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Candida albicans β-Glucan Exposure Is Controlled by the Fungal *CEK1*-Mediated Mitogen-Activated Protein Kinase Pathway That Modulates Immune Responses Triggered through Dectin-1▽†

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Innate immunity to *Candida albicans* depends upon the recognition of molecular patterns on the fungal cell wall. However, the masking of major components such as β-glucan seems to be a mechanism that fungi have evolved to avoid immune cell recognition through the dectin-1 receptor. Although the role of *C. albicans* mitogen-activated protein kinase (MAPK) pathways as virulence determinants has been established previously with animal models, the mechanism involved in this behavior is largely unknown. In this study we demonstrate that a disruption of the *C. albicans* extracellular signal-regulated kinase (ERK)-like 1 (*CEK1*)-mediated MAPK pathway causes enhanced cell wall β-glucan exposure, triggering immune responses more efficiently than the wild type, as measured by dectin-1-mediated specific binding and human dendritic cell (hDC)- and macrophage-mediated phagocytosis, killing, and activation of intracellular signaling pathways. At the molecular level, the disruption of *CEK1* resulted in altered spleen tyrosine kinase (Syk), Raf-1, and ERK1/2 activations together with IκB degradation on hDCs and increased dectin-1-dependent activator protein 1 (AP-1) activation on transfected cells. In addition, concurring with these altered pathways, we detected increased reactive oxygen species production and cytokine secretion. In conclusion, the *CEK1*-mediated MAPK pathway is involved in β-glucan exposure in a fungal pathogen, hence influencing dectin-1-dependent immune cell recognition, thus establishing this fungal intracellular signaling route as a promising novel therapeutic target.

Candida albicans is an opportunistic fungal pathogen that lives as a commensal on mucosal surfaces. In immunocompromised individuals this microorganism can behave as a pathogen, causing localized or disseminated candidiasis (41, 48). *C. albicans* is a polymorphic fungus able to change from the unicellular (yeast) to the mycelial (filamentous) form of growth, a process called dimorphic transition (3). Adaptation to a changing environment and coping with host defenses are essential for pathogen survival, and fungal mitogen-activated protein kinase (MAPK)-mediated signal transduction pathways are important for performing this function (45). A disruption of the *CEK1*-mediated pathway by the deletion of the *CEK1* (*Candida albicans* extracellular signal-regulated kinase [ERK]-like 1) MAPK or the upstream *HST7* MAPK kinase (MAPKK) genes causes defects in invasive growth (32), and *cek1* mutants are less virulent in some animal models of candidiasis (10, 26). This route has also been related to cell wall biogenesis, as *cek1* mutants displayed hypersensitivity to agents disturbing the cell wall (15, 47). However, the molecular mechanisms mediating these effects are not clarified.

The *C. albicans* cell wall is a complex dynamic structure based on a core structure of β-(1,3)-glucan covalently linked to β-(1,6)-glucan and chitin and an outer layer or matrix composed mainly of mannose-glycosylated proteins (42). The fungal cell wall surface represents the interface between the host and the infective pathogen. It is a valuable therapeutic target, as its highly conserved pathogen-associated molecular patterns (PAMPs) are recognized by different pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) (34) and C-type lectins (4, 23). Recognition by these PRRs mediates microbial uptake and killing as well as antigen presentation and the production of proinflammatory cytokines (48).

Dectin-1 is a C-type lectin receptor (28), expressed predominantly by myeloid cells, that specifically binds β-(1,3)-glucan (5, 7), a potent proinflammatory molecule that is normally hidden by the mannoprotein coat presumably to allow fungal escape from immune cell recognition (20, 58). As the main nonopsonic receptor involved in fungal uptake (27), dectin-1 ligation initiates intracellular signaling through the Syk- and CARD9-dependent pathways (25, 57), triggering different protective responses (22, 33, 36, 44). A recent study demonstrated that dectin-1 also signals through Raf-1 and that Syk- and Raf-1-dependent pathways converge at the level of NF-κB activation to control adaptive immunity to fungi (23). However, the generation of protective cytokine responses appears to require the simultaneous activation of TLR2 (6, 19). Although the role of dectin-1 in antifungal immunity *in vivo* is still controversial (50, 54), there are strong evidences supporting its involvement in the control of *C. albicans* infection (43). Most of the *in vitro* studies of dectin-1 engagement have used iso-

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lated fungal components, particulate cell wall extracts (zymosan), or heat-killed cells as stimuli, which could not reflect the true complexity of the response to intact live fungi. Moreover, little is known about the relevance of β-glucan recognition by dectin-1 for the activation of the host defense in primary human cells, especially using whole live yeast cells.

In this study, we show for the first time that a disruption of the *CEK1*-mediated MAPK pathway leads to altered β-glucan exposure, triggering enhanced dectin-1-mediated immune responses and further supporting a relevant role for this pathway in *C. albicans* immune evasion mechanism.

MATERIALS AND METHODS

***Candida albicans* strains and growth conditions.** Unless otherwise stated, *cek1*, *hst7*, and *cek2* indicate homozygous Ura^+ strains CK43B-16 (10), CDH9 (32), and BEC73 (15, 38), respectively, while CAF2 (16) and RM100 (1) were used as wild-type (wt) strains. Yeast cells were grown in YPD rich medium (2% glucose, 2% peptone, 1% yeast extract, 2% agar [if required]) at 30°C, and stationary-phase cells were used. Fluorescein isothiocyanate (FITC)-labeled yeast cells were obtained as described previously (52). For UV inactivation, 2×10^8 cells in phosphate-buffered saline (PBS) were exposed to single-dose UV radiation (1.2×10^5 $\mu J/cm^2$) in a UV-DNA cross-linker. For heat inactivation, 2.5×10^7 cells were boiled in PBS (20 min at 98°C).

Cell lines. Human embryonic kidney 293 T (HEK293T) and mouse macrophage (MΦ) RAW 264.7 cell lines (American Type Culture Collection [ATCC]) were cultured in complete Dulbecco's modified Eagle's medium (DMEM) (10% heat-inactivated fetal calf serum [FCS], 1% penicillin-streptomycin, 4 mM glutamine; Gibco). The K562 cell line (ATCC) was cultured in complete RPMI medium (Gibco). K562 cells stably expressing dectin-1 (K562–dectin-1 cells) or DC-SIGN (see below) were maintained with 0.7 mg/ml neomycin.

Transmission electron microscopy (TEM). Stationary-phase yeast cells were grown and harvested by centrifugation and washed in PBS, and pellets were prefixed in a glutaraldehyde fixative (3% glutaraldehyde in 0.1 M phosphate buffer [pH 7.4]) for 1 h. Next, a mix with an agar solution (1.5%) was carried out, followed by a new glutaraldehyde fixation step for 30 min. After extensive washing with the phosphate buffer, postfixation was carried out with 1% osmium tetroxide for 1 h. Pellets were embedded in Durcupan resin. Ultrathin sections were stained with 2% uranyl acetate. Samples were imaged with a Zeiss EM 900 transmission microscope, and the images were recorded with a Show Scan charge-coupled-device (CCD) camera (TRS).

