips (Ø12mm) for 1 hour after which they were exposed to *A. fumigatus spores* at a MOI of 3:1 at 37°C for 1 hour. After stimulation the coverslips were washed twice with PBS to remove medium and non-phagocytosed spores and cells were fixed on the coverslips for 15 minutes in 4% paraformaldehyde. Subsequently the coverslips were washed with PBS followed by a fixation in ice cold methanol for 10 minutes in −20 °C after which cover slips were stored in PBS at 4°C until immunofluorescence staining was performed.

Microorganisms and culture conditions

The *Aspergillus fumigatus* strains Af293, ATCC 46645 and the GFP-*Aspergillus fumigatus* strain (kind gift of KA Marr) were used in this study. All isolates were grown on YAG agar plates for 3 days 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 mycelium, counted by a hemacytometer, and suspended at a concentration of 10^8 spores/ml. Swollen spores of *A. fumigatus* were obtained following growth in liquid RPMI 1640 media for 4 h to 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 (5×10^7/2 ml of 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-0.1% Tween 20.

Enzymatic digestion of β-glucan in swollen spores of *A. fumigatus* was performed by using b-1–3-D-glucanase (Sigma). *A. fumigatus* spores were incubated overnight in a water bath with 100 U/ml of β-glucanase at a temperature of 55°C and pH 5, 0. 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 monoclonal antibody. Inactivation of fungi was done by heat exposure (30 min, 65 C) or exposure to 1% paraformaldehyde (PFA; 4°C, overnight) following by treatment with glycine (100 mm/PBS) and three washes in PBS. PFA-inactivation of *A. fumigatus* spores had no effect on β-glucan surface exposure as evidenced by immunostaining.

Immunofluorescence staining

For immunofluorescence imaging, cells were seeded in coverslips pretreated with poly lysine, fixed with 4% PFA for 15 min in room temperature following by 10 min of fixation with ice cold methanol in −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 1h with a mouse monoclonal antibody to LC3 (1:50, Nanotools), washed twice in PBS plus 2% BSA, stained by a secondary AlexaFluor^{555} goat anti-mouse Ab (1:500, Molecular Probes), followed by DNA staining with 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), LCS Lite software (Leica), and a 40× Apochromat 1.25 NA oil objective using identical gain settings. A low fluorescence immersion oil (11513859; Leica) 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.

Phagosome acidification was assessed by use of the acidotropic dye LysoTracker Red DND-99 according to Invitrogen instructions (Invitrogen). Briefly, THP-1 cells were seeded on coverslips in 24 well flat bottom plates and differentiated to macrophages following 48h exposure to PMA (100 μg/ml) in RPMI-10% FCS media. Cells were preloaded with LysoTracker (diluted 1:20,000 [vol/vol] in RPMI complete medium) for 2 h and were subsequently infected at 4°C with FITC-labeled *A. fumigatus* conidia (MOI 5:1) in fresh medium without LysoTracker. After removal of uningested conidia by washing with warm RPMI media, medium with LysoTracker was readded to each well and conidia internalization was initiated at 37°C. Infection was stopped after 2 h, and the cells were washed with PBS, mounted on microscope slides, and examined immediately under the confocal microscope.

For β-glucan immunostaining of *A. fumigatus* live or PFA-inactivated 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 monoclonal antibody to linear-(1,3)-β-glucan (Biosupplies; 1 μg/ml) at room temperature, washed twice in PBS plus 2% BSA, stained by a secondary AlexaFluor 555 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 antibody (Nanotools), applying the preembedding gold enhancement method as described previously[25]. Primary human monocytes cultured on poly lysine pretreated coverslips were fixed with 4% paraformaldehyde (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, then exposed overnight to 0.01 mg/ml of anti-LC3 mouse monoclonal antibody or to 0.01 mg/ml of 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 (Eindhoven, The Netherlands) CM100 electron microscope.

Western Blot analysis

Primary human monocytes were stimulated with *Aspergillus fumigatus* conidia for the indicated time points at a MOI 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 phosphate-buffered saline prior to lysis in 1% NP-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 Na_3VO_4, 1mM 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 (PVDF) membranes. Western blotting was performed according to the instructions of the manufacturer using the following primary antibodies: rabbit anti-LC3 (NB100-2220, Novus), mouse anti-actin (MAB 1501, Millipore), mouse anti-tubulin (T5168, Sigma), rabbit anti-Syk (sc-1077), rabbit anti-phospho Syk (Tyr525/526, 2710 Cell Signaling), and goat anti-ATG5 (sc-8666, Santa Cruz). Secondary antibodies used in western blotting were purchased from Cell Signaling (anti-rabbit HRP, 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 dichlorofluorescein assay[26]. Stock solution of dichlorofluorescin diacetate (DCFH-DA) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 100mM. Human monocytes (2×10^5/well) were plated on 96-well round bottom plates, incubated at 37°C for the indicated time (2h) with or without hydrocortisone and accordingly stimulated for 1h with A. *fumigatus* spores in the presence of DCFH-DA added to a final concentration of 10μM. 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 (American Type Culture Collection) were maintained 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-mercaptoethanol, 4.5 g/l glucose, and 10% FCS (v/v) at 37°C (5% CO_2), with passage every 3 days. THP-1 cells were plated onto 12-well plates and allowed to differentiate in the presence of 100 μg/ml phorbol 12-myristate 13-acetate (PMA; Sigma). Cells that were adherent after 48 h were utilized in phagocytosis and killing experiments. To measure macrophage killing of conidia, PMA was removed by adding fresh media and THP-1 cells were allowed to ingest *A. fumigatus* conidia at a 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 to 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 (siRNA)

Short interfering RNA (siRNA) targeting was used to knockdown *Atg5* expression in human THP-1 monocytes. Human monocyte nucleofector kit (Amaxa, Gaithenberg, MD, USA) and Nucleofector device (Amaxa) were used for delivering siRNA into monocytes by following the instructions provided by the company. In brief, 1.5×10^6 THP-1 cells were suspended in 100 μl of human monocyte nucleofector solution (Amaxa) and transfected with siRNA at a final concentration of 100nM using the V-001 program. Transfected cells were immediately diluted with pre-warmed growth media and cultured in 12-well plates for 24h. THP-1 cells were allowed to differentiate for an additional 48h 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): ATG5 RNAi (sc-41445) and Control RNAi oligonucleotide sequences (C RNAi; 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's t test and Bonferroni's t test ($p < 0.05$ was considered statistically significant). Analysis was done in GraphPad Prism software (version V).

RESULTS

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

In order 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-labelled-*A. fumigatus* by immunostaining with the use of an LC3 specific antibody. 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 2h of infection (Figure 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 paraformaldehyde (PFA)-killed resting (dormant) or PFA-killed swollen spores of *A. fumigatus* and assessed LC3 II recruitment. Surprisingly, we noticed minimal LC3 II recruitment in phagosomes even at late (4h) time points of infection of human monocytes with PFA-killed resting spores of *A. fumigatus* (Figure 1B). Similarly, while 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 (Figure 1C), by Western-blot analysis. In contrast to PFA-killed resting spores of *A. fumigatus*, PFA-killed swollen spores triggered robust $LC3^+$ phagosome formation (Figure 1D, 1E) and pronounced LC3 II protein expression (Figure 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 antibody against LC3 only in the outer part of the phagosome membrane in immunoelectron microscopy studies (Figure 1G).

