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Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36

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Receptors involved in innate immunity to fungal pathogens have not been fully elucidated. We show that the *Caenorhabditis elegans* receptors CED-1 and C03F11.3, and their mammalian orthologues, the scavenger receptors SCARF1 and CD36, mediate host defense against two prototypic fungal pathogens, *Cryptococcus neoformans* and *Candida albicans*. CED-1 and C03F11.1 mediated antimicrobial peptide production and were necessary for nematode survival after *C. neoformans* infection. SCARF1 and CD36 mediated cytokine production and were required for macrophage binding to *C. neoformans*, and control of the infection in mice. Binding of these pathogens to SCARF1 and CD36 was β -glucan dependent. Thus, CED-1/SCARF1 and C03F11.3/CD36 are β -glucan binding receptors and define an evolutionarily conserved pathway for the innate sensing of fungal pathogens.

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Abbreviations used: abf, anti-bacterial factor; acLDL, acetylated low density lipoprotein; CHO, Chinese hamster ovary; QPCR, quantitative real-time PCR; TLR, Toll-like receptor.

Yeasts such as *Cryptococcus neoformans* and *Candida albicans* are an emerging group of infectious pathogens in patients with impaired T cell-mediated immunity, such as those with AIDS, solid organ transplant recipients, and hospitalized patients (1, 2). Although much work has been devoted in the past decade to uncovering the role of pattern recognition receptors in the innate response to bacteria and viruses, our understanding of how the innate immune system senses fungal pathogens is less clear (3). The macrophage, a central component of the innate immune system, is essential to the effective immune response to pathogenic yeast. Macrophages have direct antimicrobial activity against these organisms; they promote antigen presentation, polysaccharide sequestration, and cytokine and chemokine production (4–6). There is also evidence that persistent infection is associated with the intracellular residence of yeast cells in macrophages. Furthermore, infected circulating macrophages can transfer these pathogens and cause dissemination of these infections hematogenously (7).

Recently, the C-type lectin-like receptor Dectin-1 was shown to be a macrophage receptor for β -glucans and to bind several fungi (8–12). β -glucan is a major carbohydrate found in the fungal cell wall. Dectin-1 mediates both Toll-like receptor (TLR)-dependent and TLR-independent responses to fungi in vitro. However, the role of Dectin-1 in the host response to fungal pathogens in vivo is less clear (13–15), suggesting that additional receptors contribute to the innate immune response to fungal pathogens.

Scavenger receptors constitute a diverse family of pattern recognition receptors that recognize both endogenous and pathogen-derived ligands. These receptors are expressed on cells patrolling potential portals of pathogen entry, including macrophages, dendritic cells, microglia, and endothelial cells, and are believed to be involved in the pathogenesis of chronic inflammatory conditions such as atherosclerosis and

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Alzheimer's disease, and in the host response to some bacterial pathogens (16–21). To date, a role for scavenger receptors in the recognition of fungal and yeast pathogens has not been described.

Using an shRNA screen in mouse macrophages, we found that two evolutionarily conserved members of the scavenger receptor family, SCARF1 and CD36, and their *Caenorhabditis elegans* orthologues, CED-1 and C03F11.3, are required for the induction of protective immune responses in worms and mice to pathogenic yeast. We show that these receptors recognize β -glucans and have an essential function in antifungal immunity and host defense by mediating yeast binding and subsequent macrophage activation.

RESULTS

CED-1 mediates *C. neoformans* recognition in *C. elegans*

To identify genes involved in the innate immune response to fungal pathogens, we used the nematode *C. elegans* as a model host for infection and *C. neoformans* as a prototypical fungal pathogen, as previously described (22, 23). Because of the established role of scavenger receptors in innate immunity to bacterial pathogens (19–21), we explored whether this family of pattern recognition receptors also plays a role in innate immunity to fungal pathogens. Using a BLAST screen of the Wormbase database WS196, we searched for *C. elegans* orthologues of the mammalian scavenger receptors *SR-A1*, *SR-A2*, *MARCO*, *SCARF1*, *CD36*, *SCARB1*, *SCARB2*, *CD68*, *CXCL16*, and *Lox-1*. Interestingly, we found that only four of these receptors, *SCARF1*, *CD36*, *SCARB1*, and *SCARB2*, have possible *C. elegans* orthologues. As previously described, we found that *SCARF1* is a *CED-1* orthologue (24). We also identified four potential *CD36* orthologues in the *C. elegans* genome (*C03F11.3* > *F11C1.3* > *R07B1.3* > *F07A5.3*; ranked by amino acid alignment score to mouse *CD36*), one potential *SCARB1* orthologue (*Y49E10.20*), and one potential *SCARB2* orthologue (*Y76A2B.6*; available from the Wormbase database at <http://wormbase.org>) (25, 26). We analyzed the role of each of these receptors in the pathogenesis of *C. neoformans* infection in *C. elegans*.

Gene expression profiling of WT *C. elegans* infected with *C. neoformans* revealed that the *SCARF1* orthologue *CED-1*, a scavenger receptor that mediates engulfment of cell corpses by *C. elegans* (24), is up-regulated approximately fourfold compared with uninfected worms (Fig. 1 a). This finding raised the interesting possibility that *CED-1* may be involved in the innate immune response to fungal pathogens. To explore this possibility, we obtained CED-1 mutants that do not express the receptor on the cell surface (strains MT4930 and MT4933) (24) and infected them with *C. neoformans*. We found that CED-1 mutants were significantly more susceptible to killing by *C. neoformans* than WT nematodes (MT4933 strain shown; Fig. 1 b). By 144 h after infection, ~80% of the CED-1 mutants exposed to *C. neoformans* died compared with just ~28% of WT nematodes ($P < 0.0001$; Fig. 1 b). Similar results were obtained with a second CED-1 mutant (strain MT4930) and when CED-1 expression was down-reg-

ulated in WT nematodes using RNAi (Fig. S1 a, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>). Notably, both WT and CED-1 mutant nematodes have a normal lifespan in the presence of the nonpathogenic yeast *Cryptococcus laurentii* (unpublished data).

To determine if *CED-1* is also involved in the host response to other pathogens, we infected WT and CED-1 mutant worms with the common human pathogens *Salmonella typhimurium*, *Staphylococcus aureus*, and *Enterococcus faecalis*. By 144 h, ~56% of CED-1 mutants died in response to *Salmonella* infection as compared with 25% of WT worms (Fig. S1 b), indicating that *CED-1* is also required, at least in part, for the innate immune response of *C. elegans* to this Gram-negative bacterium. In contrast, both WT and CED-1 mutant worms died at the same rate in response to *S. aureus* and *E. faecalis* infection (Fig. S1 c and not depicted), indicating that *CED-1* is not required for host defense to these organisms.

Gene expression profiling of WT *C. elegans* infected with *C. neoformans* revealed the up-regulation of several antimicrobial peptides, including antibacterial factor (*abf*) 1 and *abf*-2 (Fig. 1 c). These peptides have been shown to be up-regulated in response to bacterial pathogens and to have antifungal activity (27, 28). To determine if induction of antimicrobial peptides is a downstream event from CED-1 binding to *C. neoformans*, we measured mRNA levels of *abf*-1 and *abf*-2 in WT and CED-1 mutants 24 h after exposure to *C. neoformans*. Exposure of WT *C. elegans* to *C. neoformans* up-regulated mRNA levels of *abf*-1 and *abf*-2 4.4- and 4.1-fold, respectively (Fig. 1c and not depicted). In contrast, CED-1 mutants exposed to *C. neoformans* up-regulated *abf*-1 and *abf*-2 levels only 1.6- and 1.9-fold, respectively (Fig. 1c and not depicted), indicating that CED-1 expression is required for maximal induction of these antimicrobial proteins. Of note, WT and CED-1 mutants up-regulated antimicrobial peptide mRNAs equally in response to infection with *S. aureus* and *E. faecalis* (unpublished data). Collectively, these data suggest that *CED-1* is critical in protecting *C. elegans* from *C. neoformans* infection, and that expression of *CED-1* is required for induction of antimicrobial peptides that may play a role in the host response to fungal pathogens.

C03F11.3, a CD36 orthologue, mediates *C. elegans* survival after *C. neoformans* infection

Our search for potential *C. elegans* orthologues of the various mammalian scavenger receptors identified that only four of these receptors have possible *C. elegans* orthologues: *SCARF1* (*CED-1*), *CD36* (*C03F11.3* > *F11C1.3* > *R07B1.3* > *F07A5.3*), *SCARB1* (*Y49E10.20*), and *SCARB2* (*Y76A2B.6*) (25, 26). Because *CED-1* is involved in the host response to *C. neoformans* infection, and because scavenger receptors have been shown to play complementary roles to each other and share the same ligands (18), we tested whether *C03F11.3*, *F11C1.3*, *R07B1.3*, *F07A5.3*, *Y49E10.20*, or *Y76A2B.6* are also involved in the nematode immune response to *C. neoformans* infection.

Silencing of the *C03F11.3* gene with siRNA led to increased susceptibility of the nematodes to *C. neoformans* infection and

decreased survival (Fig. 1, d and e). By 168 h after infection, ~24% of control siRNA-treated *C. elegans* died in response to *C. neoformans* compared with ~60% of worms treated with siRNA in response to *C03F11.3*. In contrast, *C. elegans* worms deficient in the expression of the other three potential *CD36* orthologues (*F11C1.3*, *R07B1.3*, and *F07A5.3*) survived *C. neoformans* infection, similar to WT worms (Fig. S2, a–c, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>).