Generation of stable dectin-1 transfectants in K562 cells. The dectin-1–Flag cDNA was obtained by reverse transcription (RT)-PCR amplification from peripheral blood mononuclear cell (PBMC) total RNA with specific primers for the full coding region of human dectin-1 plus the Flag epitope tag (underlined in the primer sequence) on the COOH terminus: sense primer 5′-CAG GGG CTC TCA AGA ACA ATG G-3′ and antisense primer 5′-TTA CTT GTC ATC GTC GTC CTT GTA GTC CAT TGA AAA CTT CTT CTC ACA AAT ACT ATA TGA GGG-3′. The resulting cDNA was cloned into the pcDNA3.1/V5-His TOPO TA expression kit (Invitrogen). Plasmid pcDNA3.1/V5-His-Dectin-1-Flag was transfected into K562 cells with Cell Line Nucleofector Kit V (Amaxa Biosystems), and cells were selected by using neomycin (0.8 mg/ml). K562–dectin-1–Flag cells were isolated by using a FACSAria cell sorter (BD-Biosciences) after staining with anti-Flag monoclonal antibody (MAb) (Sigma-Aldrich).

Generation of hMΦs and hDCs. Human PBMCs were isolated from healthy donor buffy coats and purified as previously described (11, 51). Briefly, monocytes were purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech) and cultured at 0.7×10^6 to 1×10^6 cells/ml for 7 days in RPMI complete medium supplemented with 1,000 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1,000 U/ml interleukin-4 (IL-4) (ImmunoTools) for human monocyte-derived dendritic cells (hDCs) or 1,000 U/ml GM-CSF for human monocyte-derived MΦs (hMΦs). The medium was replaced, and new cytokines were added every 2 days.

Soluble dectin-1 production. The cDNA for the complete extracellular region of human dectin-1 (amino acids [aa] 71 to 247, corresponding to stalk and carbohydrate recognition domains of the receptor) was cloned upstream of the human IgG1-Fc (Fc) genomic DNA in the mammalian expression vector pEF (30), bearing an amino-terminal Ig(κ) chain signal peptide. The protein was transiently expressed in HEK293T cells transfected by the calcium phosphate method. Culture cell supernatants containing Fc-tagged soluble proteins (sDectinFc) were harvested, and protein secretion was quantified by enzyme-linked immunosorbent assay (ELISA) with anti-Fc region antibodies (Abs) (Dako). Soluble protein was purified by protein A chromatography and high-performance liquid chromatography (HPLC).

Generation of anti-human dectin-1 monoclonal antibody MGD3. MAb MGD3 was generated by the immunization of BALB/c mice with a fusion protein containing the coding region for the complete extracellular domain of human dectin-1 cloned upstream of the Flag epitope (as for sDectinFc). Splenic B cells from immunized mice were then fused with murine plasmacytoma P3X63-Ag8.653 cells (ATCC) according to standard protocols (31). The MAbs were initially selected by using supernatants screened for anti-dectin-1 activity by ELISA based on the fusion protein used for immunization. Subsequently, selection was made by flow cytometry for the ability to recognize dectin-1 specifically on stably transfected K562 cells and primary hDCs (see Fig. S5 in the supplemental material for MAb specificity characterization). MAb MGD3 was purified by affinity chromatography with protein A-Sepharose (GE Healthcare) and was analyzed by surface plasmon resonance (SPR) using the biosensor Biacore 3000 (GE Healthcare).

***Candida albicans* binding assay.** Binding assays were performed as previously described (52). Briefly, cells were washed and blocked with staining PBS (PBS with 2% bovine serum albumin [BSA], 1% fetal calf serum [FCS], and 50 μg/ml of poly-human Ig) for 15 min at room temperature. When indicated, cells were pretreated with laminarin (500 μg/ml; Sigma-Aldrich) or MAb MGD3 (5 μg/ml) before the addition of fluorescein isothiocyanate (FITC)-labeled *C. albicans* (10 yeast cells:1 cell).

Phagocytic assays. The phagocytosis of yeast was performed by incubating hMΦs or hDCs in medium alone or with the indicated blocking Abs (5 μg/ml) for 30 min at 37°C. Next, cells were washed, and media were replaced with fresh RPMI medium containing *C. albicans*-FITC-conjugated yeast cells (5 yeast cells:1 phagocyte), followed by 15 min to up to 30 min of incubation at 37°C. Cells were then immediately transferred onto ice and washed vigorously with cold PBS. Cell staining was performed as previously described (37). Briefly, yeast cells were stained with polyclonal anti-*C. albicans* Ab (kindly provided by C. d'Enfert), followed by Alexa-647-labeled secondary Ab to differentiate noninternalized yeast. After fixation and permeabilization, primary cells were stained with phalloidin–Alexa-568, conjugated, mounted, and analyzed with a Leica TCS-SP confocal microscope (Leica Microsystems). The phagocytic index was assessed by counting the number of internalized yeast cells per 100 phagocytes.

Fungicidal assays with hDCs and hMΦs. Killing assays were performed as previously described (2). Briefly, yeast cells and hDCs or hMΦs were coincubated (1 yeast cell:20 phagocytes) for 4 h. After phagocyte lysis with water, serial dilutions were spread over YPD agar plates for determinations of CFU after 24 h of incubation (37°C). The killing percentage for each strain was expressed as the percent reduction of CFU from hDC- or hMΦ-yeast cocultures versus simultaneous culture containing yeast cells without phagocyte cells.

Western blot analysis. After overnight culture in serum-reduced medium, cells were treated with lysis buffer (40 mM Tris [pH 7.6], 150 mM NaCl, 1% NP-40, 1× EDTA-free protease inhibitor cocktail, and 1× PhosSTOP phosphatase inhibitor cocktail [Roche]), and cell lysates (40 μg) were resolved by SDS-PAGE under reducing conditions. ERK1/2, Syk, and Raf-1 activation was detected by using antibodies specific for anti-phospho-p44/42-MAPK/anti-p44/42-MAPK (Cell Signaling), for anti-phospho-Syk (Calbiochem)/anti-Syk MAb (Upstate), and for anti-phospho-Raf-1 (Tyr340/341)/anti-Raf-1 Abs (Upstate, Millipore). The levels of IκB-α were detected by using a polyclonal anti-IκB-α Ab (C-15; Santa Cruz).

Luciferase reporter assay. HEK293T cells were transfected with a mixture of the AP-1–Luc reporter plasmid (250 ng/10^5 cells) and pRL-Null (5 ng/10^5 cells) (Promega) bearing a promoterless *Renilla* luciferase gene used to normalize all the firefly luciferase values obtained. The reporter plasmids were transfected together with the receptor expression vector (pCDNA3.1 Dectin-1) or empty vector (pCDNA3.1; Invitrogen) with Fugene HD reagent (Roche) according to the manufacturer's instructions. Forty-eight hours later, cells were cocultured (9 to 12 h) with PBS, UV-inactivated yeast cells (50 yeast cells:1 cell), or zymosan (200 μg/ml). Luciferase activity was determined with the dual-luciferase reporter assay system (Promega) with a luminometer (Berthold Detection Systems) and is expressed as relative light units. The histograms show means ± standard deviations (SD) for five independent experiments. Each experiment was carried out in duplicate, and data are presented as values relative to data for untreated cells.