β-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-D-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-D-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 antibody. We found that enzymatic digestion of β-glucan resulted in significant reduction in $LC3^+$ *A. fumigatus* phagosome formation (Figure 2A, and 2B) following infection of human monocytes with swollen *A. fumigatus* spores. Furthermore, laminarin, a non-immunostimulatory soluble β-glucan that acts as competitive inhibitor of β-glucan receptors[28], almost completely abolished $LC3^+$ *A. fumigatus* phagosome formation (Figure 2A and 2B) and LC3 II protein induction in human monocytes stimulated with swollen *A. fumigatus* spores (Figure 2C). Notably, laminarin

treatment had no effect in LC3 II protein conversion in human monocytes stimulated with IgG coated latex beads (Figure 2D).

The cell wall of *A. fumigatus* also contains galactomannan moieties[29], and previous studies have implicated mannose- or mannan-specific receptors, including DC-SIGN 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 $LC3^+$ phagosome formation by swollen spores of *A. fumigatus*, we pretreated human monocytes with *Saccharomyces cerevisiae*–derived mannan[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 (Supplementary Figure 1).

In order 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 whole β-glucan particles (WGP) of approximately 3 μm size. Stimulation of human monocytes with curdlan particles elicited robust autophagosome formation that was blocked by pretreatment with laminarin (Figure 2E, and 2F); in contrast, laminarin had no measurable effect in autophagy induction by LPS in human monocytes (Figure 2F). In addition, stimulation of human monocytes with DTAF-labeled WGP resulted in a high degree of $LC3^+$ phagosome formation, comparable to that induced by stimulation with IgG coated latex beads (Figure 2G). Similarly, we noticed high levels of LC3 II conversion following stimulation of human monocytes with WGP, a response completely inhibited by laminarin (Figure 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 −/−) with PFA-killed resting and swollen spores of *A. fumigatus*. We found that monocytes of Dectin-1 −/− patients had significant reduction in formation of $LC3^+$ phagosomes following infection with swollen spores of *A. fumigatus* when compared to monocytes of Dectin-1 +/+ controls (Figure 3A and supplementary Figure 2). In addition, blocking of Dectin-1 receptor in monocytes from healthy individuals with the use of a specific antibody resulted in significant reduction in $LC3^+$ phagosome formation following infection with swollen spores of *A. fumigatus* (Figure 3B). Because TLR-2 and TLR-4 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 TLR-2 receptor using TLR-2 specific antibody, or TLR-4 receptor using either TLR-4 specific antibody (Figure 3B) or *Bartonella Quintana* LPS, a specific TLR-4 inhibitor Because β-glucan has been reported to activate complement receptor 3 (CR3) in human phagocytes[17], we blocked this receptor by using competitive inhibition with N-acetyl-$_D$-glucosamine (NADG;[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 NADG and subsequently infected with swollen spores of *A. fumigatus* (Supplementary Figure 3). These studies

suggest that LC3 II recruitment in *A. fumigatus* phagosomes depends mainly on activation of 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 stage specific pattern of β-glucan exposure in 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* (Figure 3C). Importantly, treatment of human monocytes with two different syk kinase inhibitors almost completely abolished LC3 recruitment in phagosomes containing swollen *A. fumigatus* spores and blocked LC3 II protein conversion by Western-blot analysis (Figure 3D, E). Similarly, treatment with syk kinase inhibitor blocked LC3 recruitment in phagosomes containing purified β-glucan particles (**WGP**; Supplementary figure 3). Of interest, syk kinase inhibitors also blocked LC3 recruitment in phagosomes containing IgG coated latex beads (Supplementary figure 3), 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 non-canonical 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 (Figure 3E, 3F) and LC3 II protein expression (Figure 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 Fcγ receptor 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 (Figure 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 to monocytes of control healthy individuals, monocytes of three CGD patients displayed almost complete abolishment of LC3+ phagosome formation following infection with *A. fumigatus* (Figure 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 to lysates of monocytes from healthy control patients infected with the fungus (Figure 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 fusion of zymosan-containing phagosomes with lysosomes[23], and defective killing of *Saccharomyces cerevisiae*[23] and *Candida* albicans[36]. In order to evaluate the role of autophagy in human macrophage effector function against *A. fumigatus*, we performed silencing of *Atg5* in THP-1 differentiated macrophages (Figure 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 (Figure 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 2h lag phase and reaches maximum levels at about 6h of infection[9,10,37]. In agreement with previous studies[35], we found that THP-1 cells prevented germination of almost 60% of *A. fumigatus* spores at 6h of infection , whereas there was little evidence of inhibition of *A. fumigatus* growth at earlier (2h) time points of infection (Figure 5E). Silencing of *Atg5* in THP-1 human macrophages had no significant effect on the uptake of fungal spores (Figure 5D), but resulted in attenuated killing of *A. fumigatus* (Figure 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 syk kinase-dependent ROS production

Prolonged treatment with corticosteroids is a major risk factor for development of invasive aspergillosis[1–3]. Seminal studies in the 1970s demonstrated that corticosteroids blocked the fusion of lysosomes with *Aspergillus*-containing phagosomes in murine macrophages and impaired killing of *A. fumigatus*[12,13]. However, the molecular mechanisms of the immunosuppressive action of corticosteroids that account for defective phagocyte effector function and development of invasive aspergillosis are currently unknown.

Since we found that components of autophagy pathway regulate phagosome maturation and *A. fumigatus* killing upon activation of Dectin-1/syk kinase signaling, we evaluated whether corticosteroids target this antifungal immune pathway. Therefore, we collected monocytes from consecutive patients with rheumatologic diseases who received intermittent infusion of a standard dose of corticosteroids (250 mg hydrocortisone equivalent), and infected them with swollen spores of *A. fumigatus* to assess the effect on $LC3^+$ phagosome formation before and 2h after intravenous administration of corticosteroids. Notably, we found a significant reduction in *A. fumigatus*-containing phagosomes following corticosteroid treatment in monocytes of all patients tested (Figure 6A and 6C). In addition, monocytes from healthy individuals pre exposed *ex-vivo* for 2h to hydrocortisone displayed significant reduction in $LC3^+$ *A. fumigatus* phagosome formation when compared to control untreated monocytes (Figure 6B).

In order to gain insight in the molecular mechanism(s) of corticosteroid-mediated inhibition of LC3 II recruitment in *A. fumigatus* phagosomes we assessed whether Dectin-1/syk kinase signaling regulating this pathway is also targeted by corticosteroids. Of interest, we found no difference in the uptake of *A. fumigatus* spores and the level of expression of Dectin-1 receptor following administration of corticosteroids in human monocytes, suggesting that corticosteroids may affect downstream signaling events following receptor activation (Supplementary figure 4). Previous studies reported that corticosteroids block T cell receptor

(TCR) signaling by affecting early phosphorylating events induced after TCR ligation[38,39]. In particular, corticosteroids inhibited phosphorylation of ITAM motifs of TCR mediated by tyrosine kinases within 1h of administration both *in vitro* and *in vivo*, an effect mediated by direct interaction of glucocorticosteroid receptor with the TCR signalosome[38,39]. Importantly, there are no studies on the direct nongenomic action of corticosteroids in innate immune receptor signaling in phagocytic cells.