As observed with *CED-1*, *C03F11.3* expression in *C. elegans* was also required for induction of the antimicrobial peptides *abf-1* and *abf-2* (Fig. 1 e and not depicted), indicating that *C03F11.3*, rather than *F11C1.3*, *R07B1.3*, or *F07A5.3*, is the *C. elegans* *CD36* orthologue. Indeed, analysis of *C03F11.3* showed that it has ~53% similarity to mammalian *CD36* and possesses two membrane-spanning domains similar to *CD36* (Fig. S3, a and b). Silencing of *SCARB1* and *SCARB2* orthologues (*Y76A2B.6*

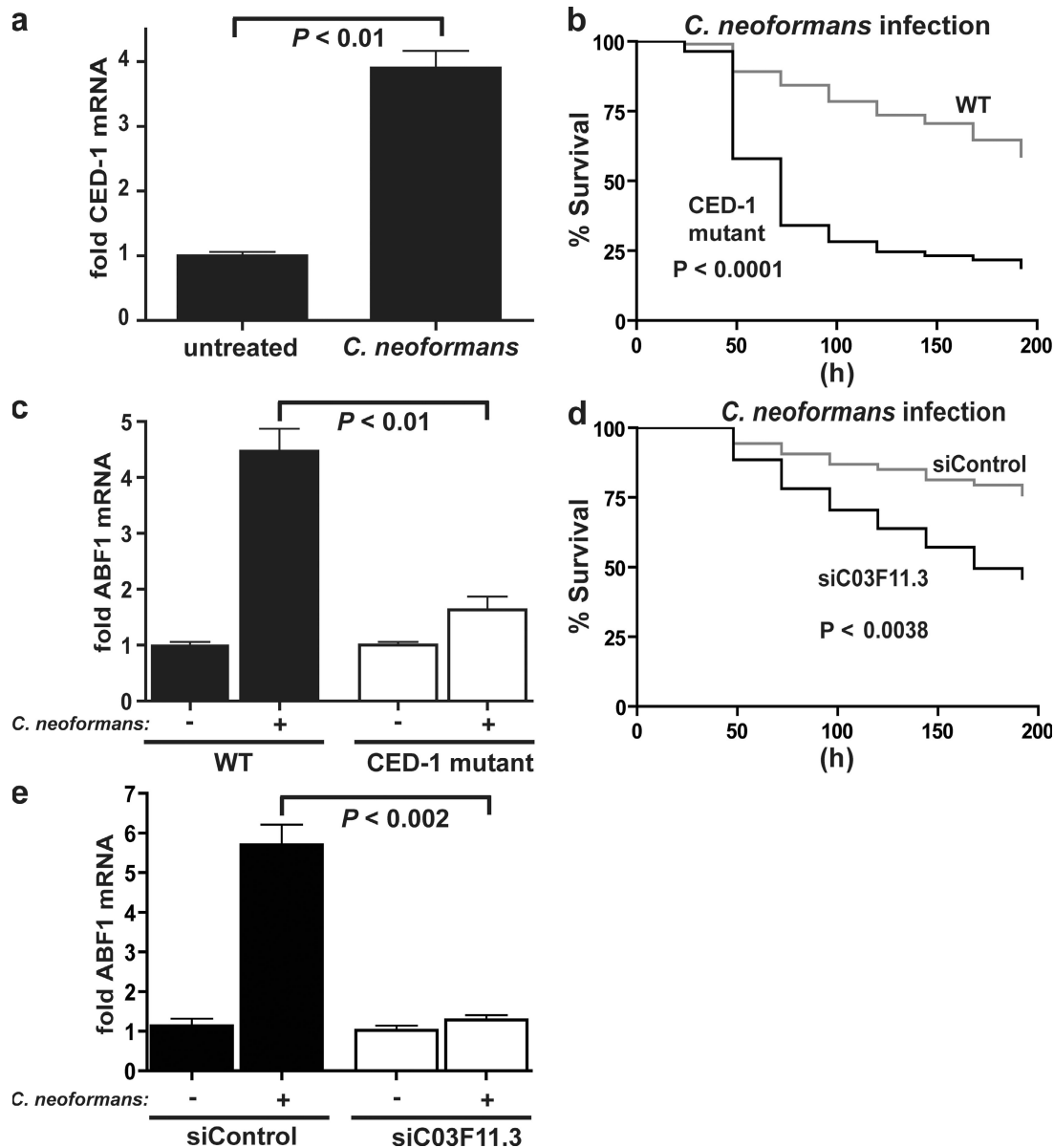


Figure 1. *CED-1* and *C03F11.3* mediate recognition of *C. neoformans* in *C. elegans*. (a) Increased *CED-1* expression in *C. elegans* infected with *C. neoformans*. mRNA levels were measured by QPCR, as described in Materials and methods, after exposure of WT nematodes to *C. neoformans* for 24 h. (b) Survival of WT N2 strain nematodes and *CED-1* mutant strain MT4933 after exposure to *C. neoformans* strain H99. Survival was determined as described in Materials and methods, and p-values were determined using the Kaplan–Meier survival statistical test ($n > 100$ worms per strain). (c) mRNA levels for the antimicrobial peptide *abf-1* were measured by QPCR 24 h after exposure of WT and *CED-1* mutant strain MT4933 nematodes to *C. neoformans*. (d) Survival of siRNA control L4440 strain and siC03F11.3 nematodes after exposure to *C. neoformans*. (e) mRNA levels for the antimicrobial peptide *abf-1* were measured by QPCR 24 h after exposure of L4440 and siC03F11.3 nematodes to *C. neoformans*. Data are representative of at least three independent experiments. Means \pm SEM are shown.

and *Y49E10.20*) had no effect on the survival of *C. elegans* in response to infection with *C. neoformans* (Fig. S2, d and e). This is the first description of C03F11.3 as a *C. elegans* membrane receptor. These data also demonstrate that the scavenger receptors CED-1 and C03F11.3 are novel members of an innate immune recognition pathway in *C. elegans* that have a direct role in the host defense against the yeast pathogen *C. neoformans*.

shRNA screen identifies receptors that mediate *C. neoformans*-induced mouse macrophage activation

To identify mammalian receptors involved in the host response to fungal pathogens, and to test if the mammalian orthologues of the nematode scavenger receptors CED-1 and C03F11.3 are also involved in the host response to *C. neoformans* infection, we used a mouse shRNA library targeting innate immune receptors to screen for genes that mediate macrophage activation in response to this fungal pathogen. The RNAi Consortium has previously stated that its lentiviral library silences genes in a diverse set of cell types, contains five shRNAs for nearly every gene in the mouse genome, and is capable of significantly reducing target mRNA levels (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>) (29). Using a subset of the mouse lentiviral shRNA library targeting TLRs, scavenger receptors including *CED-1* orthologues, c-type lectins, and other pattern recognition receptors (Fig. 2 a), we performed a screen to identify genes that mediate macrophage activation induced by *C. neoformans* stimulation using RAW 264.7 (RAW) macrophages. *C. neoformans* induced a broad spectrum of cytokines and chemokines in RAW cells, including IL-1 β and MIP-2 (unpublished data). Silencing of the scavenger receptors *CD36*, *SCARF1*, or *SCARB2* led to a significant reduction in *C. neoformans*-induced IL-1 β and MIP-2 mRNA in macrophages (Fig. 2 b and not depicted). In addition, our shRNA screen confirmed the role of genes already known to be involved in the innate response to fungal pathogens, such as *TLR2*. Importantly, this mammalian shRNA screen confirmed our *C. elegans* data by demonstrating that orthologues of *CED-1* (*SCARF1*) and *C03F11.3* (*CD36*) mediate macrophage responsiveness to *C. neoformans*. Gene silencing of *MEGF10* and *LRP1*, two other potential orthologues of *CED-1* also expressed by macrophages, had no effect on *C. neoformans*-induced macrophage activation (Fig. 2 b), indicating that *SCARF1* is the mammalian *CED-1* orthologue responsible for recognition of *C. neoformans*. Interestingly, our screen also implicated *SCARB2*, a lysosomal protein, in the cytokine response to *C. neoformans*, suggesting a novel role for lysosomal proteins in regulating cytokine responses to fungal infection.

SCARF1 mediates recognition of *C. neoformans* and *C. albicans*

SCARF1 is expressed on macrophages and endothelial cells (30), and both cell types play an important role in host interactions with *C. neoformans*. Macrophages phagocytose and kill *C. neoformans* (31), and binding of these fungi to endothelial cells initiates tissue invasion (32). To test the ability of SCARF1 to bind and phagocytose *C. neoformans*, we isolated the *SCARF1*

cDNA from a human endothelial cell cDNA library and established a stable Chinese hamster ovary (CHO) cell line expressing SCARF1 (CHO-SCARF1; Fig. 3 a, top). CHO-SCARF1 cells, but not CHO control cells, gained the ability to bind nonopsonized *C. neoformans* (Fig. 3 b). Antibodies to SCARF1 blocked the binding of *C. neoformans* to CHO-SCARF1, indicating that this binding is specific (Fig. 3 b). We also found that SCARF1 expression conferred a fivefold increase in cellular internalization of *C. neoformans* over mock-transfected control cells, indicating that SCARF1 acts as a phagocytic receptor for pathogenic yeast (Fig. 3 c). In addition, human microvascular endothelial cells or monocyte-derived macrophages incubated with *C. neoformans* for 2 h in the presence of blocking anti-SCARF1 antibodies showed an ~55–60% reduction in binding (Fig. 3 d and not depicted), confirming that SCARF1 can mediate binding of mammalian cells to this pathogen. Staining of peritoneal macrophages with anti-SCARF1 antibodies confirmed colocalization of the receptor with areas of *C. neoformans* attachment (Fig. 3 a, bottom).

To further investigate the role of SCARF1 in the recognition of *C. neoformans*, we examined the ability of several soluble fungal-derived carbohydrates and classical scavenger receptor ligands to inhibit the binding of fluorescently labeled *C. neoformans* to CHO-SCARF1 cells. β -Glucan, laminarin (a soluble fungal-derived β -glucan), acetylated low density lipoprotein (acLDL), poly I, fucoidan, and, to a lesser extent, mannan inhibited SCARF1 recognition of *C. neoformans* (Fig. 3, e and f). In contrast, addition of dextran, pullulan, or poly C had no effect. These results suggested that binding of *C. neoformans* to SCARF1 is mediated in part by the recognition of β -1,3-linked and β -1,6-linked β -glucans. Although β -glucans in encapsulated *C. neoformans* were thought to be hidden beneath the capsule layer, recent evidence suggests that β -glucans are exposed on the cell surface and released during systemic fungal infections (33, 34). To address this issue, we examined the ability of CHO cells expressing SCARF1 to directly bind zymosan (a classical β -glucan receptor ligand). We found a 3.5-fold increase in zymosan binding to SCARF1 over mock-transfected CHO cells (Fig. 4 a). The binding of fluorescently labeled zymosan to SCARF1 was blocked by the addition of unlabeled β -glucan (Fig. 4 a). Collectively, these data demonstrate that binding of *C. neoformans* by SCARF1 is mediated by the recognition of β -glucans (35). Other receptors, most notably Dectin-1, have also been shown to bind β -glucans and other yeast and fungal organisms in a similar manner (35). These data indicate that SCARF1, in addition to Dectin-1, is a receptor for β -glucans.

In our RNAi screen, we found that silencing SCARF1 and TLR2 expression significantly reduced *C. neoformans*-induced macrophage activation. Recently, SCARF1 was shown to cooperate with TLR2 in mediating the recognition and cellular response to bacterial outer membrane protein A (36). To determine if SCARF1 and TLR2 cooperate in the cellular response to *C. neoformans*, we stimulated CHO cells expressing SCARF1, TLR2, SCARF1/TLR2, SCARF1/TLR4, and SCARF1/TLR9, and measured mRNA induction of the chemokine Gro

in these cells (Fig. 4 b). We found that TLR2 but not TLR4 or TLR9 expression conferred responsiveness to *C. neoformans* and that coexpression of SCARF1 and TLR2 synergized for maximal responsiveness to *C. neoformans* stimulation (Fig. 4 b).