Measurement of ROS. A total of 10^6 hMΦs or hDCs cultured in phenol red-free complete RPMI medium (Gibco) were cocultured with different stimuli for 4 h. Intracellular reactive oxygen species (ROS) levels were measured by incubating cells with 5 μM 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCFDA; Molecular Probes) (30 min at 37°C). Cells were then washed with

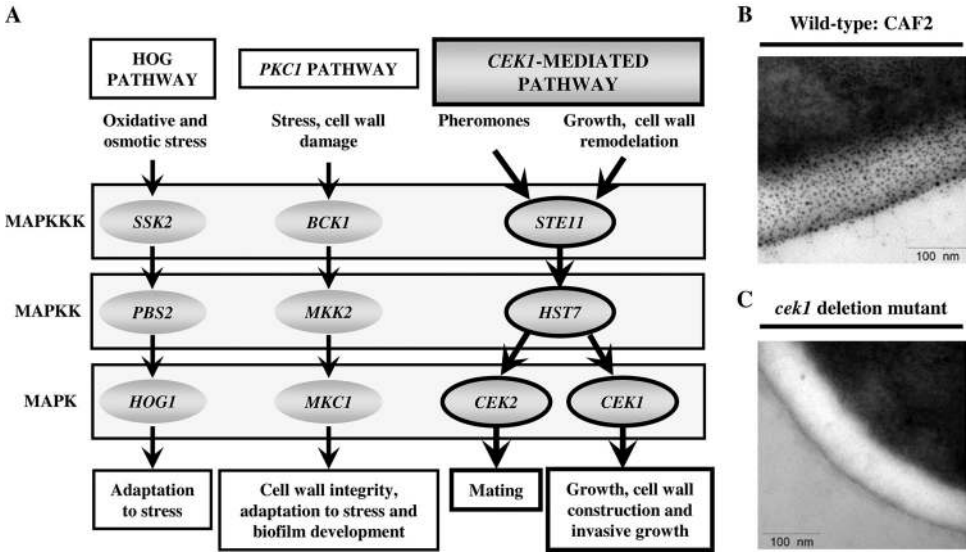

FIG. 1. *Candida albicans*-MAPK signal transduction pathways and cell wall morphology of *C. albicans* wild-type CAF2 and the *cek1* deletion mutant. (A) The main elements of MAPK signal transduction pathways in *C. albicans* are schematized, and the physiological function of each pathway is indicated. The *CEK1*-mediated MAPK pathway is highlighted. (B and C) Transmission electron micrographs of the cell wall of wt strain CAF2 (B) and the *cek1* deletion mutant (C), which lacks Cek1 MAPK. Scale bar, 100 nm.

cold PBS, and ROS production was examined by flow cytometry. Data are shown as the mean fluorescence intensities (MFIs) given by H_2DCFDA in the presence of the different *C. albicans* strains relative to the MFI in the presence of PBS.

Stimulation of cytokine production in hDCs. A total of 0.8×10^6 hDCs were cocultured with zymosan (200 μg/ml; Sigma-Aldrich) and UV-inactivated or heat-killed *C. albicans* strains (50 yeast cells:1 cell). For blocking, hDCs were preincubated (30 min at 37°C) with anti-human dectin-1 MAb (MGD3; 5 μg/ml) or a functional-grade purified IgG2a isotype control (eBiosciences) before coculture with UV-inactivated strains (10 yeast cells:1 cell). Controls included cells cultured in medium alone. Supernatants were analyzed for IL-10 and tumor necrosis factor alpha (TNF-α) by using commercial ELISA pair sets (R&D and Immunotools, respectively).

Immunofluorescence and flow cytometry. For immunofluorescence analysis, live yeast cells were allowed to adhere onto poly-L-lysine-coated coverslips (30 min at 37°C), and after washing, cells were fixed with 3.7% paraformaldehyde (10 min at room temperature). For *C. albicans* cell wall staining, live, heat-killed, or paraformaldehyde-fixed yeast cells (for immunofluorescence assays) were incubated with anti-β-(1,3)-glucan MAb (Biosupplies) or sDectinFc with an anti-Fc MAb (Jackson ImmunoResearch) followed by FITC (for cytometry) or Alexa-488 (for immunofluorescence) secondary labeled antibody (Dako and Molecular Probes, respectively). Dectin-1 cell expression was determined with MAb MGD3 followed by an FITC-labeled secondary Ab. Incubations were done at 4°C in staining PBS. Flow cytometry analyses were performed with a FACSCalibur flow cytometer using CellquestPro software (BD-Biosciences), and immunofluorescence preparations were analyzed by confocal microscopy (described above) or a conventional Leica DMR photomicroscope with QFISH software (Leica Microsystems).

Statistical analyses. The differences between groups were analyzed by the Mann-Whitney U test. The levels of significance between groups were set at a P value of <0.05, a P value of <0.001, and a P value of <0.0005. Unless otherwise stated, all experiments were performed at least five times, and the data are given as mean values ± SD.

RESULTS

Disruption of the *C. albicans CEK1*-mediated MAPK pathway alters cell wall morphology. In the fungal pathogen *C. albicans*, MAPK pathways are mechanisms by which different types of stress (oxidative, temperature, pH, and others) are sensed and an appropriate response is developed (Fig. 1A). The *cek1* mutant is defective in the Cek1 MAPK, which participates in cell wall construction (15) and becomes activated during growth-associated cell wall remodeling (47). In order to identify cell wall alterations on the *cek1* mutant compared to wild-type (wt) strain CAF2, transmission electron microscopy (TEM) analysis was initially performed. Although cell wall thicknesses were not significantly different between them (data not shown), TEM micrographs revealed a less-electron-dense cell wall on the *cek1* mutant compared to wt strain CAF2 (Fig. 1B and C). This differential cell wall density on the *cek1* mutant could account for its hypersensitivity to agents disturbing the cell wall (15) and might modify fungal detection by human immune cells.

The *C. albicans CEK1*-mediated MAPK pathway controls β-glucan exposure and dectin-1-mediated fungal recognition. Considering the *C. albicans* cell wall structure, we hypothesized that the less electron-dense cell wall of *cek1* mutants on TEM micrographs could reflect enhanced β-glucan exposure. Flow cytometry measurements revealed that β-glucan exposure is significantly higher in live *cek1* deletion mutants than in wt CAF2 yeast cells (Fig. 2A). Moreover, the recognition of the *cek1* mutant by soluble Fc-tagged dectin-1 recombinant protein (sDectinFc) was also considerably elevated (Fig. 2A), thus demonstrating that a *CEK1* mutation leads to enhanced β-glucan exposure and dectin-1 recognition. Assays with heat-killed (HK) fungi, which show increased β-glucan exposure at the cell surface (20, 58), were used as positive controls (Fig. 2A). Confocal microscopy analysis indicated that β-glucan was found over the entire surface of the mutant, whereas wt fungi showed a restricted pattern corresponding to the mother-daughter septal regions (20) (Fig. 2B). A similarly increased level of β-glucan staining was seen in the upstream MAPKK Hst7 mutant (*hst7*), further confirming the implication of the *CEK1*-mediated MAPK route for β-glucan exposure (see Fig. S1A and S1B in the supplemental material). A tridimensional analysis of the yeast cell wall revealed an even staining over the