Because activation of C-type lectin receptors in myeloid cells via phosphorylation of ITAM motifs resembles activation of TCR signaling, we reasoned that corticosteroids might as well inhibit phosphorylation of syk tyrosine kinase in human monocytes. Therefore, human monocytes exposed at different time points to hydrocortisone at a dose that blocked $LC3^+$ phagosome formation were stimulated for 10 min with swollen *Aspergillus* spores, and subsequently lysed and assessed for phosphorylation of syk kinase by Western-blot analysis. Importantly, we found that hydrocortisone administration caused a rapid block in phosphorylation of syk kinase, without affecting cytoplasmic levels of syk within minutes of administration, an effect that was more pronounced following 1h of pre-exposure (Figure 6D).

Corticosteroids have been previously shown to inhibit ROS production in murine macrophages following infection with *A. fumigatus*[10]. Because we found that in human monocytes ROS production in response to *A. fumigatus* infection is dependent on phosphorylation of syk kinase, we hypothesized that a blockade of syk kinase activation following hydrocortisone receipt would result in defective ROS production and subsequent defective LC3 II phagosome recruitment. Indeed, human monocytes treated with corticosteroids at doses that blocked LC3 II recruitment displayed a significant reduction in ROS production following infection with *A. fumigatus* (Figure 6E). These studies demonstrate that corticosteroids target autophagy protein recruitment in *A. fumigatus* phagosomes via inhibiting syk dependent ROS production, and provide a mechanistic explanation for their direct immunosuppressive properties in phagosome maturation and killing of *Aspergillus spp*.

DISCUSSION

In the present work we shed light in the signaling pathway regulating *A. fumigatus* phagosome maturation and uncover an important mechanism for development of invasive fungal disease. In particular, we found that activation of Dectin-1/syk kinase signaling upon exposure of β-glucan in *A. fumigatus* spores triggers the recruitment of autophagy protein LC3 II in fungal phagosomes. LC3 II recruitment requires syk kinase-dependent ROS production and is abolished in monocytes of patients with CGD, who display unique susceptibility for invasive aspergillosis. 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 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 on 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*, suggesting 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 recruitment in fungal phagosomes. By using different assays, including β-glucan enzymatic digestion and competitive inhibition with the use of the soluble β-glucan laminarin, we found that $LC3^+$ *A. fumigatus* phagosome formation was dependent on sensing β-glucans. As a further proof-of-principle, purified β-glucan particles triggered robust $LC3^+$ phagosome formation. 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 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 −/−). Indeed, we found a significant reduction in recruitment of LC3 protein in monocytes of Dectin-1 −/− patients when compared to control Dectin-1 +/+ monocytes infected with *A. fumigatus*. Similarly, blocking Dectin-1 receptor in monocytes of healthy individuals with the use of a specific antibody resulted in significant reduction in $LC3^+$ *A. fumigatus* phagosomes, whereas blocking TLR-2 and TLR-4 did not affect LC3 recruitment. In addition, blocking of mannose, mannan receptors and the lectin binding site of CR3 had no significant effect in $LC3^+$ *A. fumigatus* phagosome formation in human monocytes. Our findings corroborate a recent study reporting that in murine dendritic cells and macrophages Dectin-1 activation was required for LC3 II recruitment in *Candida* albicans phagosomes[43]. Thus, our study links activation of Dectin-1 receptor with autophagy protein recruitment in the phagosome of human phagocytes.

In addition, we assessed the role of syk kinase in LC3 recruitment in *A. fumigatus* phagosomes. Pharmacologic inhibition of syk kinase almost completely abolished LC3 protein recruitment in *Aspergillus* phagosomes. Notably, inhibition of raf-1 kinase that also activates an alternative signaling pathway downstream of Dectin-1 had no impact on $LC3^+$ 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. For example, LC3 recruitment following stimulation of primary human monocytes with IgG coated beads, a response dependent on activation of Fcγ receptors, was also dependent on syk kinase. Although there are no previous studies on the role of syk kinase in phagosome maturation, conditional inactivation of syk in murine neutrophils resulted in impaired clearance of bacterial pathogens without a significant effect on phagocytosis[42].

NADPH oxidase-derived ROS production was recently shown to regulate recruitment of autophagy proteins in phagosomes of murine macrophages containing TLR or Fc-gamma receptor 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* is lethal in mice[44] and human patients with homozygous loss-of-function mutations in *Atg5* have not been described, it has been difficult to assess the direct *in vivo* role of autophagy in *Aspergillus* 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. Because the main immunosuppressive effect of corticosteroids in phagocyte effector function is mediated via inhibition of fusion of phagosomes with the lysosomes[2,3,12,13], a process regulated by autophagy proteins, we reasoned that corticosteroids may possess a direct inhibitory action on recruitment of autophagy proteins in fungal phagosomes. Surprisingly, we found that administration of a relatively low dose of corticosteroids blocked LC3 recruitment in *A. fumigatus* phagosomes within 2h of exposure. This effect was consistent in all patients tested and was highly reproducible following *ex vivo* administration of hydrocortisone in monocytes of healthy individuals.

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. Since corticosteroids have been shown to block tyrosine kinase phosphorylation within minutes of exposure in T cells[39,40] and another study on B-cell chronic lymphocytic leukemia reported inhibition of p-syk kinase by methylprednisolone via activation of phosphatase PTP1B[45], we focused on their effects in phosphorylation of syk kinase in monocytes. Notably, we found that hydrocortisone almost completely inhibited phosphorylation of syk kinase within 10 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 IP_3-mediated calcium signaling[46]. Thus, our studies reveal a selective property of corticosteroids to inhibit LC3 recruitment in fungal phagosomes, which is regarded as a specialized form of autophagy.

Collectively, we discovered a new antifungal immunity pathway that links dectin-1/syk kinase signaling with intracellular elimination of *A. fumigatus*. This pathway has major

physiologic relevance for the control of fungal infection by human phagocytes as evidenced by impaired autophagy protein recruitment in *A. fumigatus*-containing phagosomes in two distinct groups of patients with increased susceptibility for invasive aspergillosis. Therefore, our studies unravel a new pathway with important physiologic relevance in fungal disease and provide a mechanistic explanation for the defective phagocyte function of individuals with susceptibility for invasive aspergillosis. Moreover, future studies are warranted to explore the therapeutic potential of autophagy induction in these patients.