To determine if SCARF1 mediates recognition of *C. neoformans* in vivo, we instilled fluorescently labeled *C. neoformans* in the lungs of WT mice in the presence or absence of neutralizing anti-SCARF1 or control antibodies. 2 h later, the

mice were killed and alveolar macrophages were harvested by alveolar lavage. We chose a pulmonary challenge for the in vivo antibody experiment instead of the systemic one, because the duration of the lethality experiment in vivo (up to 12–14 d) would require frequent injections of large amounts of anti-SCARF1 antibodies injected i.v., complicated by spleen- or liver-mediated first-pass clearance of the antibodies. In contrast, the lung offers a relatively contained space that

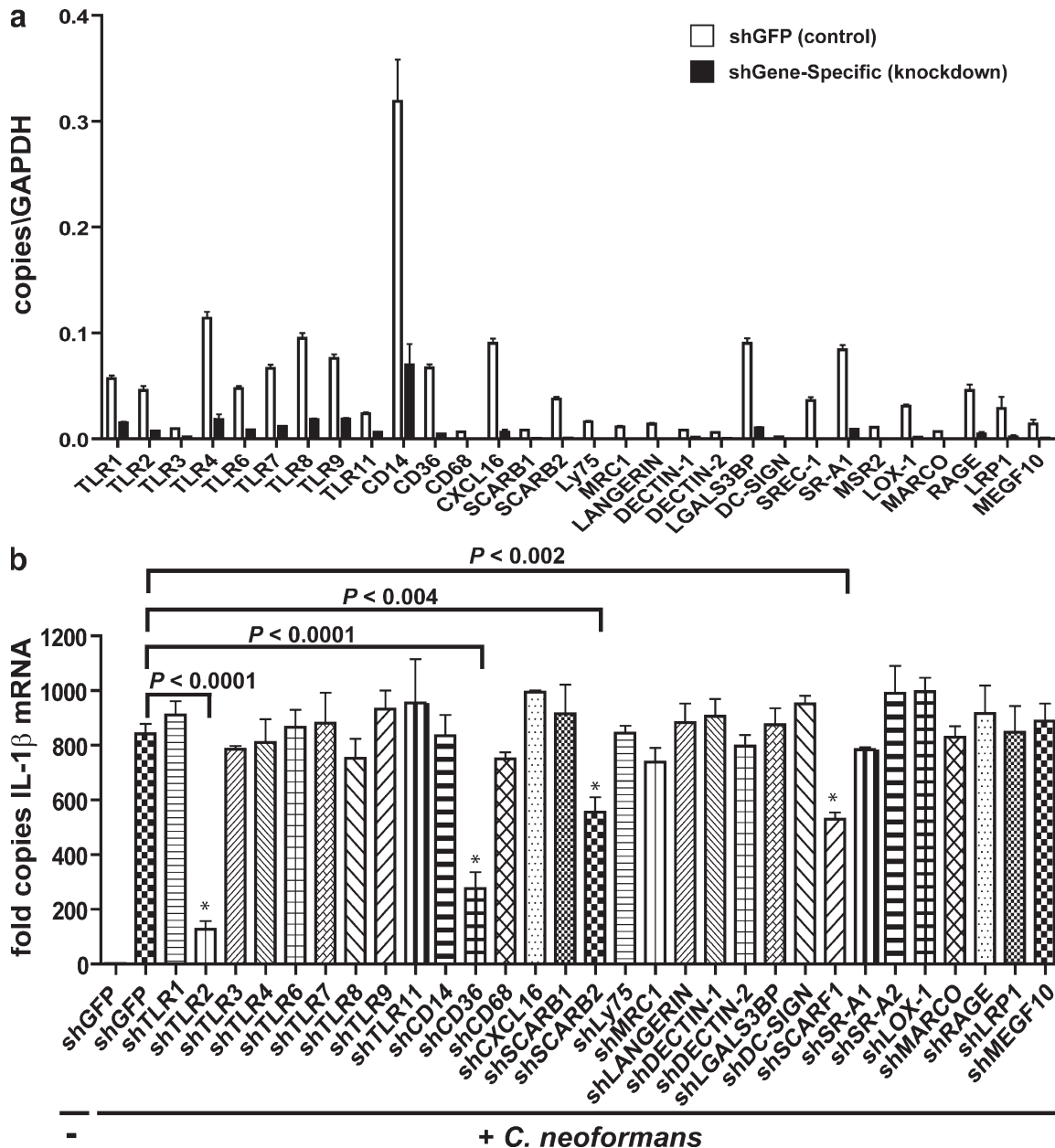


Figure 2. Expression and shRNA knockdown of pattern recognition receptors on RAW mouse macrophages stimulated with *C. neoformans*. (a) RAW macrophages were transduced with lentivirus encoding gene-specific shRNAs to 30 pattern recognition receptors or with a control shRNA directed against GFP. Gene expression was measured by QPCR, and three to five individual shRNAs per gene were identified that significantly reduced mRNA expression. (b) Pattern recognition receptor expression was knocked down in individual pools of RAW macrophages, which were then stimulated with *C. neoformans*. After 4 h, total RNA was isolated from the cells, and the expression of IL-1 β was measured by QPCR. Data are expressed as means \pm SEM of three independent experiments. *, $P < 0.007$ versus control using the Student's t test.

allows performance of the experiment within hours, before diffusion and/or clearance of the antibodies. We found that *C. neoformans* binding in vivo to alveolar macrophages was in-

hibited $\sim 32\%$ by the presence of anti-SCARF1 antibodies (Fig. 4 c). These data indicate that SCARF1 is involved in the recognition of fungal pathogens in vivo.

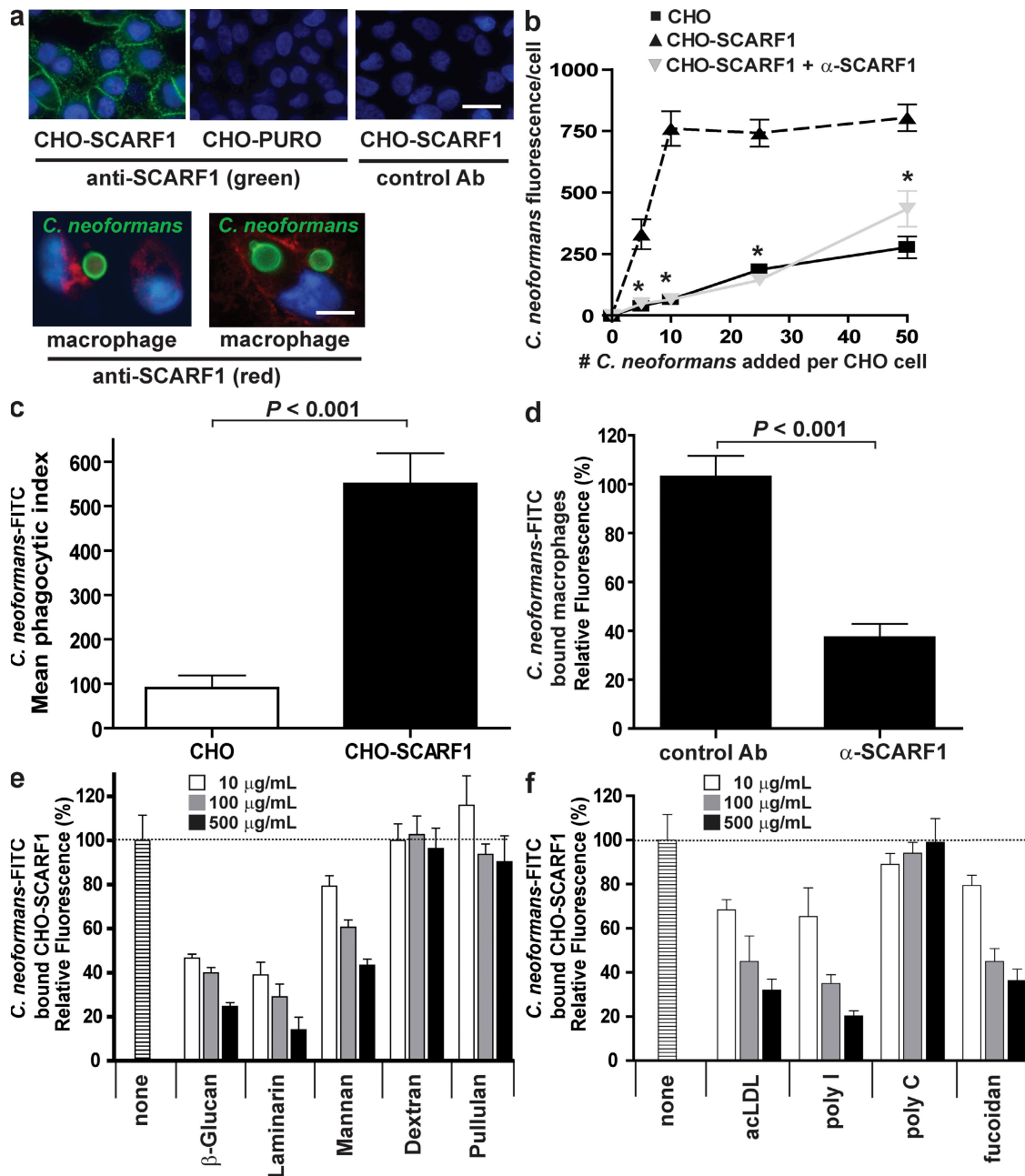


Figure 3. SCARF1 mediates binding to *C. neoformans*. (a, top) CHO cells transfected with the CED-1 orthologue SCARF1 (CHO-SCARF1), but not vector transfected cells (CHO-PURO), stain with fluorescently labeled anti-SCARF1 antibodies (green). CHO-SCARF1 cells do not stain with control antibodies. (bottom) Peritoneal macrophages were incubated with FITC-labeled *C. neoformans* (green) and stained with anti-SCARF1 antibodies (red). Nuclei were stained with DAPI (blue). Bars: (top) 10 μ m; (bottom) 1 μ m. (b) CHO-SCARF1 cells bind unopsonized fluorescently labeled *C. neoformans*, as measured by flow cytometry. Anti-SCARF1 antibodies block binding of unopsonized *C. neoformans* to CHO-SCARF1. *, $P < 0.01$ versus CHO-SCARF1. (c) Flow cytometry measurement of cell internalization of *C. neoformans* using the Trypan blue quenching assay described in Materials and methods. *C. neoformans* internalization is increased fivefold in SCARF1-transfected CHO cells relative to mock transfected cells. (d) Anti-SCARF1 but not isotype control antibodies block binding of unopsonized *C. neoformans* to human monocyte-derived macrophages, as measured by flow cytometry. (e and f) CHO-SCARF1 cells were pretreated with 10, 100, or 500 μ g/ml of carbohydrates or other classical scavenger receptor ligands before the addition of fluorescently labeled *C. neoformans* (20 per cell). *C. neoformans* binding was quantified on a fluorescent microplate reader and is expressed relative to an uninhibited control. Background binding of *C. neoformans* to control CHO cells was normally ~ 15 – 20% . Dotted lines indicate the 100% level. Data are representative of at least three independent experiments. Means \pm SEM are shown.