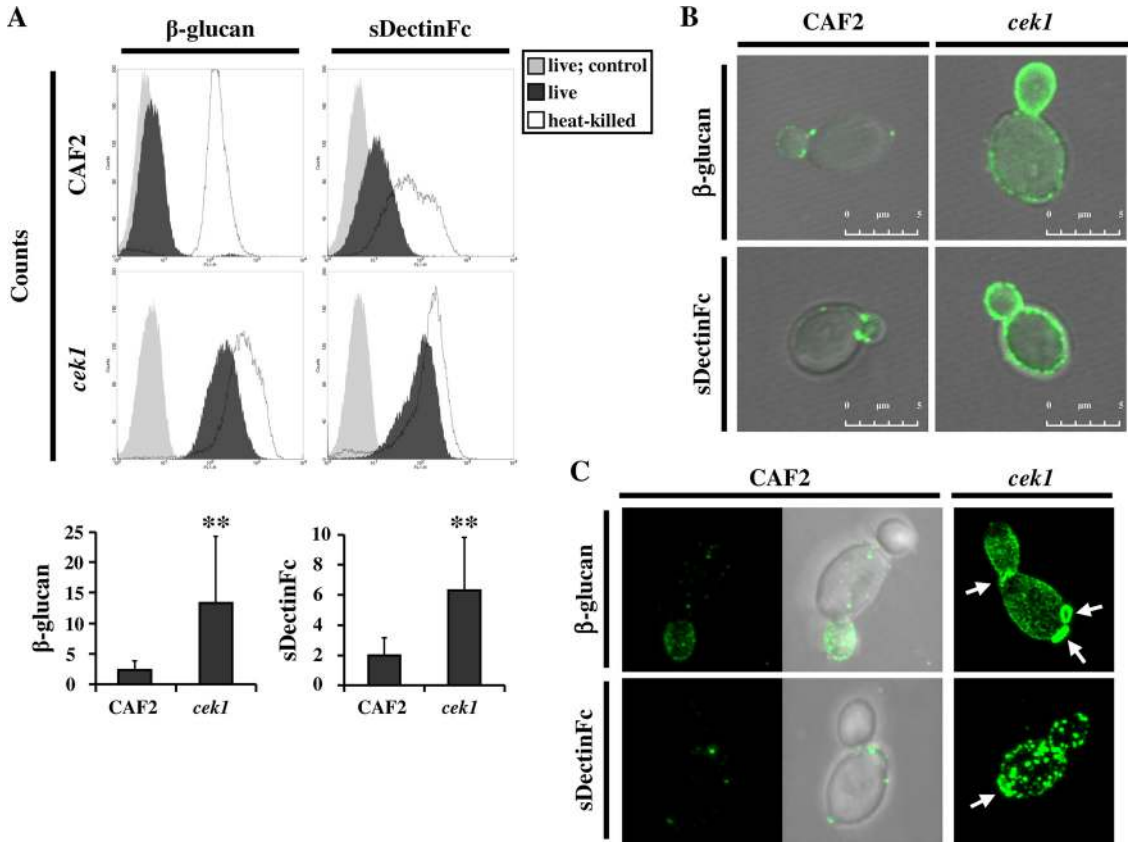

FIG. 2. Deletion of the *CEK1*-mediated MAPK pathway results in cell wall β-glucan exposure and increased dectin-1 recognition. (A) Representative flow cytometry analysis of β-glucan exposure on live *C. albicans* wt CAF2 and *cek1* deletion mutants. Light-gray-filled histograms correspond to control antibody (Ab), dark-gray-filled histograms correspond to anti-β-glucan MAb or soluble dectin-1–Fc (sDectinFc), and the empty histogram represents heat-killed positive-control yeast cells. Statistical analysis of these data is shown below and represents the mean fluorescence intensity (MFI) ± SD (after the subtraction of control Ab MFI). **, $P < 0.001$. (B) Confocal immunofluorescence analysis of individual yeast cells showing representative differential interference contrast (DIC) (Nomarski) microscopy and fluorescence images overlaid. Fungi were stained alternatively with anti-β-glucan MAb or sDectinFc followed by Alexa-488-labeled secondary Abs. (C) Confocal immunofluorescence analysis of individual yeast cells stained as described above (B) showing a tridimensional reconstruction of *z*-stack fluorescence images. Arrows indicate stronger-stained patches corresponding to bud scars. For CAF2 yeast cells, DIC and fluorescence image overlays are also shown.

entire surface of the *cek1* mutant when β-glucan MAb was used, whereas a patched recognition pattern was detected with sDectinFc (Fig. 2C). In addition to the above-described delocalized pattern, bud scars were strongly stained, showing that β-glucan is exposed in these surface structures (Fig. 2C). For wt fungi, the tridimensional reconstruction confirmed the restricted pattern shown in Fig. 2B. Finally, in order to analyze whether the mannoprotein content was also different on the surface of the *C. albicans* strains used, concanavalin A (ConA) staining of both the wt and the *cek1* mutant was performed, and no significant differences were found (see Fig. S2A and S2B in the supplemental material), further supporting the specific increase of β-glucan exposure on *cek1* mutants. Taken together, these data demonstrate that the disruption of the *CEK1*-mediated MAPK pathway leads to enhanced β-glucan exposure, causing increased dectin-1-mediated recognition.

Dectin-1-mediated binding to *C. albicans* is increased specifically in *cek1* mutants. To extend the above-described findings at the cellular level, binding assays were performed with K562 cells stably overexpressing dectin-1 (K562–dectin-1). As shown in Fig. 3A, K562–dectin-1 cells bound to the *cek1* mutant at a significantly higher percentage than to wt yeast. Moreover, the binding of the *cek1* mutant was specifically inhibited by both the anti-human dectin-1 MAb (MGD3) (see Materials and Methods) and the soluble glucan laminarin, confirming the specificity of dectin-1 binding to the *cek1* mutant. In contrast, and although both Cek1 and Cek2 MAPKs are potential targets of the upstream Hst7 MAPKK (Fig. 1A), K562–dectin-1 binding to *cek2* mutants was not significantly distinct from that exhibited by the corresponding wt strain (RM100) (Fig. 3B). Taken together, and since all strains tested were bound by DC-SIGN to a similar extent (see Fig. S3 in the supplemental material), these data indicate that the enhancement of the β-glucan on the yeast surface results in a specific increase of dectin-1 recognition and confirm the involvement of the Cek1 MAPK in masking β-glucan within the *Candida* cell wall.

Deletion of *CEK1* augments phagocytosis by and susceptibility to human phagocytes. Macrophages (MΦs) and dendritic cells (DCs) are strategically located at the sites of *Candida* entry (mucosal surfaces and skin), being essential to initiate antifungal innate immune responses. Phagocytosis facilitates the removal and killing of pathogens and primes the adaptive