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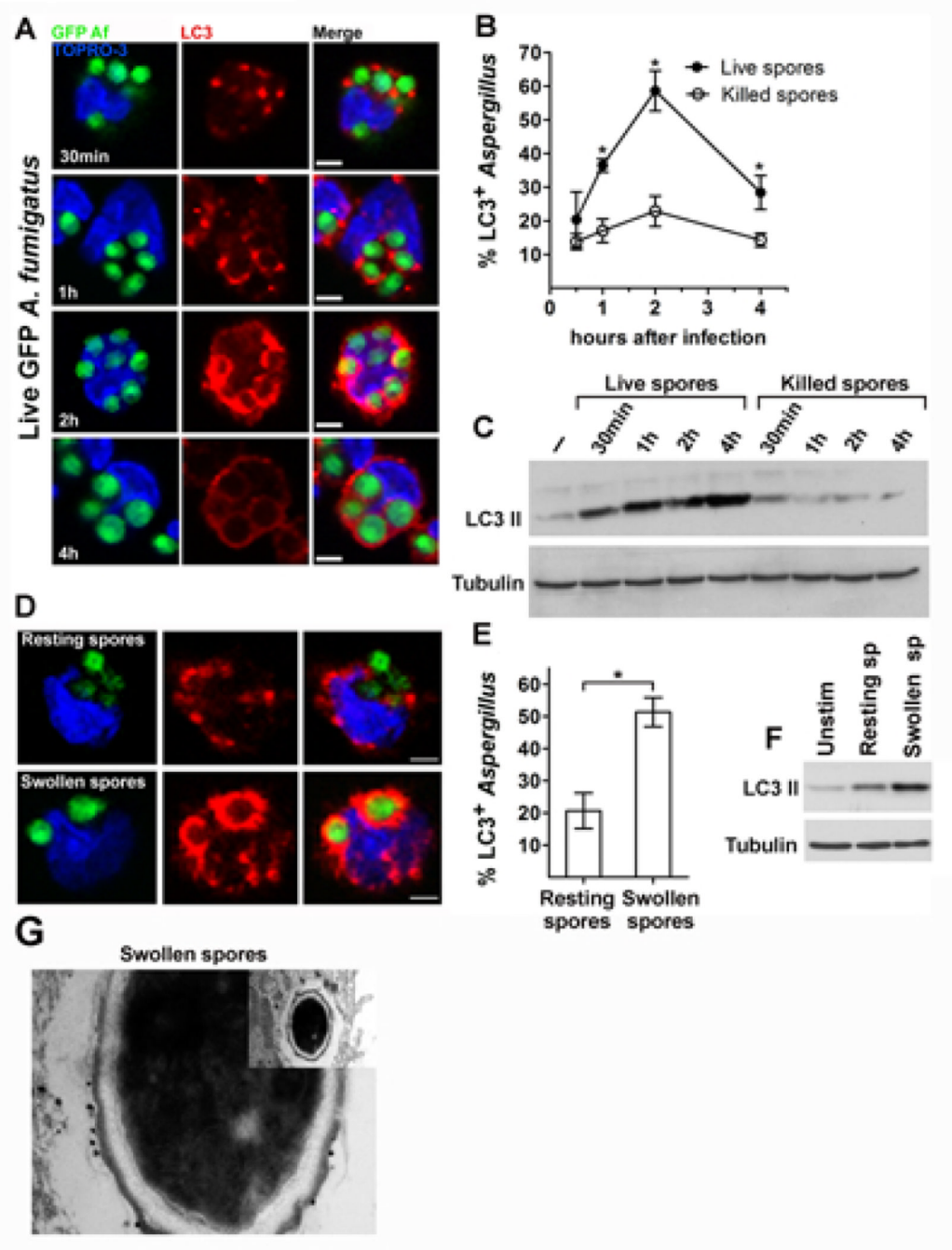

Figure 1. LC3 II is selectively recruited to phagosomes of primary human monocytes during cell wall swelling of*A. fumigatus*

A-B. Primary human monocytes (2×10^5 cells/condition) isolated from healthy individuals were infected with live GFP *A. fumigatus* (GFP Af; A, B) or PFA-killed GFP *A. fumigatus* (B) at a MOI 5: 1 for the indicated times. Cells were fixed, permeabilized, stained for LC3 II 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 LC3-associated *A. fumigatus*-containing phagosomes (LC3$^+$*Aspergillus*; n > 150 per group) at all time points were quantified by measuring the number of LC3$^+$*Aspergillus*-containing phagosomes out of the total number of engulfed *Aspergillus* spores and data are presented as

mean + S.E.M. of 3 independent experiments. *, $P < 0.0001$, paired Student's *t* test. Bar, 5 μm. C. Primary human monocytes (2×10^6 cells/condition) were infected with live GFP *A. fumigatus* or PFA-killed GFP *A. fumigatus* as in A-B for the indicated times. Cell lysates were prepared and levels of LC3 II protein were determined by immunoblotting. Levels of tubulin in the same lysates were determined by immunoblotting as loading controls. D-E. Primary human monocytes were stimulated for 1h with PFA-killed dormant or PFA-killed swollen spores of GFP *A. fumigatus*, fixed and stained as in A. 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 5 independent experiments. *, $P < 0.0001$, paired Student's *t* test. Bar, 5 μm. F. Primary human monocytes (2×10^6 cells/condition) were left untreated (unstim) or stimulated with either PFA-killed dormant or PFA-killed swollen spores of *A. fumigatus*. Cell lysates were prepared and LC3 II and tubulin protein levels were determined by immunoblotting. G. Representative immunoelectron micrograph in which LC3 II was labeled in primary human monocytes stimulated for 1h with PFA-killed *A. fumigatus* swollen spores with 1.4-nm gold particles.