Because fungal pathogens share several cell-wall molecules, we tested whether SCARF1 could mediate binding to other fungal pathogens. We found that CHO-SCARF1 cells bind *C. albicans* in a β -glucan-dependent manner (Fig. 4 d). As expected, control CHO cells bound a negligible number of organisms. Therefore, CED1/SCARF1 are involved in binding and the innate response to *C. neoformans* and *C. albicans*. These data demonstrate that these receptors are components of a previously uncharacterized, evolutionarily conserved pathway for fungal pathogen recognition and innate immunity.

CD36 but not TLR2 expression is required for *C. neoformans* cell binding

CD36 is a class B scavenger receptor that is a sensor for endogenous molecules (i.e., β -amyloid and modified LDL) and

microbial products (i.e., bacterial diacylated lipoproteins and lipoteichoic acid) that signal via TLR2. As shown in Fig. 2 b, down-regulation of *CD36* or *TLR2* expression by shRNA in RAW macrophages significantly reduced cytokine induction in response to *C. neoformans*. These results suggest that, like its *C. elegans* orthologue C03F11.3, CD36 is also involved in the interaction of mammalian macrophages with fungal pathogens. To test whether mammalian CD36 also mediates binding to yeast pathogens, we used CHO cells expressing CD36 (CHO-CD36) and found that these cells gained the ability to bind *C. neoformans* in contrast to control CHO cells (Fig. 5 a). Because TLR2 expression on macrophages has previously been shown to be required for *C. neoformans*-induced cytokine production (9, 11, 37), we generated CHO cells stably expressing human CD36, TLR2, or CD36/TLR2. These

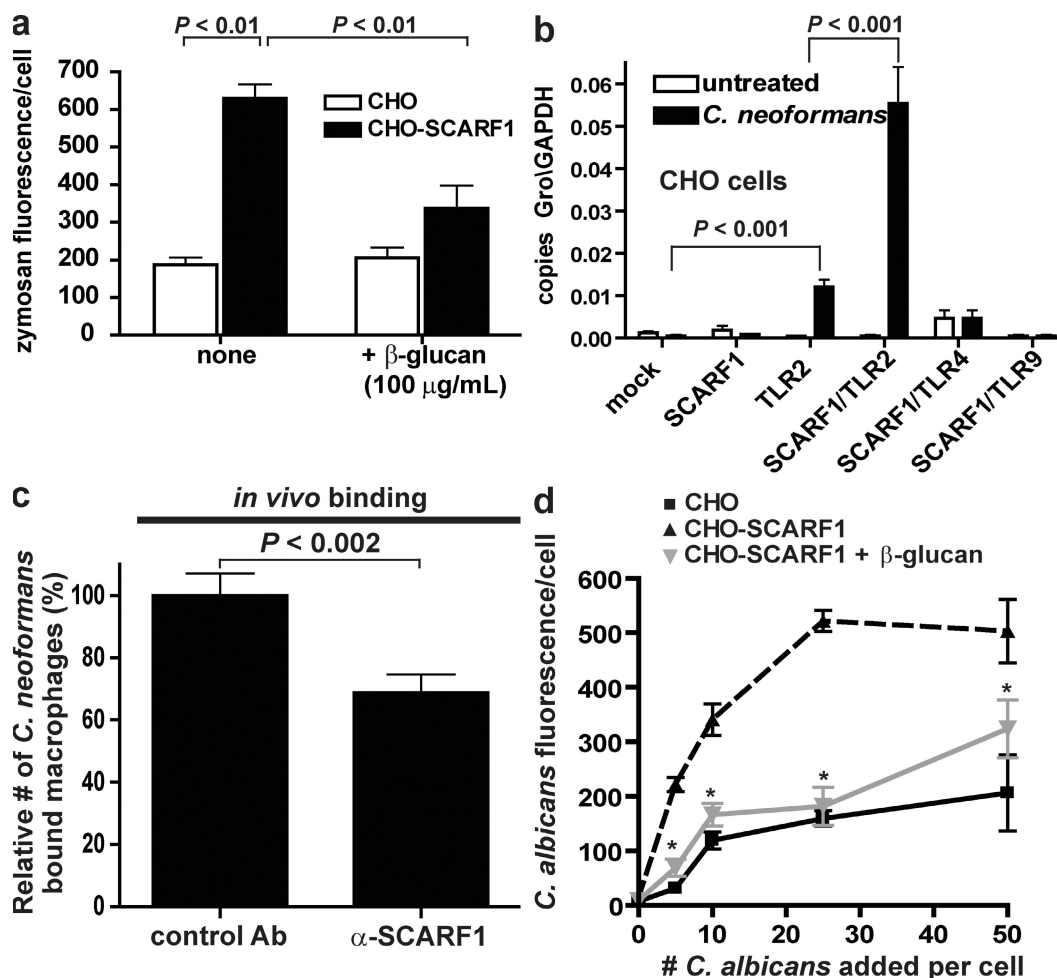


Figure 4. SCARF1 mediates binding of β -glucan and *C. neoformans* in vitro and in vivo, and cooperates with TLR2 to mediate cell activation. (a) 50 μ g/ml of FITC-labeled zymosan was added to CHO and CHO-SCARF1 cells in the presence or absence of 100 μ g/ml of unlabeled β -glucan. Zymosan binding was quantified on a fluorescent microplate reader. (b) Total RNA was isolated from 10^6 SCARF1, TLR2, SCARF1/TLR2, SCARF1/TLR4/MD2, SCARF1/TLR9, or control CHO cells stimulated with *C. neoformans* at a ratio of 20:1 for 4 h. Expression of Gro was determined by QPCR and is depicted as the number of copies of Gro mRNA per copies of the control *GAPDH* mRNA. (c) Fluorescently labeled *C. neoformans* was instilled in the lungs of WT mice ($n = 8$) in the presence of anti-SCARF1 or isotype control antibodies. 2 h later, alveolar macrophages were harvested and analyzed for *C. neoformans* binding by flow cytometry. (d) Binding of fluorescently labeled *C. albicans* to CHO and CHO-SCARF1 cells was measured by flow cytometry in the presence or absence of 100 μ g/ml β -glucan. Data are representative of at least three independent experiments. Means \pm SEM are shown. *, $P < 0.01$ versus CHO-SCARF1.

cells were then tested for their ability to bind unopsonized *C. neoformans*. We found that CD36 but not TLR2 expression was required for optimal binding of *C. neoformans* (Fig. 5 b). In addition, we found that a CD36-Fc fusion protein, but not a control RAGE-Fc fusion protein or neutralizing antibodies to TLR2, inhibited *C. neoformans* cell binding to CHO/CD36/TLR2-expressing cells (Fig. 5 c). These results demonstrate that CD36 but not TLR2 expression is required for binding *C. neoformans*.

Recently, CD36 was also shown to act as a coreceptor for TLR2 in mediating responses to diacylated lipoproteins and lipoteichoic acid isolated from *S. aureus* (38–40). In addition, TLR2 has been shown to collaborate with Dectin-1 to mediate responsiveness to β -glucans and live fungal pathogens (9, 11). To determine the relative contribution of CD36 and TLRs to *C. neoformans*-induced cell activation, we stimulated CHO cells expressing CD36, TLR2, CD36/TLR2, CD36/TLR4, or CD36/TLR9 with *C. neoformans* and measured induction of the mRNA for the chemokine Gro in these cells (Fig. 5 d). Expression of TLR2 alone but not of CD36 led to a modest up-regulation of Gro in transfected CHO cells stimulated with *C. neoformans*. Interestingly, coexpression of CD36 and TLR2 dramatically increased Gro mRNA expression in response to *C. neoformans* (Fig. 5 d). In contrast, coexpression of CD36 with TLR4 or TLR9 did not affect *C. neoformans* activation of these cells. These data indicate that CD36 and TLR2 but not TLR4 or TLR9 collaborate in mediating the innate immune response to yeast infection.

To further characterize the role of CD36 in the recognition of *C. neoformans*, we examined the ability of several fungal-derived carbohydrates, and the well-established scavenger receptor ligands modified LDL and fucoidan, to inhibit the binding of fluorescently labeled *C. neoformans* to CHO-CD36 cells. β -Glucan, laminarin, fucoidan, and modified LDL inhibited CD36 binding to *C. neoformans* (Fig. 5 e). In contrast, the addition of mannan, dextran, or pullulan had minimal or no effect. These results suggest that binding of *C. neoformans* to CD36 is mediated in part by the recognition of β -glucans. To determine if CD36 directly binds β -glucan, we examined the ability of CHO cells expressing CD36 to bind zymosan. We found a 4.2-fold increase in zymosan binding to CD36 over mock-transfected CHO cells (Fig. 5 f). The binding of fluorescently labeled zymosan to CD36-expressing CHO cells was inhibited by addition of unlabeled β -glucan (Fig. 5 f). Collectively, these results demonstrate that binding of *C. neoformans* by CD36 is mediated in part by the recognition of β -glucans.

CD36 expression on macrophages is required for *C. neoformans* and *C. albicans* binding and cytokine production

To determine whether CD36 expression mediates binding to *C. neoformans* on primary cells, we isolated macrophages from WT and CD36^{-/-} mice and stimulated them with *C. neoformans* in vitro. We found that CD36-deficient macrophages had a marked reduction in binding to *C. neoformans* (Fig. 6, a and b). In addition, we found that CD36-deficient macro-

phages showed a 50–60% impairment in the cellular internalization of *C. neoformans* relative to WT macrophages, indicating that CD36 mediates internalization of pathogenic yeast (Fig. 6 c). We also found that a CD36-Fc fusion protein, but not a control fusion protein encoding RAGE-Fc, inhibited macrophage binding to *C. neoformans* (Fig. 6 d). Furthermore, neutralizing antibodies to TLR2 had no effect on macrophage binding to *C. neoformans*. These data indicate that CD36, but not TLR2 or RAGE, is required for optimal binding of *C. neoformans* to macrophages.

To determine if CD36 expression on macrophages mediates *C. neoformans*-induced cytokine production, we stimulated macrophages isolated from WT and CD36^{-/-} mice and measured IL-1 β expression by quantitative real-time PCR (QPCR). As a control, we isolated macrophages from RAGE^{-/-}, SR-A^{-/-}, and LOX-1^{-/-} mice. We found that CD36 expression on macrophages was required for *C. neoformans*-induced IL-1 β expression, whereas macrophages from RAGE^{-/-}, SR-A^{-/-}, and LOX-1^{-/-} mice responded similarly to WT macrophages (Fig. 6 e). Moreover, we found that *C. neoformans* induced the expression of IL-1 β , TNF, IL-12_{p40}, IFN- γ , MIP-2, MIP-1 α , MIP-1 β , RANTES, and IP-10 from WT macrophages (Fig. 6 f). In contrast, CD36^{-/-} macrophages stimulated with *C. neoformans* had a significant reduction in the expression of IL-1 β , TNF, IL-12_{p40}, MIP-2, MIP-1 α , MIP-1 β , and RANTES, whereas the induction of IFN- γ and IP-10 was similar to WT macrophages (Fig. 6 f). TLR2 ligation is not required for induction of the IFN pathway or production of IFN-induced chemokines such as IP-10. In this context, because CD36 appears to be collaborating with TLR2 in mediating *C. neoformans* responses, our results are not surprising and support the published literature (41, 42). To determine whether the increases in mRNA levels were accompanied by protein production, we stained WT and CD36^{-/-} macrophages stimulated with *C. neoformans* with fluorescently labeled anti-TNF and anti-MIP-1 α antibodies, and measured protein expression of these two cytokines by intracellular flow cytometry. The results were consistent with the mRNA data. *C. neoformans* stimulation induced MIP-1 α and TNF protein production in WT macrophages but not in CD36^{-/-} macrophages (Fig. 6 g). In contrast, bacterial LPS induced the production of MIP-1 α and TNF equally in WT and CD36^{-/-} macrophages (unpublished data). These data show that CD36, but not SR-A, RAGE, or LOX-1, is a major macrophage receptor mediating macrophage activation by *C. neoformans*.