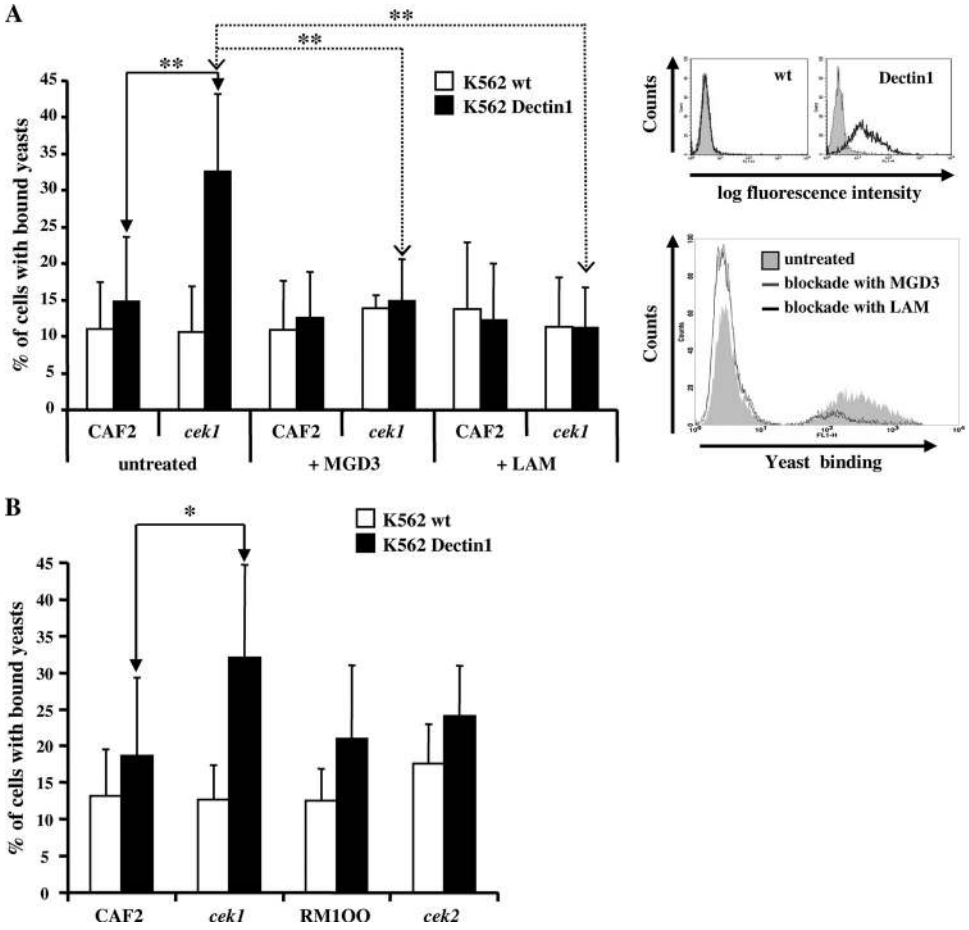

FIG. 3. Dectin-1-dependent binding to *C. albicans* is enhanced on *cek1* mutants. (A) Binding of wt CAF2 or *cek1* mutant yeast cells to wt K562 and transfected K562–dectin-1 cells. Cells were incubated with FITC-labeled *C. albicans* strains (10 yeast cells:1 cell) and analyzed by flow cytometry. Bars represent percentages of cells with bound fungi (means ± SD for eight independent experiments). Statistical analyses compared untreated K562 cell binding to wt CAF2 with binding to the *cek1* mutant (solid arrows) and also untreated K562 cell binding to the *cek1* mutant with MGD3 MAb- or laminarin (LAM)-pretreated cell binding to this mutant (dotted arrows). **, $P < 0.001$. The inset at the top shows flow cytometry expression levels of dectin-1 on wt or stably transfected K562 cells (filled histogram, control MAb; empty histogram, anti-dectin-1 MAb). The inset below shows a representative *C. albicans cek1* mutant binding profile for K562–dectin-1 cells (light-gray-filled histogram) and blocking profiles with MGD3 (gray empty histogram)- or laminarin (black empty histogram)-pretreated cells. (B) Comparative binding of K562–dectin-1 stably transfected cells to *cek1* and *cek2* deletion mutants and the corresponding wt strains, CAF2 and RM100, respectively. Differences between data were not significant, unless otherwise indicated. *, $P < 0.05$.

immune response (29). To evaluate the physiological relevance of *cek1*-dependent enhanced β-glucan exposure, the phagocytosis of both strains (the *cek1* mutant and wt CAF2) by human monocyte-derived DCs and MΦs (hDCs and hMΦs, respectively) was analyzed, and the phagocytic index was determined through immunomicroscopical procedures to discriminate between bound and internalized fungi (Fig. 4A and B). For both types of phagocytes, the phagocytic index of *cek1* was approximately twice that of wt CAF2, with hDCs exhibiting a higher phagocytic index than hMΦs (Fig. 4C and D). Strikingly, the anti-dectin-1 MAb (MGD3) inhibited phagocytosis by both hDCs and hMΦs to a similar extent (Fig. 4C and D), although it was slightly more effective in hDCs. Moreover, it seems that MGD3 pretreatment did not affect CAF2 uptake, while it specifically inhibited *cek1* phagocytosis. These data demonstrate a critical role for dectin-1 in *cek1* phagocytosis by both hDCs and hMΦs. Regarding pathogen viability after exposure to immune cells, killing assays revealed that the *cek1* mutant strain was up to three times more susceptible to hDCs than the corresponding wt strain (Fig. 4E). In contrast, both strains are equally susceptible to hMΦ killing (Fig. 4F), in agreement with previously reported results (2). Altogether, this set of data indicates that the deletion of *CEK1* increases β-glucan exposure on the *Candida* cell wall, giving a boost to dectin-1-dependent fungal recognition and phagocytosis by both hDCs and hMΦs and increasing killing by hDCs.

The *C. albicans cek1* mutant promotes phosphorylation of Syk, Raf-1, and ERK kinases inducing IκB degradation in hDCs and triggers activator protein 1 (AP-1) activation through dectin-1. To determine the molecular basis for the differential handling of both strains by human mononuclear phagocytes, the dectin-1-dependent signaling pathways were