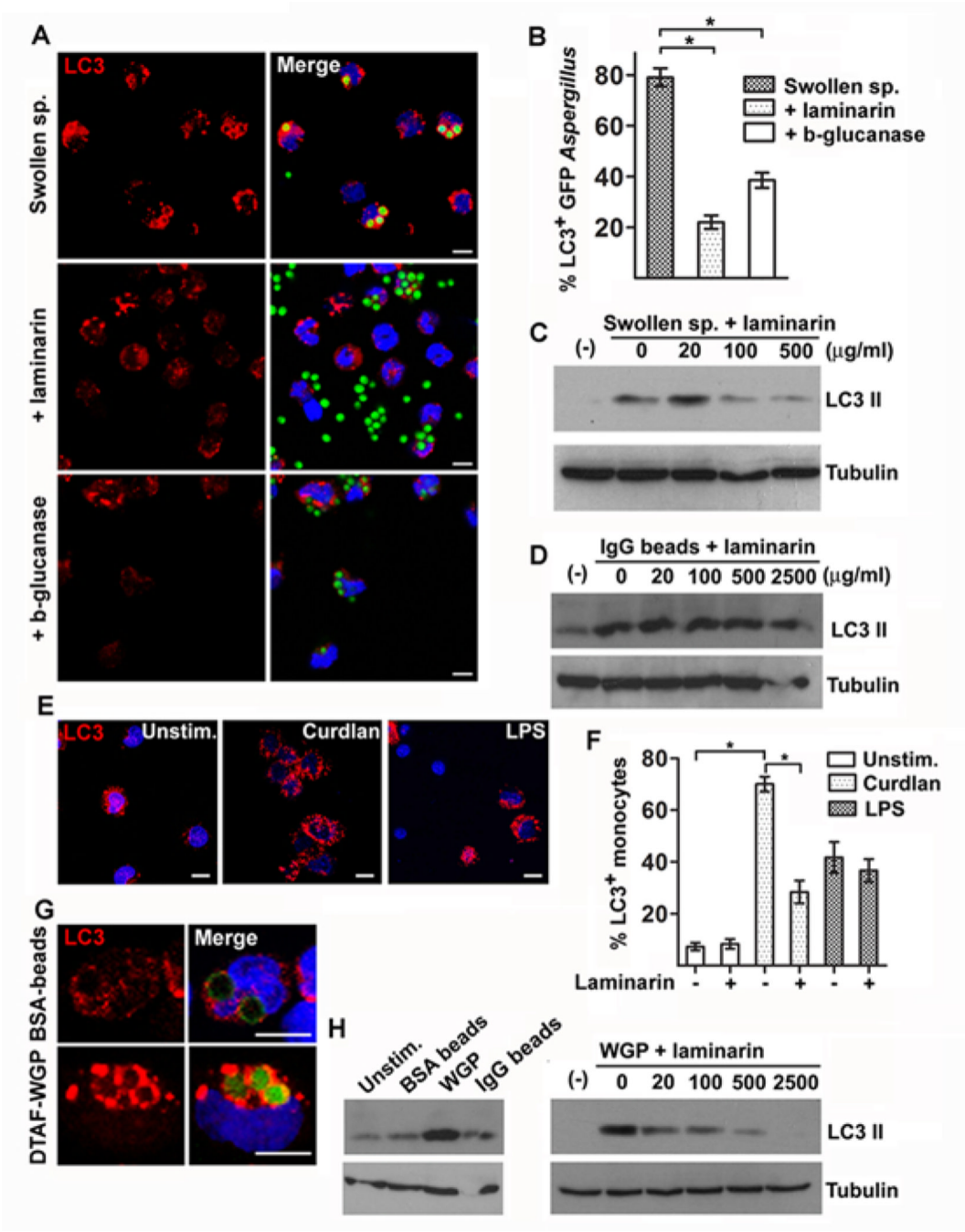

Figure 2. β-glucan surface exposure in swollen spores of *A. fumigatus* triggers LC3 II recruitment in fungal phagosomes

A. Primary human monocytes (2×10^5 cells/condition) isolated from healthy individuals were infected with GFP *A fumigatus* swollen spores with or without laminarin (500 μg/ml) or swollen spores following overnight enzymatic digestion of β-glucan (β-glucanase) at a MOI 5: 1 for 1h. Cells were fixed, permeabilized, stained for LC3 II with the use of an Alexa^{555} secondary antibody (red) and TOPRO-3 (blue, nuclear staining) and analyzed by immunofluorescence confocal microscopy. Bar, 5 μm. B 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 3 independent experiments. *, $P < 0.0001$, paired

Student's *t* test. C 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. Cell lysates were prepared and levels of LC3 II protein were determined by immunoblotting. Levels of tubulin in the same lysates were determined by immunoblotting as loading controls. E-F. Primary human monocytes (2×10^5 cells/condition) were left untreated or stimulated with purified β-gucan (curdlan, 100 μg/ml) or LPS (100 ng/ml) with or without pretreatment with laminarin (500 μg/ml). The percentages of human monocytes containing autophagosomes as indicated by punctuate LC3 staining ($LC3^+$ monocytes; n > 150 per group) were quantified and data are presented as mean + S.E.M. of 2 independent experiments. *, $P < 0.0001$, paired Student's *t* tes. Bar, 5 μm (G). Primary human monocytes (2×10^5 cells/condition) were stimulated with FITC-labeled BSA beads or DTFA-labeled WGP at a MOI 5: 1 for 1h. Cells were processed as in A and analyzed by immunofluorescence confocal microscopy. Bar, 5 μm. (H). Primary human monocytes (1×10^6 cells/condition) were left untreated or stimulated with BSA coated-beads, IgG coated-beads or WGP with or without pretreatment with increasing concentration of laminarin at a MOI of 10:1 for 1h. Cell lysates were prepared and levels of LC3 II and tubulin were determined by immunoblotting.

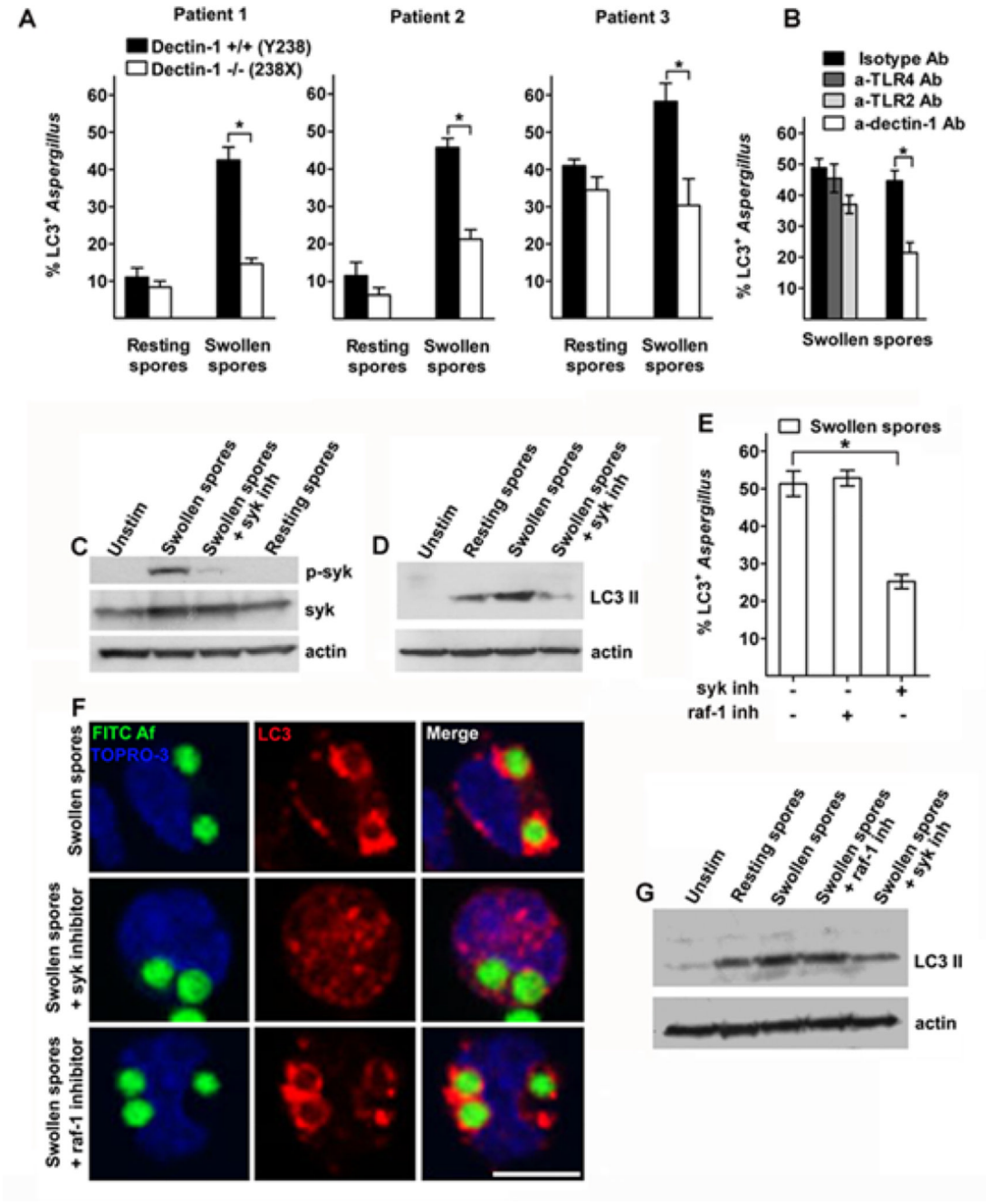

Figure 3. Dectin-1/syk kinase signalling regulates LC3 II recruitment in *A. fumigatus* phagosomes