To determine the relative contribution of CD36 and TLR2 expressed on primary WT macrophages in the response to *C. neoformans*, we stimulated these cells in the presence or absence of recombinant CD36-Fc and/or neutralizing anti-TLR2 antibodies. As a control, WT macrophages were stimulated with *C. neoformans* in the presence of RAGE-Fc or isotype control antibody. CD36-Fc and anti-TLR2 individually blocked ~45–55% of *C. neoformans*-induced IL-1 β expression. Combining both inhibitors blocked >75% of IL-1 β induction (Fig. 6 h). In contrast, control antibody and

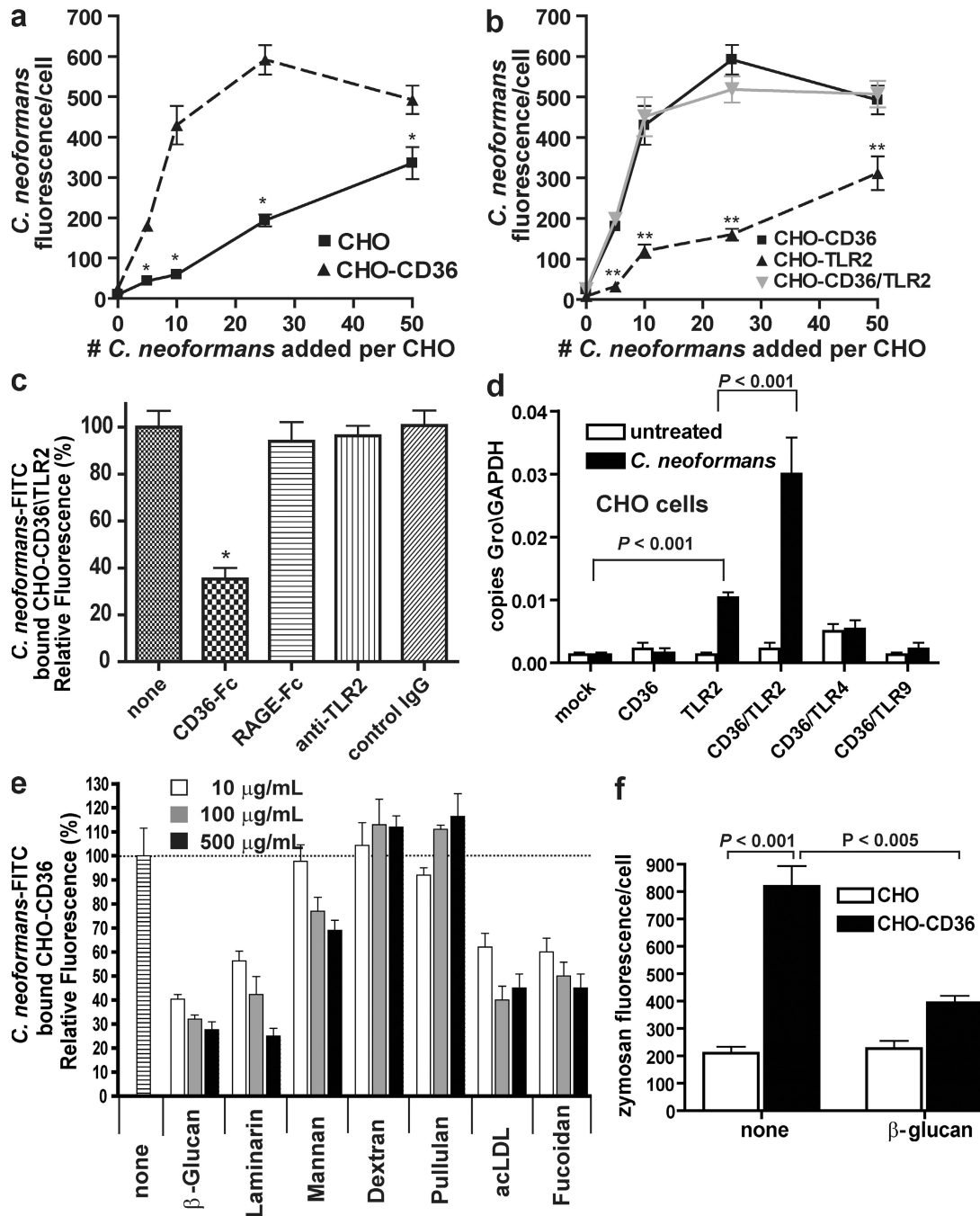


Figure 5. CD36 is required for *C. neoformans* cell adhesion, and TLR2 cooperates with CD36 to mediate *C. neoformans*-induced cell activation. (a and b) Fluorescently labeled *C. neoformans* were incubated with CHO-CD36, CHO-TLR2, CHO-CD36/TLR2, or control CHO-NEO cells for 1 h, washed, and subjected to flow cytometry. *, $P < 0.01$ versus CHO; **, $P < 0.01$ versus CHO-CD36. (c) Fluorescently labeled *C. neoformans* were incubated with CHO-CD36/TLR2 cells in the presence or absence of 25 μ g/ml CD36-Fc, 25 μ g/ml RAGE-Fc, 50 μ g/ml anti-TLR2, or 50 μ g/ml of control IgG, as indicated. *C. neoformans* binding was quantified on a fluorescent microplate and is expressed relative to an uninhibited control. *, $P < 0.001$. (d) Total RNA was isolated from 10^6 CD36, TLR2, CD36/TLR2, CD36/TLR4, CD36/TLR9 or control CHO cells stimulated with *C. neoformans* at a ratio of 20:1 for 4 h. Expression of Gro was determined by QPCR and is depicted as the number of copies of Gro mRNA per copies of the control GAPDH mRNA. Data are representative of four similar experiments. (e) CHO-CD36 cells were pretreated with 10, 100, or 500 μ g/ml of carbohydrates, fucoidan, or acLDL before addition of fluorescently labeled *C. neoformans* (20 per cell). *C. neoformans* binding was quantified on a fluorescent microplate reader and is expressed relative to an uninhibited control. Background binding of *C. neoformans* to control CHO cells was normally ~ 15 –20%. The dotted line indicates the 100% level. (f) 50 μ g/ml of FITC-labeled zymosan was added to CHO and CHO-CD36 cells in the presence or absence of 100 μ g/ml of unlabeled β -glucan. Zymosan binding was quantified on a fluorescent microplate reader. Data in e and f are representative of four independent experiments with similar results. Means \pm SEM are shown.

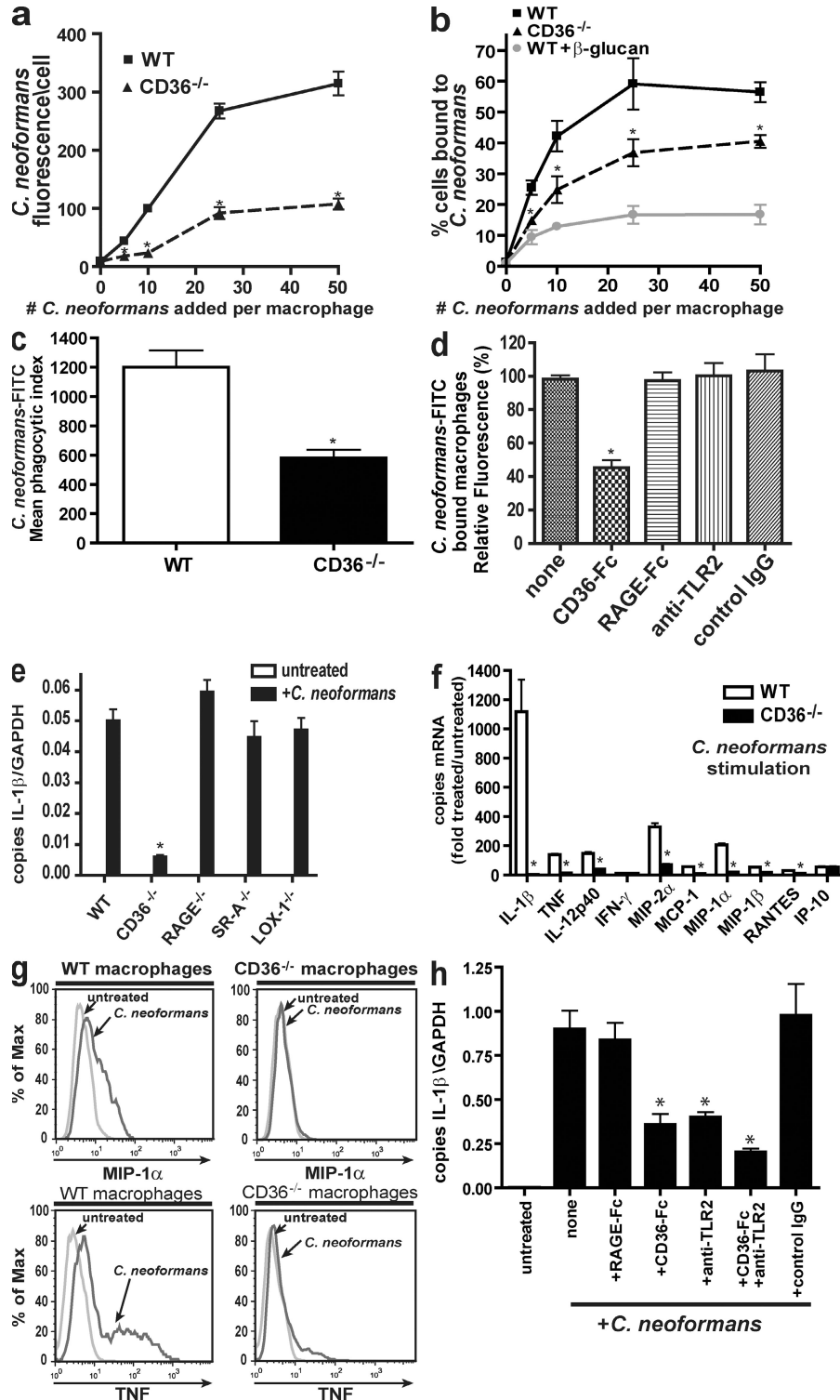


Figure 6. CD36 expression mediates macrophage recognition of *C. neoformans*. (a and b) Fluorescently labeled *C. neoformans* were incubated with macrophages isolated from WT and CD36^{-/-} mice for 1 h and washed; cell association was measured by flow cytometry. *, P < 0.01 versus WT. (c) Flow cytometry measurement of cell internalization of *C. neoformans* using the Trypan blue quenching assay described in Materials and methods. *C. neoformans* internalization was increased threefold in CD36-transfected CHO cells relative to mock transfected cells. *, P < 0.001. (d) Fluorescently labeled *C. neoformans* were incubated with WT mouse macrophages in the presence or absence of 25 μ g/ml CD36-Fc, 25 μ g/ml RAGE-Fc, 50 μ g/ml anti-TLR2, or 50 μ g/ml of control IgG, as indicated. *C. neoformans* binding was quantified on a fluorescent microplate and is expressed relative to an uninhibited control. *, P < 0.001. (e and f) Total RNA was extracted from 10⁶ macrophages isolated from WT, CD36^{-/-}, RAGE^{-/-}, SR-A^{-/-}, and LOX-1^{-/-} mice stimulated with

RAGE-Fc had no effect on *C. neoformans*-induced IL-1 β expression. These data confirm our shRNA data and demonstrate that expression of both CD36 and TLR2 are necessary for maximal responsiveness to *C. neoformans*.