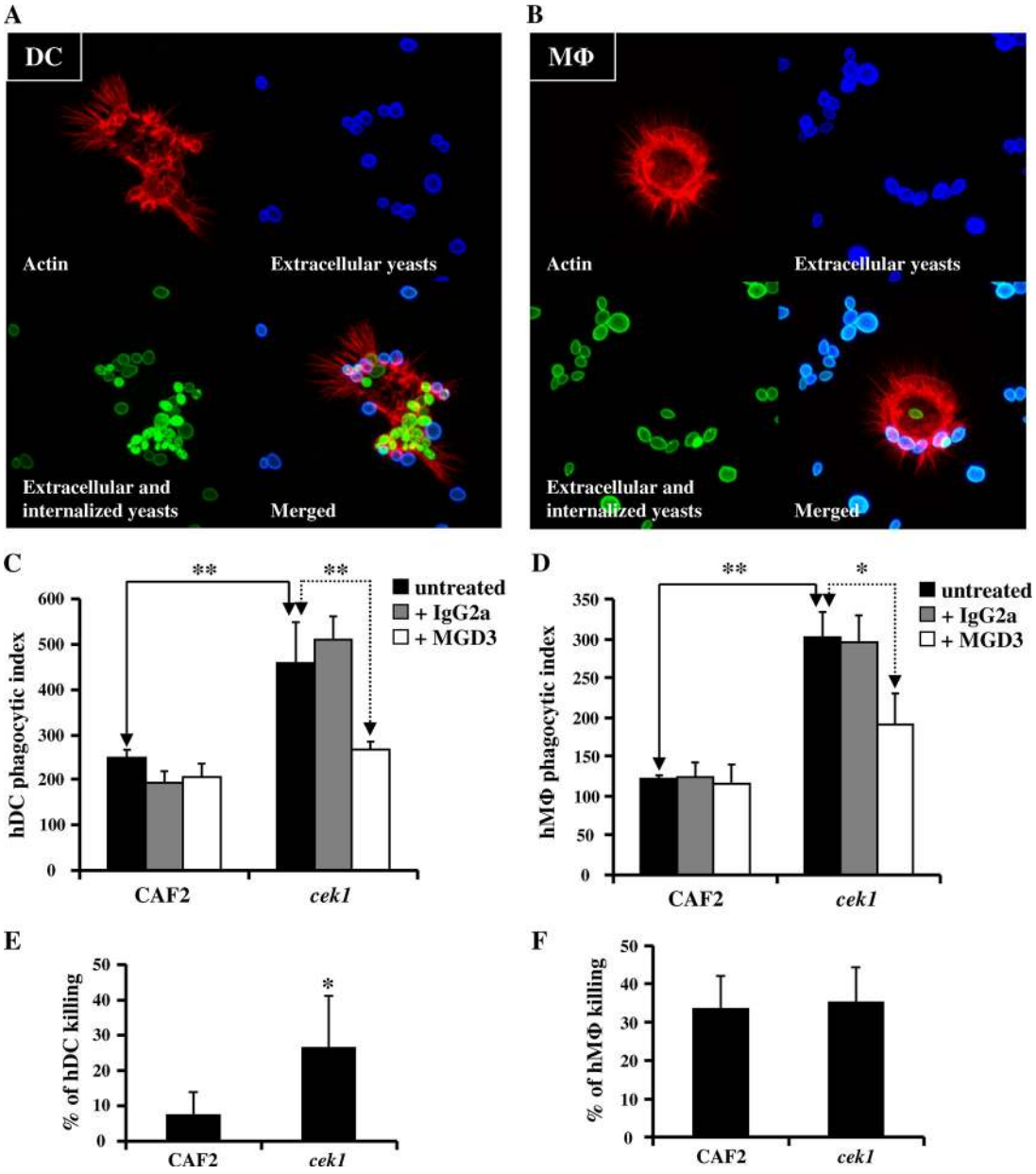

FIG. 4. Phagocytosis and killing of *C. albicans* by human monocyte-derived dendritic cells (hDCs) and macrophages (hMΦs). (A and B) Confocal microscopy images of hDCs (A) and hMΦs (B) showing extracellular yeast cells (blue), extracellular and internalized yeast cells (green), and actin (red). The images shown are not representative of the results but of the method used to quantify them. (C and D) The fungal phagocytic indices of hDCs (C) and hMΦs (D) exposed to wt CAF2 and the *cek1* deletion mutant were measured by differential immunofluorescence labeling. The phagocytic index was assessed by counting the number of internalized yeast cells per 100 phagocytes (at least 10 fields for each immunofluorescence carried out in duplicate). Data for phagocytic assays were collected in at least three independent experiments. *, $P < 0.05$; **, $P < 0.001$. (E and F) Killing of wt CAF2 and *cek1* strains by hDCs (E) and hMΦs (F) after 4 h of coincubation (1 yeast cell:20 phagocytes). The killing percentage for each strain was expressed as the percent reduction of CFU from hDC- or hMΦ-yeast cocultures versus simultaneous cultures containing yeast cells without phagocyte cells. Data are expressed as means ± SD for at least five independent experiments with different donors. Statistical analyses compared the phagocytic index of hDCs or hMΦs for CAF2 versus the *cek1* mutant (solid arrows) and the phagocytic index after treatment or nontreatment of primary cells with MAb MGD3 for the *cek1* mutant (dotted arrows).

evaluated with hDCs exposed to wt and mutant yeast cells. As shown in Fig. 5A, Syk phosphorylation was observed only after stimulation with the *cek1* mutant. Moreover, and unlike wt yeast cells, which stimulated early but short ERK phosphorylation, the *cek1* mutant triggered stronger and sustained ERK activation (Fig. 5A). Furthermore, and in agreement with data from a recent report on dectin-1-dependent Raf-1 activation (23), the *cek1* mutant activated the Raf-1-dependent signaling pathway more strongly than did the wt. Dectin-1–*C. albicans* engagement ultimately activates the transcription factor NF-κB through Syk- and Raf-1-dependent signaling pathways. To indirectly analyze the activation levels of NF-κB, we thus

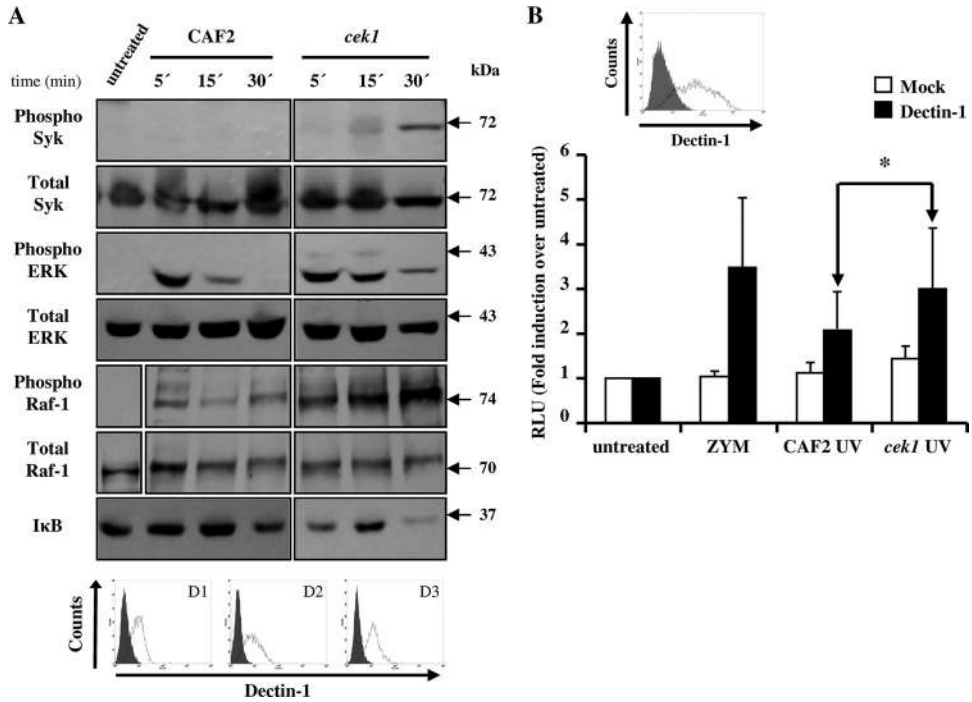

FIG. 5. Deletion of *CEK1* triggers Syk, ERK, and Raf-1 phosphorylation eliciting IκB degradation on hDCs and induces dectin-1-dependent AP-1 activation on transfected cells. (A) Western blot of cell lysates from hDCs cocultured with live *C. albicans* strains (10 yeast cells:1 cell) blotted with anti-phospho-Syk, -ERK, and -Raf-1 Abs versus anti-total Syk, -ERK, and -Raf-1 Abs, respectively. The bottom shows IκB degradation assayed by blotting with anti-IκB-α Ab. Below, flow cytometry histograms show dectin-1 expression levels of hDCs from three representative donors (D1 to D3) (filled histogram, control Ab; empty histogram, MGD3 MAb). (B) Luciferase activity of HEK293T cells transiently transfected with the indicated plasmids after incubation with zymosan (ZYM) or *C. albicans* UV-inactivated yeast cells. Luciferase activity was calculated as firefly/*Renilla* luciferase activities and normalized versus the control (untreated). Bars represent relative light units (RLU) as means ± SD for five independent experiments, each carried out in duplicate. Statistical analyses compared the luciferase activity induction of HEK293T–dectin-1-transfected cells exposed to the wt versus the mutant strain. *, $P < 0.05$. The inset shows the flow cytometry expression profile of HEK293T–dectin-1-transfected cells (filled histogram, control Ab; empty histogram, dectin-1 staining).

studied IκB-α degradation on hDCs exposed to different strains. Only *cek1* yeast cells were able to induce a late (30 min) degradation of IκB, indirectly indicating NF-κB activation.