A. Primary human monocytes (2×10^5 cells/condition) isolated from homozygous patients with the stop codon mutation *238X* (Dectin-1 $^{-/-}$) and healthy controls (Dectin-1 $^{+/+}$) were infected with FITC-labeled resting or swollen spores of *A. fumigatus* at a MOI 5: 1 for 1h at 37 °C. Cells were fixed, permeabilized, and stained for LC3 II as in Figure 1A. The percentages of LC3$^+$*A. fumigatus*-containing phagosomes (LC3$^+$*Aspergillus*; n > 100 per group) were quantified and data are presented as mean + S.D. for each patient. *, $P <$ 0.0001, paired Student's *t* test. B. Primary human monocytes from healthy individuals were stimulated with FITC-labeled swollen spores of *A. fumigatus* following 30 min pre-

incubation with blocking antibodies for Dectin-1 (10 μg/ml), TLR-2 (10 μg/ml), or TLR-4 (10 μg/ml) or the indicated isotype control antibodies (10 μg/ml) at a MOI 5: 1 for 1h at 37 °C. Cells were processed for immunofluorescence microscopy as in Fig 1A. C. Primary human monocytes (2×10^6 cells/condition) from healthy individuals were either left untreated, or stimulated with resting spores of *A. fumigatus*, or swollen spores of *A. fumigatus* with or without 30 min pretreatment with syk inhibitor (1μM) at a MOI 10:1 for 10 min at 37 °C. Cell lysates were prepared and levels of phospho-syk activity were determined by immunoblotting. Levels of tubulin and total syk in the same lysates were determined by immunoblotting as loading controls. D. Primary human monocytes (2×10^6 cells/condition) were stimulated as in C for 1h at 37 °C and levels of LC3 II and tubulin were determined in cellular lysates by immunoblotting. E-F. Primary human monocytes (2×10^5 cells/condition) were stimulated with FITC-labeled swollen spores of *A. fumigatus* with or without 30 min pretreatment with syk inhibitor (1μM) or raf-1 inhibitor (40μM) at a MOI of 5:1 for 1h and processed for immunostaining as in A. 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 four independent experiments. *, $P < 0.0001$, paired Student's *t* test. Bar, 5 μm. G. Primary human monocytes (2×10^6 cells/condition) stimulated as in E and levels of LC3 II and tubulin were determined in cellular lysates by immunoblotting.

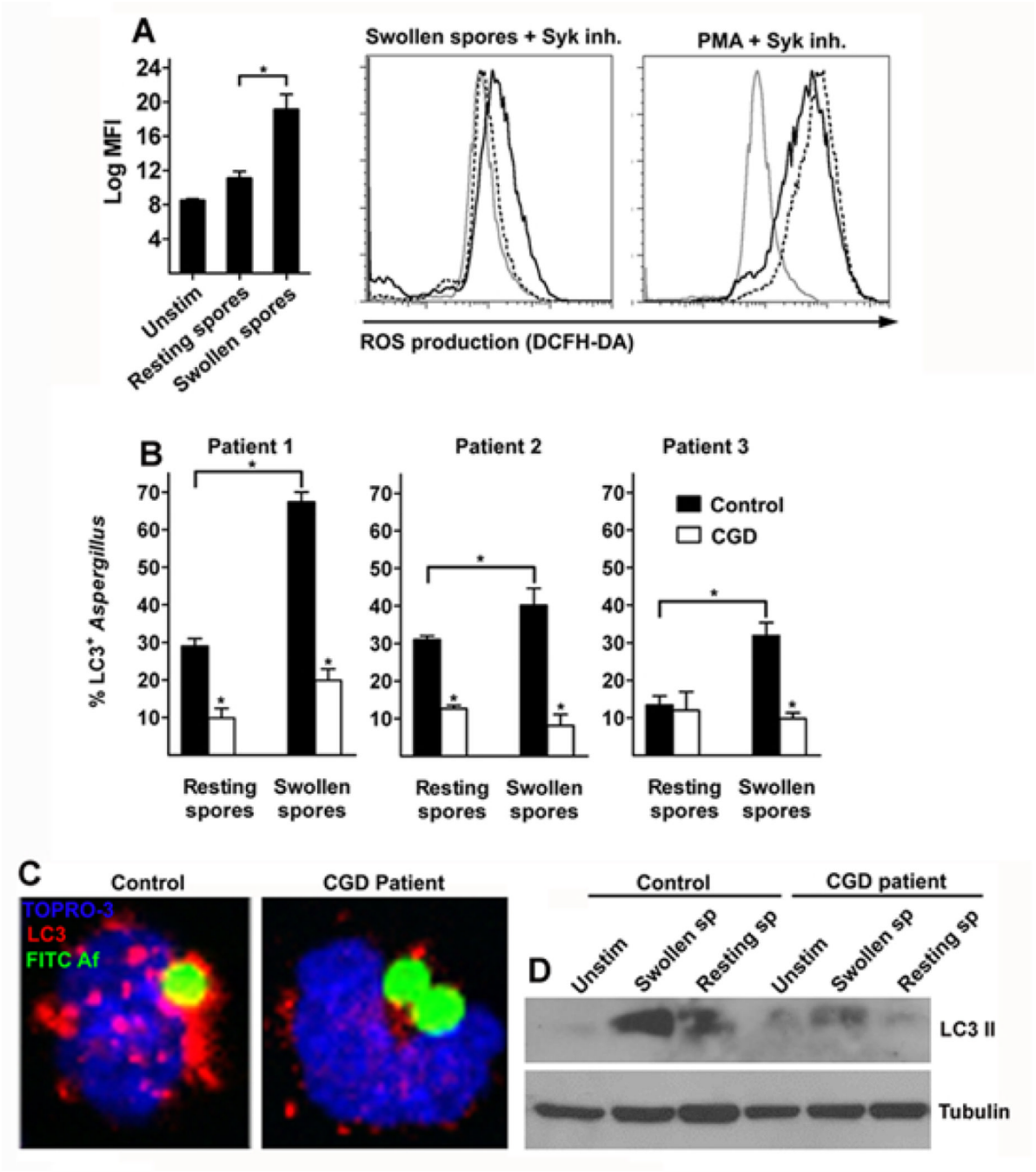

Figure 4. Syk kinase dependent ROS production regulates formation of LC3$^+$ *Aspergillus* containing phagosomes

A. Primary human monocytes (2×10^5 cells/condition) were left unstimulated or infected with resting spores or swollen spores of *A. fumigatus* at a MOI of 5:1 with or without 30 min pretreatment with syk inhibitor (1 μM) or stimulated with PMA (100 ng/ml) with or without 30 min pretreatment with syk inhibitor (1 μM) for 1h at 37 °C. DCFH-DA was added during the last 30 min of stimulation and intracellular ROS production was determined by measurement of relative fluorescent intensity at the FL1 channel (log MFI). Differences in ROS production between experimental groups were quantified and data are presented as mean + S.E.M. from 4 independent experiments. *, $P < 0.005$, paired Student's *t* test.

Representative FL1 histograms from human monocytes left untreated (gray solid line), stimulated with either swollen spores of *A. fumigatus* alone or PMA alone (black solid line) or in the presence of syk inhibitor (black dashed lines) are shown. B-C. Primary human monocytes (2×10^5 cells/condition) isolated from CGD patients and healthy controls were infected with FITC-labeled resting or swollen spores of *A. fumigatus* at a MOI 5: 1 for 1h at 37 °C. Cells were fixed, permeabilized, and stained for LC3 II as in Figure 1A. The percentages of $LC3^+$*A. fumigatus*-containing phagosomes ($LC3^+$*Aspergillus*; n > 100 per group) were quantified and data are presented as mean + S.D. for each patient. *, $P < 0.0001$, paired Student's *t* test. Representative immunofluorescence image of $LC3^+$ phagosomes containing FITC-labeled swollen spores of *A. fumigatus* in monocytes obtained from healthy control and CGD patient. D. Primary human monocytes (1×10^6 cells/condition) from a representative CGD patient and the corresponding healthy control were left untreated (unsitm) or stimulated with resting spores or swollen spores of A. fumigatus at a MOI 10:1 for 1h at 37 °C and levels of LC3 II and tubulin were determined in cellular lysates by immunoblotting.