To test whether CD36 could recognize other fungal pathogens, we stimulated WT and *CD36*^{-/-} macrophages with *C. albicans* and measured cytokine and chemokine induction by QPCR. We found that *C. albicans* induced the expression of IL-1 β , TNF, IL-12_{p40}, IFN- γ , MIP-2, MIP-1 α , MIP-1 β , RANTES, and IP-10 from WT macrophages (Fig. S5 a, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>). In contrast, *CD36*^{-/-} macrophages stimulated with *C. albicans* had a marked reduction in the expression of IL-1 β , TNF, IL-12_{p40}, MIP-2, MIP-1 α , MIP-1 β , and RANTES, whereas the induction of IFN- γ and IP-10 was similar to WT macrophages (Fig. S5 a). We also found that CHO-CD36 cells bind *C. albicans* in a β -glucan-dependent manner (Fig. S5 b). Finally, we found that CD36-deficient macrophages showed an \sim 50% reduction in binding *C. albicans* relative to WT macrophages (Fig. S5 c). Collectively, these data demonstrate that *C. elegans* C03F11.1 and its mammalian orthologue CD36 are components of a previously uncharacterized, evolutionarily conserved pathway for fungal pathogen recognition.

Effects of CD36 deficiency after in vivo infection with *C. neoformans*

To study the role of CD36 in fungal infections in vivo, WT and *CD36*^{-/-} mice were infected i.v. with the virulent *C. neoformans* strain H99. The i.v. route of infection was used because it mimics the hematogenous spread observed when the organism disseminates, and because it is more reproducible and easier to accurately inoculate the same amount into each mouse. To assess the course of *C. neoformans* infection, the spleen and liver were harvested from WT and *CD36*^{-/-} mice on day 5 after i.v. infection. *CD36*^{-/-} mice displayed significantly increased numbers of CFUs in the spleen and liver at day 5 compared with WT mice (Fig. 7, a and b). To visualize the number of yeast in the liver samples, we stained liver sections with calcofluor. We found significantly more *C. neoformans* in liver sections from *CD36*^{-/-} mice compared with WT mice (Fig. 7, c and d), confirming results obtained from our microbiological assay.

To further confirm the yeast CFU counts, we designed primers to the cryptococcus capsule A gene and determined the number of copies in each organ by QPCR. The cryptococcal capsule plays an important role in the pathogenesis of cryptococcal infection, and expression of this mRNA reflects

the presence and viability of the organism (43). As expected, mRNA expression of the capsule A gene paralleled the number of CFU counts (Fig. 7, e and f).

To test whether the fungal load differences seen in WT and *CD36*^{-/-} mice correlated with the induction of proinflammatory mediators, we determined the level of cytokines and chemokines induced in the spleen and liver 5 d after i.v. infection with *C. neoformans*. We found that several chemokines (MIP-2, MCP-1, MIP-1 α , and MIP-1 β) and cytokines (IL-1 β , TNF, and IL-12_{p40}) were induced in the organs of *C. neoformans*-infected WT mice (Fig. 7, g and h). In contrast, all of these cytokines were markedly reduced in the organs of *CD36*^{-/-} mice (Fig. 7, g and h). To analyze whether loss of CD36 expression affects the susceptibility of mice to *C. neoformans*, we infected WT and *CD36*^{-/-} mice i.v. with *C. neoformans*. We found that *CD36*^{-/-} mice succumbed to *C. neoformans* (2.5×10^5 CFU) infection significantly faster than WT mice (Fig. 7 i). When lower doses (10^5 CFU) of *C. neoformans* were used, similar results were obtained. However, at lower doses of *C. neoformans*, 100% mortality in the *CD36*^{-/-} mice was delayed by 5 d compared with the higher doses. In all experiments, we found that *CD36*^{-/-} mice succumbed at a significantly faster rate than WT mice regardless of the dose. Of note, expression of the other known β -glucan receptors Dectin-1, Dectin-2, mannose receptor, and TLR2 were not affected in *CD36*^{-/-} macrophages (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>). Collectively, these data show that CD36 expression is required for *C. neoformans*-induced cytokine and chemokine production in vivo, and demonstrate that CD36 is a cellular receptor for strains of *C. neoformans* that are pathogenic to humans and mice, and plays a crucial role in the host response to this organism.

DISCUSSION

Opportunistic yeasts cause significant morbidity and mortality in patients with impaired immune systems. Several yeast cell-wall proteins are recognized by the innate immune system in mice and humans; however, the molecular mechanisms and receptors used by immune cells to bind and trigger cell activation have not been fully elucidated. In this paper, we show that the *C. elegans* receptors CED-1 and C03F11.3, and their mammalian orthologues, the scavenger receptors SCARF1 and CD36, mediate host defense against pathogenic yeast. In the absence of CED-1 or C03F11.1, nematodes succumb much faster to fungal infection and fail to induce the production of antimicrobial peptides. In mammalian cells, SCARF1 and CD36 mediate binding and the subsequent induction of inflammatory cytokines and chemokines after exposure to

C. neoformans at a ratio of 20:1 for 4 h. Expression of cytokines and chemokines was determined by QPCR and is depicted as the number of copies of mRNA per copies of the control GAPDH mRNA (e) or fold treated over untreated (f). *, $P < 0.001$. (g) WT and *CD36*^{-/-} macrophages were stimulated with *C. neoformans* (20:1) for 6 h. Protein expression of the cytokine TNF and the chemokine MIP-1 α was determined by intracellular flow cytometry. (h) Total RNA was isolated from WT macrophages (10^6 cells) stimulated with *C. neoformans* at a ratio of 20:1 for 4 h in the presence or absence of CD36-Fc, RAGE-Fc, anti-TLR2, or control IgG. Expression of IL-1 β was determined by QPCR and is depicted as the number of copies of IL-1 β mRNA per copies of the control mRNA GAPDH. Data are representative of four similar experiments. Means \pm SEM are shown. *, $P < 0.001$.

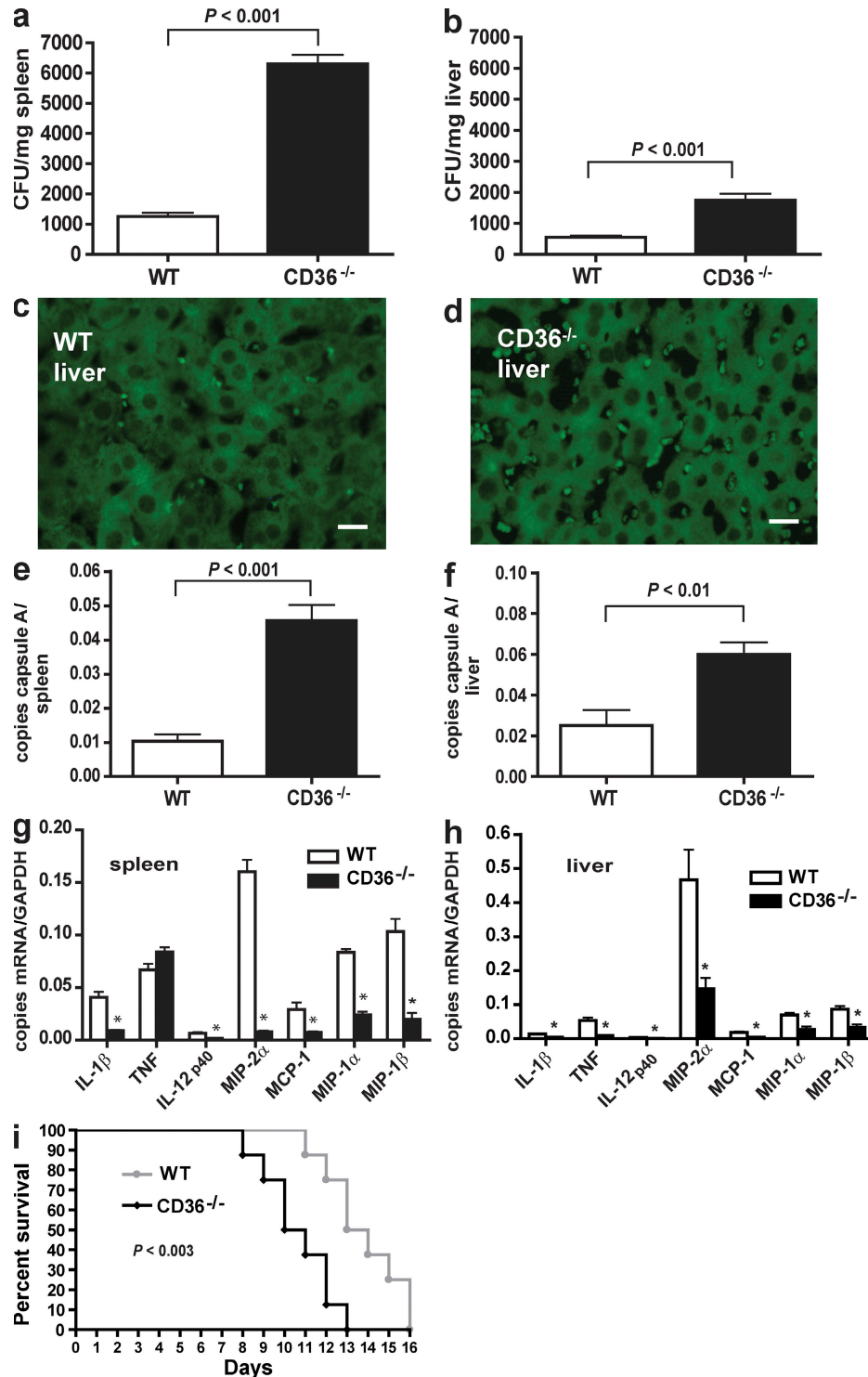


Figure 7. Fungal burden, cytokine expression, and mortality of WT and CD36^{-/-} mice after infection with *C. neoformans*. (a and b) WT and CD36^{-/-} mice were infected i.v. with 10⁵ *C. neoformans* organisms. Mice were killed 5 d after infection, and the numbers of CFUs in the spleen and liver were determined. Data are expressed as CFU per milligram of tissue. The figure represents the combined results of two independent experiments, and the results are expressed as means \pm SEM of eight mice per group. p-values are as indicated (Student's *t* test). (c and d) WT and CD36^{-/-} mice were infected i.v. with 10⁵ *C. neoformans* organisms. Mice were killed 5 d after infection, and 7- μ m liver sections were fixed in 100% methanol for 5 min and stained with a Fungi-Fluor kit. Photos were taken using a Nikon DXM 1200C camera and Nis-Elements AR software. Bars, 5 μ m. (e and f) Total RNA was extracted from a portion of the spleen and liver isolated from WT and CD36^{-/-} mice infected i.v. with 10⁵ *C. neoformans* organisms 5 d after infection. The expression of the cryptococcus capsule A gene was determined by QPCR. p-values are as indicated (Student's *t* test). (g and h) QPCR was used to measure the