Finally, previous studies reporting AP-1 activation via the dectin-1/Syk pathway during fungal infection (18, 56) led us to analyze the effect of *cek1* on the dectin-1-dependent activation of AP-1. To avoid a yeast-to-hypha transition during the experiment and to minimize cell killing, we used single-dose UV-inactivated fungi (sufficient to inactivate the fungus while avoiding cell wall disruption and consequent β-glucan unmasking [see Fig. S4 in the supplemental material]). Luciferase reporter assays with transfected HEK293T–dectin-1 cells showed that the *cek1* mutant induced AP-1 activation more efficiently than did wt CAF2 cells (Fig. 5B). As expected, *C. albicans* or zymosan was unable to induce AP-1 activation in HEK293T cells, which are devoid of dectin-1 expression. Therefore, and in agreement with its higher dectin-1 binding ability, the *cek1* mutant promotes a stronger activation of dectin-1-initiated intracellular signaling pathways.

Deletion of the *CEK1* gene leads to an augmented respiratory burst and enhanced dectin-1-dependent cytokine synthesis. In addition to binding, phagocytosis, killing, and intracellular signaling, the interaction of *C. albicans* with phagocytes ultimately leads to respiratory burst activation and cytokine release (48). Thus, we tested the abilities of the *cek1* mutant and the wt strain to induce the phagocyte respiratory burst in hMΦs and hDCs. The *cek1* mutant promoted a slight but significant increase in ROS secretion compared to that of the wt (Fig. 6A and B) in both types of cells. In addition, and in accordance with the ERK activation observed after intracellular ROS production (17), a stronger phosphorylation of ERK in RAW 264.7 MΦ cells exposed to the *cek1* mutant was observed (data not shown).

Finally, since the activation of the dectin-1-dependent Syk pathway on hDCs leads to ERK phosphorylation (Fig. 5A) and the production of cytokines (13, 53), the effect of the *cek1* mutant on hDC cytokine synthesis was evaluated. UV-inactivated fungi induced lower levels of TNF-α secretion than heat-killed fungi (Fig. 6C), concurring with previous observations of heat treatment-induced cell wall disturbances (20, 22, 58). Nonetheless, when the *C. albicans* cell wall was undisturbed (single-dose UV-treatment [see Fig. S4 in the supplemental material]), the *cek1* mutant promoted a slight but significant increase in TNF-α secretion compared to that produced by wt yeast cells (Fig. 6C). This cytokine secretion was dectin-1 dependent, as it was blocked by anti-human dectin-1 MAb MGD3 (Fig. 6D). In addition, we also observed that Cek1-deficient fungi induced a later and higher level of dectin-1-mediated IL-10 production than wt yeast (Fig. 6D), and this effect was significantly inhibited in the presence of MAb MGD3. Therefore, the deletion of the Cek1 kinase in *C. albi-*

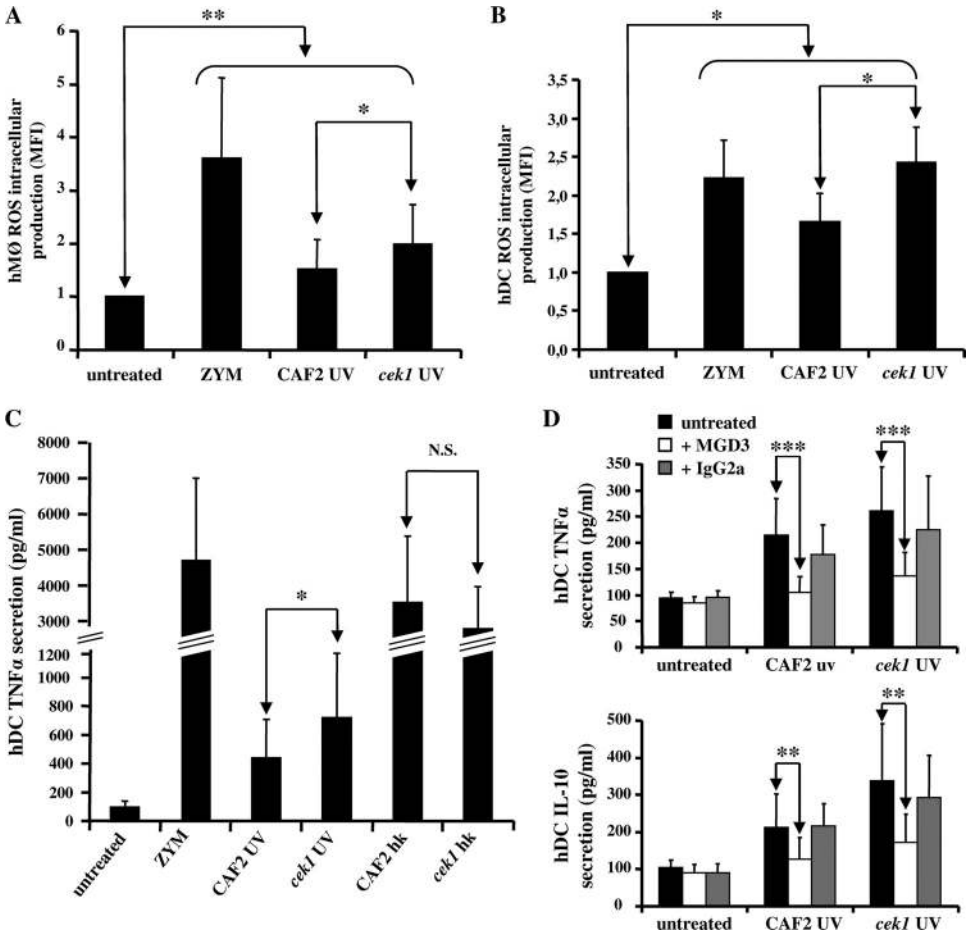

FIG. 6. The *C. albicans cek1* mutant enhances respiratory burst and dectin-1-dependent cytokine secretion. (A and B) ROS secretion analysis of hMΦs (A) and hDCs (B) cocultured with UV-inactivated strains (10 yeast cells:1 cell). Asterisks indicate significant differences between untreated and treated cells (square brackets) or between *cek1* mutants and wt strain CAF2 (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0005$). ROS intracellular production is represented as the means ± SD for at least five independent experiments. (C) Mean levels of TNF-α secretion by hDCs stimulated with zymosan (ZYM) or UV-inactivated or heat-killed *C. albicans* strains (50 yeast cells:1 hDC). Data are representative of data from nine independent healthy donors (means ± SD). (D) Mean levels of TNF-α (6 h) and IL-10 (18 h) in stimulated hDCs (10 yeast cells:1 hDC) in the presence or absence of anti-human dectin-1 MAb (MGD3). ELISA results for test conditions were averaged for six independent healthy donors and are expressed as means ± SD (pg/ml). Data are not significant unless otherwise indicated.

cans results in an enhancement of all the known cellular responses previously ascribed to dectin-1 ligation, establishing this signaling route as a potential target for the modulation of antifungal immune responses.

DISCUSSION

Candida albicans is the most common fungus causing nosocomial infections and remains a major diagnostic and therapeutic challenge to the clinician (49). As the unmasking of β-glucan increases the recognition of this fungus by innate immune cells (22, 58), the identification of genes involved in this process has attracted research interest for the development of new anticandidal therapies. Here we report the involvement of a MAPK signaling pathway in β-glucan masking in a pathogenic fungus. We demonstrated that a Cek1 deficiency enhanced β-glucan exposure on the *C. albicans* cell wall, leading to greater innate immune responses triggered through dectin-1.