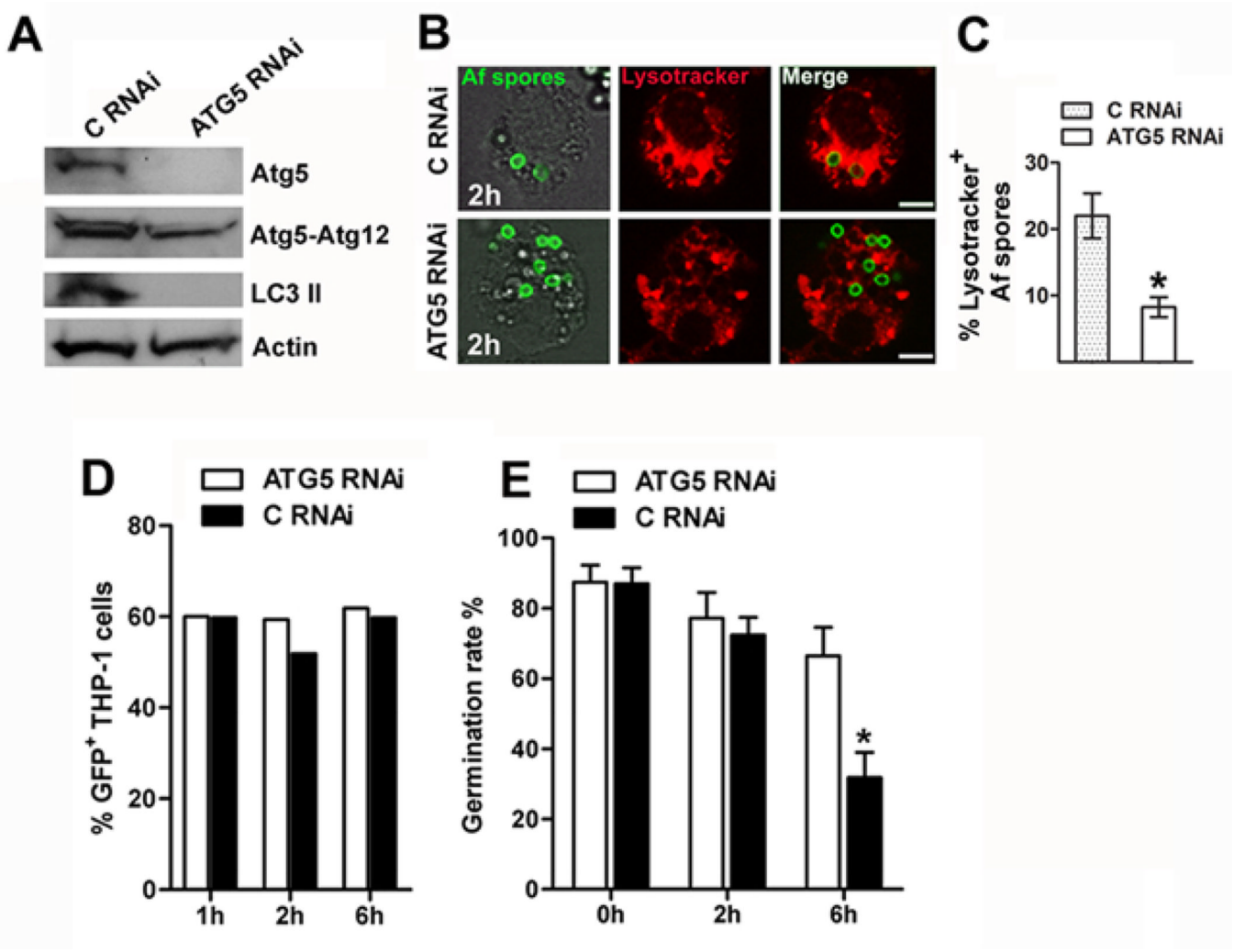

Figure 5. Conditional inactivation of *Atg5* in THP-1 human macrophages results in attenuated phagolysosomal fusion and killing of *A. fumigatus*

A. THP-1 cells (1×10^6 cells/condition) were transfected with RNAi sequences targeting ATG5 vs. scramble control RNAi (C RNAi) by Amaxa electroporation. Cell lysates were prepared 48 h following transfection and levels of LC3 II, Atg5, and Atg5-Atg12 proteins were determined by immunoblotting. Levels of actin in the same lysates were determined by immunoblotting as loading controls. B-C. LysoTracker staining in THP-1 cells transfected with ATG5 RNAi or C RNAi and differentiated to macrophages with addition of PMA (25 ng/ml) following 2h of infection with FITC-labeled *A. fumigatus* spores. Data are presented as mean + S.E.M. of three independent experiments. *, $P < 0.0001$, paired Student's *t* test. Bar, 5 μm. D. Degree of association (uptake) of GFP *A. fumigatus* spores with THP-1 cells transfected with ATG5 RNAi or C RNAi and differentiated to macrophages in the presence of PMA (25 ng/ml) at different time points of infection (1h, 2h, 6h), assessed by FACS analysis. Results are representative of two independent experiments. E. THP-1 cells transfected by Amaxa nucelofection with ATG5 RNAi or C RNAi, were seeded in 12 well plates (5×10^5 cells/condition), differentiated with PMA (25 ng/ml) for 48 h and infected with *A. fumigatus* spores at a MOI of 1:10 at 37 °C. Medium containing nonadherent, nonphagocytosed conidia was removed at 1 h, and wells were washed three times using warm PBS. Macrophages were then allowed to kill conidia for 2 h and 6 h before

intracellular conidia were harvested. The percentage of germinating spores in the culture well after 6 to 8 h of incubation at 37°C was assessed under a microscope. The percentage of germination rate (number of germinated spores per 100 counted conidia) of *A. fumigatus* spores following different time points of infection (1 h, 2 h, 6 h) was calculated and data are expressed as mean + S.E.M. of three independent experiments; * $P = 0.0003$, paired Student's *t* test.