C. neoformans and *C. albicans*. Furthermore, binding of *C. neoformans* and *C. albicans* to SCARF1 and CD36 is β -glucan dependent. The role of CD36 in these responses appears to be nonredundant in vivo, because the number of yeast cells in the spleen and liver of *C. neoformans*-infected $CD36^{-/-}$ mice were significantly higher than WT mice, and $CD36^{-/-}$ mice died at an accelerated rate after *C. neoformans* infection, indicating an essential role of CD36 in controlling the infection. Furthermore, the expression of proinflammatory cytokines in the organs of *C. neoformans*-infected $CD36^{-/-}$ mice were significantly reduced compared with WT mice. Collectively, these data demonstrate that the scavenger receptors SCARF1 and CD36 are nonredundant components of an evolutionarily conserved pathway for fungal recognition and innate immunity that is necessary for controlling fungal infections in vivo. SCARF1 and CD36 do not appear to signal on their own in response to fungal binding; instead, they collaborate with TLR2 but not TLR4 or TLR9 for induction of cytokines. It is possible that collaboration of different scavenger receptors with different TLRs is determined by the ligand at hand. Different ligands may trigger different collaborations between these two classes of pattern recognition receptors.

An important aspect of our findings is reflected by the role of *CED-1* and *C03F11.3* genes in *C. elegans*. We have previously established *C. elegans* as a simple model host in which yeast pathogenesis can be studied. *CED-1* is a phagocytic receptor known to mediate the engulfment of dying cells. Our studies identify a novel role for *CED-1* in host defense. Nematodes deficient in *CED-1* are more rapidly killed by the yeast pathogen *C. neoformans* and the Gram-negative bacteria *S. typhimurium* than WT worms, and fail to produce the antimicrobial peptides *abf-1* and *abf-2* in response to these infections. To our knowledge, this is the first demonstration of a role for *CED-1* in fungal recognition and host defense. Recently, Haskins et al. demonstrated that *CED-1* was required for the survival of *C. elegans* after infection with the human pathogen *S. enteric* strain 1344 (44). Collectively, these data demonstrate that *CED-1* is critical in the *C. elegans* immune response to live yeast and Gram-negative bacteria, and confirm the role of this receptor as an innate immune receptor. *CED-1* does not appear to act alone as an innate immune receptor, as *C03F11.3* also plays a nonredundant role in the nematode response to fungal infection. We are currently investigating if these two receptors play complementary roles in host defense against fungal as well as bacterial pathogens. Our data show that there is a remarkable similarity between the role of *C. elegans* and mammalian scavenger receptors in innate immunity. Indeed, just as *C. elegans* scavenger receptors are necessary for antimicrobial peptide production, mammalian scavenger receptors are required for cytokine production in response to fungal pathogens.

Identifying the receptors responsible for pathogen recognition is essential to understanding the innate immune system. Several classes of pathogen recognition receptors have now been described, each capable of recognizing and responding to conserved microbial structures. Scavenger receptors are cell-surface proteins present on a variety of cell types. Although scavenger receptors have been primarily studied for their ability to bind endogenous modified LDL and β -amyloid, they also recognize and respond to pathogens (19, 20). Scavenger receptors recognize a myriad of microbial structures, including LPS, lipoteichoic acid, DNA, and RNA. A role for scavenger receptors in the recognition of fungal pathogens has not been established. Recently, Rice et al. found indirect evidence for the involvement of scavenger receptors in the innate sensing of fungal pathogens. They showed that acLDL, a common ligand for many scavenger receptors, inhibits β -glucan binding to monocytes (45). Our data show that SCARF1 and CD36, both of which bind acLDL, serve a nonredundant role as receptors for β -glucans. Interestingly, although the role of other β -glucan receptors in fungal pathogens in vivo remains unclear (13, 15), our data indicate that SCARF1 and CD36 play important roles in vivo in controlling fungal infection.

In our shRNA screen, we also found that TLR2 was required for maximal cytokine and chemokine production from macrophages stimulated with live *C. neoformans*. Several TLRs (TLR2, TLR4, TLR6, and TLR9) have been shown to be involved in triggering macrophage activation by *C. neoformans*, *C. albicans*, or fungal-derived molecules (46–49). In vitro experiments have shown that CD14 cooperates with TLR2 and TLR4 in binding to the major component of the *C. neoformans* capsule, polysaccharide glucuronoxylomannan. However, CD14, TLR2, and TLR4 appear to only play relatively minor roles in vivo in the host response to *C. neoformans* (37, 49). TLR2–6 heterodimers are involved in recognition of zymosan; however, cytokine production in TLR6-deficient macrophages is only moderately reduced in response to zymosan (50). Similar to TLR6, the absence of TLR9 on macrophages results in only a moderate reduction in cytokine production after stimulation with fungal pathogens (51). These data are consistent with our findings that silencing the expression of TLR2, but not TLR4, TLR6, or TLR9, in macrophages resulted in reduced cytokine production in response to live *C. neoformans* stimulation.

Other receptors, including Dectin-1, mannose receptor, Galectin-3, and Mincle, have been shown to bind yeast or to components of its capsule or cell wall (i.e., β -glucan and mannans) (8, 9, 52, 53). Dectin-1 cooperates with TLR2 for the production of inflammatory cytokines in response to β -glucan stimulation (11, 12). However, Nakamura et al. did not find any difference in cytokine production in response to *C. neoformans*

expression of cytokines and chemokines in the spleen and liver of *C. neoformans*-infected mice. Data are representative of three similar experiments using eight mice per group. *, $P < 0.01$. (i) Survival of WT and $CD36^{-/-}$ mice after i.v. infection of 2.5×10^5 *C. neoformans*. Survival was determined as described in Materials and methods, and p-values were determined using the Kaplan-Meier survival statistical test ($P < 0.003$). Data are representative of three similar experiments using eight mice per group.

infection between WT and Dectin-1 KO mice (14). We confirmed these results and found that RAW macrophages silenced for Dectin-1 or Dectin-2 did not exhibit a reduction in cytokine induction after stimulation by *C. neoformans*. Our results are possibly caused by the low expression of these receptors on RAW cells. However, it is likely that CD36 and SCARF1 rather than Dectin-1 play a more prominent role in cytokine production than Dectins in response to fungal pathogens. The mannose receptor binds *C. neoformans* through the recognition of mannosylated proteins present in the fungal cell wall. After *C. neoformans* infection, mannose receptor-deficient mice died significantly faster than WT mice (54). Interestingly, no significant difference in survival between mannose receptor-deficient and WT mice infected with *C. albicans* was observed, and both mice exhibited competence in inflammatory cell recruitment and antibody production (55). Because SCARF1 appears to bind fungal pathogens in a mannan-dependent manner, it is possible that SCARF1 and the mannan receptor play complementary roles in innate immunity to *C. albicans*. The role of scavenger receptors in innate immunity is only beginning to be uncovered. Our data strongly support such a role in response to fungal infections.

MATERIALS AND METHODS

Reagents. The extracellular domain of mouse CD36 was cloned into the Fc fusion mammalian expression vector pFUSE-IgG1-Fc2 (InvivoGen). This plasmid fuses the extracellular scavenger receptor domain to the Fc region of IgG and contains the IL-2 signal sequence to allow for the secretion of the Fc fusion proteins. The construct was transfected into HEK 293 cells and stable high expressing clones were isolated. The Fc fusion protein was purified by a single-step protein G affinity chromatography. RAGE-Fc fusion protein was purchased from R&D Systems. Ultrapure LPS was purchased from InvivoGen. The anti-mouse TLR2 and isotype control antibodies were purchased from eBioscience. All carbohydrates were obtained from Sigma-Aldrich.

Mice. *CD36*^{-/-} mice were generated by targeted gene disruption in mouse embryonic stem cells by homologous recombination (56). *CD36*^{-/-} mice, *CD36*^{+/+} littermate controls, and WT C57BL/6 mice purchased from the Jackson Laboratory were used for the *in vitro* and *in vivo* experiments. Animal protocols were reviewed and approved by the Massachusetts General Hospital subcommittee on research animal care, and conform to the United States Department of Agriculture animal welfare act and the Public Health Service policy on the humane care and use of laboratory animals.

***C. neoformans* and *C. albicans*.** The highly virulent encapsulated *C. neoformans* strain H99 (American Type Culture Collection) and the *C. albicans* strain (American Type Culture Collection) were grown on yeast-peptone-dextrose agar (Difco) at 30°C. For each infection, a fresh culture of *C. neoformans* or *C. albicans* was started from -80°C stocks. For experiments, a fresh colony was isolated from an agar plate and grown in YPD media for 24 h at 30°C. Before inoculation, the yeast cells were washed three times in PBS, counted, and adjusted to the appropriate concentration. *S. typhimurium* was a gift from E. Hohmann (Massachusetts General Hospital, Boston, MA) and was propagated in Brain Heart Infusion media (Difco) or plates.

shRNA lentiviral infections. Plasmids encoding lentiviruses expressing shRNAs were obtained from the RNAi Consortium library TRC-Mm1.0 (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>) (29). Plasmids were purified using a miniprep kit (QiaPrep; QIAGEN). Plasmids were then transfected into HEK 293T cells with a three-plasmid system to produce lentivirus. Infection conditions were optimized in 96-well plates. 20,000 macrophages were placed in each well of 96-well tissue cul-

ture dishes and infected using 5 μ l of shRNA lentiviral supernatant and 7.5 μ g/ml polybrene. The cells were spun for 30 min at 2,000 rpm and incubated for 24 h. To select for shRNA integration, the infected cells were placed in 3 μ g/ml puromycin and RPMI 1640 containing 10% FBS. The cells were tested 1–2 wk after infection.