In the present study we detected β-glucan exposure over the entire cell wall surface on *cek1* and *hst7* deletion mutants, whereas on wt yeast cells, β-glucan was found only in discrete patches. The unmasking of β-glucan increased dectin-1-mediated recognition, uptake, killing, and signaling by phagocytic cells. In this regard, the activations of Syk-, Raf-1-, and ERK-dependent pathways along with IκB degradation in hDCs were enhanced, as was dectin-1-dependent AP-1 activation in dectin-1-transfected cells. As a consequence of this signaling activation, we detected enhanced ROS production and cytokine secretion. It is noteworthy that these data suggested that this enhanced dectin-1-mediated immune response could explain the attenuated virulence of *cek1* mutants in animal models of disseminated or localized candidiasis (10, 26). On the other hand, it was previously described that under certain conditions, the Cek1 MAPK participates in filamentation and invasion (32). This route is also involved in cell wall biogenesis, as either *cek1* mutants or mutants defective in their phosphorylation (*hst7* or *sho1*) are sensitive to compounds such as Congo red,

calcofluor white, caspofungin, or zymolyase (15, 46, 47). Therefore, it could be hypothesized that the hypersensitivity to agents perturbing the cell wall and the increased exposure of β-glucan shown by the *cek1* mutant reflected an altered cell wall organization that could lead to a lower level of resistance to candidacidal mechanisms. Accordingly, we found these mutants to be more susceptible to hDC-mediated uptake and killing. Moreover, the phagocytic index was notably reduced in the presence of an anti-dectin-1 MAb, suggesting a major role of this receptor in *cek1* uptake. Outstandingly, although the phagocytic index of the *cek1* mutant by hMΦs was twice that of wt CAF2, the viabilities of both strains in response to hMΦ killing were similar. These monocyte-derived hMΦs were not previously elicited; therefore, the MΦ cytocidal function could be only partially activated, thus explaining the reduced hMΦ phagocytic index compared to that of hDCs. Moreover, although dectin-1 confers the ability to phagocytose yeasts, the level of its expression on the surface of primary hMΦs could be lower than that on hDCs, since MGD3 blocking is less significant. This lower level of surface expression together with the attenuated cytocidal function may not be sufficient to initiate the specifically enhanced *cek1* killing mechanisms observed for hDCs. In addition, MΦs express higher levels of TLRs than DCs (39) that could mask the specific *cek1* killing mediated by dectin-1. The mosaic of PRRs that is expressed by each of these cell types ultimately determines the type of response elicited following the recognition of *C. albicans*. However, further research into the specific regulation of yeast-killing mechanisms will shed light on this. Nonetheless, a *CEK1* disruption could result in additional phenotypes that cannot be excluded as a cause of their virulence defects. These data allow us to speculate that dectin-$1^{-/-}$ mice should be more susceptible than wt mice to infection with Cek1-deficient strains. However, the outcome of experimental infections is influenced by several mechanisms, including PRR recognition and cytokine production, as well as other factors such as adherence to host cells and the growth rate of the mutants. Further *in vivo* studies to evaluate the relevance of β-glucan unmasking on the immune response would thus be of interest. Regarding this issue, a recent study by Wheeler et al. (59) showed that β-glucan is progressively unmasked during infection, further supporting a major role of dectin-1 in protective antifungal immunity.

Dectin-1 engagement by the cell wall extract zymosan in DCs triggers Syk and ERK pathway activation (13, 44). Regarding Syk phosphorylation, we observed activation only after the stimulation of hDCs with the *cek1* mutant. Additionally, *cek1*-induced ERK activation was sustained in comparison to that of wt yeast. These data concur with previous observations showing ERK phosphorylation coupled to dectin-1/Syk signaling (35, 53). We also showed that the *cek1* mutant induced the Raf-1 signaling pathway (23) more strongly than the wt. Although Syk activation was induced exclusively through dectin-1, Raf-1 activation is induced through both dectin-1 and DC-SIGN (24), explaining the baseline activation of hDCs exposed to wt CAF2 cells. Finally, previous studies showed that dectin-1 activates NF-κB (19, 25) and AP-1 (56) via Syk. It thus appears that the enhanced β-glucan exposure in the *cek1* mutant induces increased dectin-1 recognition, leading to Syk/ERK/Raf-1 phosphorylation, which triggers NF-κB and AP-1 activation and ultimately leads to ROS production and cytokine synthesis. In this regard, the *cek1* mutant elicited slight but significant TNF-α secretion. In addition, significant IL-10 production from hDCs was detected, which, at later stages of the infection, may be beneficial to resolving an inflammatory process (13, 48). The weak cytokine response elicited by *C. albicans* wt cells may prevent the recruitment of effector cells and the elimination of the pathogen, explaining its persistence as a commensal. Nonetheless, the biological relevance of the slight increase in cytokine synthesis induced by the *cek1* mutant is unclear, and this increase alone might not account for the loss of virulence, while the enhanced phagocytosis and killing observed for hDCs may help the transition from commensalism to infection.

During *C. albicans* infection, both yeast and filamentous forms can be found in infected tissues, and DCs discriminate between them, eliciting a protective response against yeast and tolerance to hyphae through different PRRs (14). An integrated model of fungal recognition has been proposed, in which immune sensing of *C. albicans* requires the cooperative recognition of mannans and glucans by PRRs (39). The outcome of an immune response to *C. albicans* thus depends on the balance of the signals generated through TLR2, TLR4, dectin-1, MR, and DC-SIGN, among others, each of which recognizes a different PAMP of the fungal cell wall. Both TLR2 and TLR4 collaborate with dectin-1 to induce an inflammatory response (6, 12, 19, 40). Herein we showed that dectin-1-specific activation by *C. albicans* occurs only when β-glucan is exposed, concurring with data from previous studies (20, 59). The enhanced uptake, killing, and cytokine synthesis observed for hDCs exposed to the *cek1* mutant were thus inhibited by dectin-1 MAb MGD3, suggesting a dectin-1-dependent elicitation. Nonetheless, we cannot exclude the participation of other PRRs in this process (8, 9, 21, 24). Further studies are needed to evaluate the complex cross talk between dectin-1 and other PRRs in immunity to *C. albicans*, mainly in the context of the *in vivo* recognition of intact fungi by primary human immune cells. In this regard, we observed that responses elicited by live or UV-inactivated fungi were weaker than those triggered by heat-killed yeast (Fig. 6B), indicating the need for studies using whole live or suitable UV-inactivated *C. albicans* yeasts to avoid misleading conclusions (see Fig. S4 in the supplemental material). Moreover, conventionally used isolated fungal components or zymosan could not reflect the true complexity of the response against intact live fungi. Therefore, the preservation of cell wall structure is of outstanding importance in elucidating the immune cell-fungus interaction.

Finally, as we have demonstrated that increased *cek1* mutant β-glucan exposure led to enhanced innate immune activation, the *CEK1*-mediated MAPK pathway can be proposed as a valuable antifungal target. Moreover, drug-induced β-glucan exposure could target fungi for recognition by natural anti-β-glucan antibodies, which are detected in patients with progressive fungal infection (42, 55), rendering the pathogen more susceptible to the host immune system.

In conclusion, the present study demonstrated that the *CEK1*-mediated MAPK pathway has a key role in β-glucan masking in *C. albicans* and that, given the high degree of conservation in MAPK pathways, this phenomenon may be a general mechanism of fungi to evade host recognition. Addi-

tionally, our study highlights the value of fungal MAPK pathways as potential therapeutic targets in modulating the host immune response to a pathogen.

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