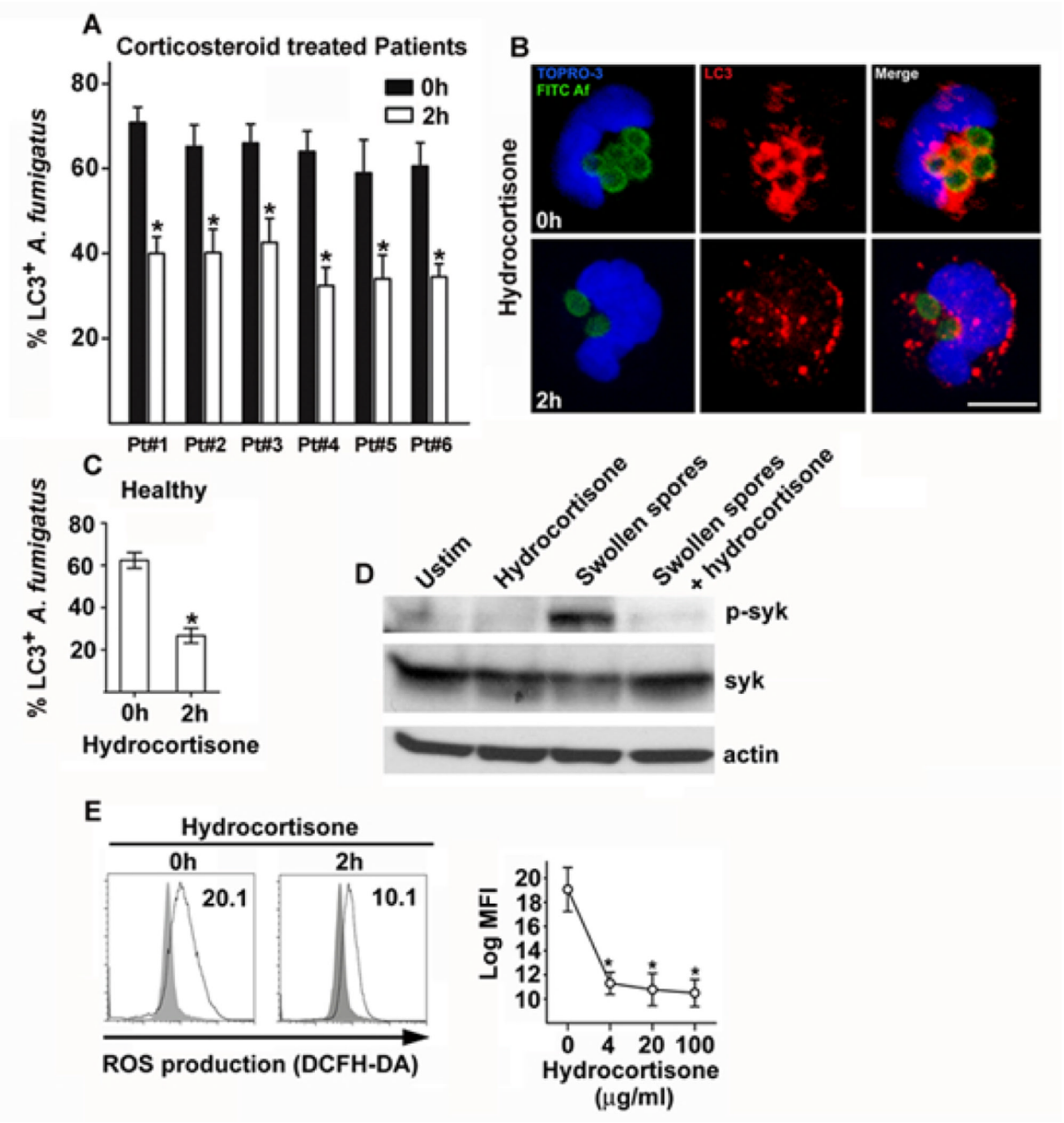

Figure 6. Corticosteroids block LC3 II recruitment in *A. fumigatus* phagosomes via inhibiting phosphorylation of syk kinase and downstream ROS production

A. Primary human monocytes (2×10^5 cells/condition) from six consecutive patients with rheumatologic diseases were collected before and 2h after intravenous administration of corticosteroids (250 mg hydrocortisone) and stimulated with swollen spores of *A. fumigatus* at a MOI 5:1 at 37 °C. Cells were fixed, permeabilized, stained for LC3 II 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 LC3$^+$*A. fumigatus*-containing phagosomes (LC3$^+$*Aspergillus*; n > 150 per group), before (0h) and after (2h) corticosteroid treatment, were quantified and data are presented as mean + S.D. for each patient. *, *P* <

0.05, paired Student's *t* test. B. Representative immunofluorescence image of $LC3^+$ phagosomes containing FITC-labeled swollen spores of *A. fumigatus* in monocytes obtained before (0h) and after (2h) administration of corticosteroids. Bar, 5 μm. C. Primary human monocytes (2×10^5 cells/condition) from healthy individuals (n = 4) were stimulated before (0h) and after (2h) *ex vivo* exposure to hydrocortisone (20 μg/ml), fixed and processed as in A; data are presented as mean + S.E.M. of four independent experiments. $P < 0.05$, paired Student's *t* test. D. Primary human monocytes (2×10^6 cells/condition) from healthy individuals were either left untreated with or without 1h exposure to hydrocortisone (20 μg/ml), or stimulated with swollen spores of *A. fumigatus* with or without 1h pre-exposure to hydrocortisone ((20 μg/ml) at a MOI 10:1 for 10 min at 37 °C. Cell lysates were prepared and levels of phospho-syk activity were determined by immunoblotting. Levels of tubulin and total syk in the same lysates were determined by immunoblotting as loading controls. E. Primary human monocytes (2×10^5 cells/condition) were left unstimulated or infected with swollen spores of *A. fumigatus* at a MOI of 5:1 for 1 h with or without pre-exposure (2 h) to increasing concentrations of hydrocortisone at 37 °C. DCFH-DA was added during the last 30 min of stimulation and intracellular ROS production was determined by measurement of relative fluorescent intensity at the FL1 channel (log MFI). Representative FL1 histograms from human monocytes left untreated (gray area), or stimulated with swollen spores of *A. fumigatus* (black solid line) with or without pre-exposure to hydrocortisone (20 μg/ml) are shown. Differences in ROS production between experimental groups were quantified and data are presented as mean + S.E.M. from 4 independent experiments. *, $P < 0.0001$, paired Student's *t* test.

Table 1

Clinical and demographic characteristics of patients with rheumatologic diseases who received iv corticosteroids

Patient Initials (Pt#)	Sex	Age	Underlying Disease	Disease Status	Co-morbidities	Other Immunosuppressive agents
D.T. (Pt#1)	F	62	Rheumatoid arthritis	Active disease	Multiple sclerosis	Methotrexate, rituximab (anti- CD20)
O.D (Pt#2)	F	68	Rheumatoid arthritis	Active disease, extra articular manifestations (rheumatoid lung)	Hepatitis B	Leflunomide, rituximab (anti- CD20), receipt of anti-TNFa monoclonal antibody in the past 12 months
S.A. (Pt#3)	F	63	Systemic lupus erythematoso us	In remission	Cirrhosis (autoimmune hepatitis)	Azathioprine, hydroxychloroquine, rituximab (anti- CD20)
L.S. (Pt#4)	F	46	Polymyositis	In remission	Parkinson disease, pulmonary embolism	Methotrexate, low dose prednisone (5mg daily) in the past 6 months
K.M. (Pt#5)	F	56	Rheumatoid arthritis	Active disease, extra articular manifestations (pericarditis)	N/A	Methotrexate, rituximab (anti- CD20), receipt of anti-IL6 monoclonal antibody in the past 6 months
A.M. (Pt#6)	F	61	Rheumatoid arthritis	In remission	Chronic obstructive pulmonary disease	Methotrexate, low dose prednisone (2.5 mg daily) in the past 6 months

All patients received corticosteroids (methyl prednisone or hydrocortisone) at a standard dose of 250mg of hydrocortisone equivalent as premedication for prevention of infusional reactions associated with the use of rituximab (anti-CD20 monoclonal antibody). Blood was drawn before (0h) and after (2h) iv treatment with corticosteroids.