Infection and survival assays in *C. elegans*. *C. elegans* strain N2 Bristol was maintained at 15°C. All *C. elegans* strains were propagated on *Escherichia coli* strain OP50 using established procedures, as previously described (22, 23, 57–59). *C. elegans* survival assays in the presence of *C. neoformans*, *S. typhimurium*, *S. aureus*, and *E. faecalis* were performed as previously described (22, 23, 57, 58). We performed the *C. elegans* survival assays using males as well as hermaphrodite nematodes, because we have previously observed that some pathogens such as *C. neoformans* and *E. faecalis* can induce the internal hatching of eggs in some hermaphroditic worms, leading to matricide (22, 23, 57, 58). Because no differences were seen between the responses of male worms or hermaphrodites, the differences observed in survival between WT and mutants are caused by infection and not by this potential matricidal effect. For these studies, we used the well characterized CED-1 mutant strains MT4930 and MT4933, obtained from the Caenorhabditis Genetics Center (CGC) database. In some experiments, we used siRNA to down-regulate *C. elegans* scavenger receptor orthologues (Table S2, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>). For the studies examining the potential *C. elegans* SCARB1, SCARB2, and CD36 orthologues, we used three worm strains from the CGC database: RB1227 (mutant for the *Y49E10.20/SCARB1* gene), RB973 (mutant for the *Y76A2B/SCARB2* gene), and RB1409 (mutant for the *R07B1.3/CD36* gene). The other three genes for the remaining CD36 orthologues (*C03F11.3*, *F11C1.3*, and *F07A5.3*), for which mutants did not exist, were down-regulated by siRNA (Table S2). *C. elegans* strain L4440 was used as the control strain for the siRNA experiments, and the N2 strain was used as the control for the mutant strains. Each survival experiment was repeated using at least 100 nematodes divided in 3 different plates per strain. Data shown are from a representative experiment. Each experiment was repeated three times with similar results.

***In vivo* infections.** For *i.v.* infections, the mice received 0.1 ml containing 100,000 yeast cells resuspended in PBS, injected in the lateral tail vein. The actual number of CFUs administered was confirmed using colony plate counts. For assessment of lethality, WT and *CD36*^{-/-} mice were injected with *C. neoformans* and monitored every 12 h for moribund state or death. Moribund animals were killed by CO₂ asphyxiation.

***C. neoformans* staining.** 7- μ m frozen liver sections were fixed in 100% methanol for 5 min. Staining was performed using a Fungi-Fluor kit (Polysciences) according to the manufacturer's protocol. Sections were rinsed and stained for 30 s in hematoxylin. Sections were mounted using Vectashield (Vector Laboratories). Photos were taken at 20 \times using a camera (DXM 1200C; Nikon) and AR software (Nis-Elements).

Cloning of human SCARF1 and generation of stable CHO-SCARF1 cell lines. Human SCARF1 was cloned from a human microvascular endothelial cell-derived cDNA library in the PEAK-8 vector (Edge Biosystems) using an expression cloning strategy. In brief, the cDNA library was divided into pools of 5,000 individual cDNA clones, and each pool was transfected into HEK 293 cells. Cells that bound fluorescently labeled acLDL were selected and individual clones were identified (17). One of these clones had 100% homology to the published sequence of human SCARF1 (available from GenBank/EMBL/DDBJ under accession no. NM_003693). Stably transfected CHO-SCARF1 and CHO-PURO cells were generated by transfecting SCARF1 cDNA or the empty PEAK8 vector, and selecting high expressors by culturing in 2 μ g/ml puromycin and carrying out FACS of the surviving cells.

Immunofluorescence staining. Anti-SCARF1 rabbit polyclonal antibodies were generated against the synthetic peptide RGTQGSELDPKGQHVC from the human extracellular domain of SCARF1. CHO or CHO-SCARF1

cells, or mouse peritoneal macrophages were plated on multispot glass slides and incubated with anti-SCARF1 or control antibodies for 2 h (1:500 dilution). FITC- or rhodamine-labeled donkey anti-rabbit antibodies were added, and the cells were visualized by fluorescence microscopy (Eclipse ME600; Nikon) and digitally photographed (DXM 1200C camera). Nuclei were stained with DAPI (blue).

Yeast binding assays. Yeast cells were labeled with FITC according to published protocols (20). FITC-labeled yeast cells were added in serum-free medium at an organism/cell ratio of 1:1, 5:1, 10:1, 25:1, or 50:1, and binding was allowed to occur for 1–2 h at 4°C. Unbound yeasts were removed by extensive washing, and the cells were fixed in 2% paraformaldehyde for microscopy and digital imaging or detached in divalent cation-free buffer and then fixed in 2% paraformaldehyde for flow cytometry, or fluorescence was measured in a microplate reader (Fluoroskan Ascent FL; Thermo Fisher Scientific). The number of yeast cells that bound to cells was determined as previously described (22, 23, 57–59). For anti-SCARF1, control antibody, Fc-fusion proteins, or carbohydrate competition experiments, the cells were preincubated with these reagents for 30 min, and the yeasts were added for an additional 1–2 h. Data shown are the means of three determinations from a representative experiment. Each experiment was repeated three times with similar results. Statistical analysis was performed using one-way analysis of variance provided in the Prism 4 graphics and statistics software (GraphPad Software, Inc.).

Isolation of primary macrophages and bone marrow-derived macrophages. Thioglycollate-elicited peritoneal macrophages were harvested from 8–12-wk-old CD36^{-/-} and CD36^{+/+} mice, as previously described (20). Cells were confirmed to be >95% macrophages by staining with F4/80, CD11b, CD11c, and anti-GR1 antibodies. Bone marrow-derived macrophages were prepared by culturing the bone marrow isolated from the femur and tibia of mice in 10 ng/ml M-CSF. On day 4, the cells were washed and placed in fresh media with M-CSF. On day 7, the adherent macrophages were detached, counted, and passed into the appropriate size dish for in vitro experiments.

Assessment of *C. neoformans* internalization. Thioglycollate-elicited peritoneal macrophages were harvested as described in the previous section. CHO, CHO-SCARF1, WT, or CD36^{-/-} peritoneal macrophages were plated in 24-well plates at 2×10^5 cells per well in 1 ml DMEM supplemented with 10% fetal calf serum and incubated overnight. The cells were washed with PBS, and labeled *C. neoformans* were added at 20 CFU per macrophage for 2 h at 37°C. Phagocytosis was stopped by the addition of cold PBS. The fluorescence of extracellular yeast was quenched by incubation with PBS containing 1.0 mg/ml Trypan blue for 10 min at room temperature, as previously described (20). Intracellular fluorescence of phagocytosed yeast was measured by flow cytometry using a flow cytometer (FACSCalibur; BD).

Intracellular cytokine staining. Macrophages were stimulated for 30 min with *C. neoformans*. Next, 1 μ l/ml GolgiPlug (BD) was added to the culture media, and after an additional 5.5 h, the macrophages were harvested and resuspended in 4% paraformaldehyde for 10 min, spun down at 450 g, and resuspended in PBS containing 0.1% saponin, anti-mouse MIP-1 α -FITC (R&D Systems), and anti-mouse TNF-PE (eBioscience). 30 min later, the cells were washed and immediately run on a FACSCalibur.

QPCR. Total RNA was extracted using the RNeasy kit according to the manufacturer's protocol (QIAGEN), and each sample was reverse transcribed using multiscribe RT (Applied Biosystems). Oligonucleotide primers were designed using Primer Express software (version 1.0; Applied Biosystems) or Primer Bank (available at <http://pga.mgh.harvard.edu/primerbank/>; Table S3, available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>) (60). The 25- μ l QPCR reaction contained 2 μ l of cDNA, 12.5 μ l of 2 \times SYBR green master mix (Applied Biosystems), and 500 nM of sense and antisense primers. A series of standards was prepared by performing 10-fold serial dilutions of full-length cDNAs in the range of 20 million copies to 2 copies per

QPCR reaction. Subsequent analysis was performed on the data output from the MX4000 software (Agilent Technologies) using Excel 2002 (Microsoft). In this study, we modified a protocol described by Amjad et al. to quantify viable *C. neoformans* in the spleen and liver of *C. neoformans*-infected mice by a two-step QPCR amplification of the capsule (CAP10) gene mRNA (43). The forward primer was CAP10F 5'-TTCTCCTCTCCTGACCGACT-3', and the reverse primer was CAP10R 5'-CTCTTCTTGTACTCGGCAACG-3'. In addition, we used QPCR to measure cell activation of TLR2- and CD36-transfected CHO cells. In preliminary experiments, we found that stimulation of CHO cells induced the activation of NF- κ B and mRNA production of the neutrophil chemoattractant GRO (available from GenBank/EMBL/DBJ under accession no. J03560). The forward primer was CHOGRO1F 5'-GTGCCTGCAGACCATGACAG-3', and the reverse primer was CHOGRO1R 5'-GACTTCGGTTTGGGTGCAGT-3'.

Online supplemental material. Fig. S1 shows the effects of silencing *CED-1* on *C. elegans* survival in response to *C. neoformans* infection, and the effects of *CED-1* deficiency on the survival of *C. elegans* infected with *S. typhimurium* or *S. aureus*. Fig. S2 shows the effects of silencing various *C. elegans* CD36 orthologues and deficiency in two *SCARB1* orthologues on survival after infection with *C. neoformans*. Fig. S3 displays the alignment of *C. elegans* *CO3F11.1* and mouse and human *CD36*. Fig. S4 shows the shRNA knockdown efficiency and specificity for *TLR4*, *TLR7*, and *TLR9*. Fig. S5 shows that CD36 mediates binding of *C. albicans* to macrophages in a β -glucan-dependant manner, and activation of these cells to produce proinflammatory chemokines and cytokines. Fig. S6 shows that targeted deletion of *CD36* does not affect expression of other β -glucan receptors in macrophages. Tables S1 and S2 provide the gene identifiers and shRNA target sequences for *CD36*, *TLR2*, *SCARB2*, *SCARF1*, and their *C. elegans* orthologues. Table S3 contains the sequences of the QPCR primers used in this study. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20082109/DC1>.

T.K. Means and J. El Khoury designed and performed experiments, and wrote the paper. E. Mylonakis, E. Tampakakis, E. Seung, and R. Pukkila-Worley performed *C. elegans* experiments and in vivo *C. neoformans* experiments. L. Puckett performed gene expression and binding experiments, S.E. Hickman performed cell isolations and generated stable cell lines, and K.J. Moore and C.R. Stewart performed *C. neoformans* experiments and provided CD36^{-/-} mice. R.A. Colvin and M.F. Tai performed immunohistochemistry and fluorescence microscopy. N. Hacohen generated and provided the mouse shRNA library and protocols for using the library. S.B. Calderwood and A.D. Luster gave valuable suggestions and discussions regarding the design of the experiments, and helped edit the manuscript.

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