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TLR9 Is Actively Recruited to Aspergillus fumigatus Phagosomes and Requires the N-Terminal Proteolytic Cleavage Domain for Proper Intracellular Trafficking

Pia V. Kasperkovitz,*‡ Michael L. Cardenas,* and Jatin M. Vyas,*‡

TLR9 recognizes unmethylated CpG DNA and induces innate immune responses. TLR9 activation is a multistep process requiring proteolytic cleavage and trafficking to endolysosomal compartments for ligand-induced signaling. However, the rules that govern the dynamic subcellular trafficking for TLR9 after pathogen uptake have not been established. In this study, we demonstrate that uptake of Aspergillus fumigatus conidia induced drastic spatial redistribution of TLR9 to the phagosomal membrane of A. fumigatus-containing phagosomes but not to bead-containing phagosomes in murine macrophages. Specific TLR9 recruitment to the fungal phagosome was consistent using A. fumigatus spores at different germination stages and selected mutants affecting the display of Ags on the fungal cell surface. Spatiotemporal regulation of TLR9 compartmentalization to the A. fumigatus phagosome was independent of TLR2, TLR4, and downstream TLR signaling. Our data demonstrate that the TLR9 N-terminal proteolytic cleavage domain was critical for successful intracellular trafficking and accumulation of TLR9 in CpG-containing compartments and A. fumigatus phagosomal membranes. Our study provides evidence for a model in which A. fumigatus spore phagocytosis by macrophages specifically induces TLR9 recruitment to A. fumigatus phagosomes and may thereby mediate TLR9-induced antifungal innate immune responses. The Journal of Immunology, 2010, 185: 7614–7622.

Mammalian TLRs detect microbial products and initiate immune responses to infection. The different members of the TLR family recognize and signal to a broad range of microbial ligands, such as bacterial and fungal cell wall components, bacterial lipoproteins, and bacterial and viral nucleic acids. The nucleic acid-sensing TLRs, TLR3, 7, 8, and 9, are localized to intracellular compartments, whereas TLR1, 2, 4, 5, and 6 are expressed at the plasma membrane. TLR activation and signal transduction are regulated by subcellular compartmentalization of receptors and downstream signaling components, and the intracellular localization of nucleic acid-sensing TLRs appears to facilitate self versus nonself discrimination.

The subcellular localization of TLR9, which engages and signals to unmethylated CpG DNA, is tightly regulated and receptor activation is a multistep process. TLR9 is translated into the endoplasmic reticulum (ER) in its mature, full-length form and then passes through the Golgi to the endolysosomal compartment where the ectodomain is proteolytically cleaved to generate a functional receptor. In the endolysosomal compartment, ligand binding to preassembled TLR9 dimers induces a conformational change that allosterically initiates signal transduction. While the truncated form of TLR9 can be found in the endolysosomal compartment of unstimulated cells, several reports have indicated that TLR9 trafficking is a highly regulated, dynamic process. The extent to which there is dynamic movement of TLR9 between subcompartments and the underlying processes regulating TLR9 trafficking remain poorly understood.

Although the best known ligand for TLR9 is unmethylated bacterial and viral CpG-rich DNA, TLR9 has also been implicated in antifungal defense. A. fumigatus is a common environmental fungus capable of invasive infection in immunocompromised persons and particularly in patients who have undergone allogeneic hematopoietic stem cell transplantation. Additionally, A. fumigatus spore forms, including the asexual conidia, can exacerbate allergic and asthmatic disease. Evidence for the importance of innate immune mechanisms in fungal defense is mounting. The fungal β-glucan receptor dectin-1 is essential in pulmonary defense against A. fumigatus and collaborates with TLR2 and TLR4 in providing critical immune signals. Although most studies have focused on the importance of TLR2 and TLR4 in defense against A. fumigatus, a polymorphism study associated increased susceptibility to ABPA with a polymorphism in the TLR9 gene, and intranasal CpG had a therapeutic effect during established murine fungal asthma. However, the cell biological processes underlying TLR9-mediated A. fumigatus immune responses are still largely unresolved. TLR9-mediated recognition of A. fumigatus DNA by human and murine cells induced proinflammatory cytokines, but the intracellular processes that enable A. fumigatus Ag recognition by TLR9 in professional APCs remain uninvestigated.

To gain insight into the intracellular fate of TLR9 when immune cells are exposed to A. fumigatus conidia, we investigated the spatiotemporal regulation of TLR9 compartmentalization after

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Abbreviations used in this paper: ABPA, allergic bronchopulmonary aspergillosis; BafA, bafilomycin A1; DIC, differential interference contrast; ER, endoplasmic reticulum; RAW, RAW 264.7; WT, wild-type.

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phagocytosis of *A. fumigatus* conidia by murine macrophages. We found that the presence of *A. fumigatus* phagosomes resulted in a dramatic change of the subcellular distribution of TLR9 to a bright, ring-shaped compartment around the *A. fumigatus* conidia. TLR9 recruitment was specifically induced by *A. fumigatus*-but not bead-containing phagosomes, indicating that the composition of the phagocytosed content dictates recruitment of TLR9 to the phagosomal membrane. We demonstrated that *A. fumigatus*-induced TLR9 recruitment was independent of *A. fumigatus* spore germination stage. Expression of TLR2, TLR4, and the TLR signaling adaptors MyD88 and TRIF were not required for successful *A. fumigatus* phagosomal TLR9 recruitment. Further investigation of the requirements for proper intracellular trafficking of TLR9 revealed that the TLR9 N-terminal proteolytic cleavage domain was critical for accumulation of TLR9 in CpG-containing compartments and *A. fumigatus* phagosomal membranes.

**Materials and Methods**

**Reagents**

All products used for cell culture were from Invitrogen (Carlsbad, CA). The CpG phosphorothioate oligodeoxynucleotide 1826 (5'-TCCATGACGTT-CCTGAGCATT-3') with Alexa Fluor 647 conjugated to the 3' end was purchased from Integrated DNA Technologies (Coralville, IA). OVA Alexa Fluor 488 conjugate (O34781) was purchased from Invitrogen. Dynasore and bafilomycin A1 (BafA) were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell lines and cell culture**

RAW 264.7 (RAW) and HEK293T cells were purchased from American Type Culture Collection (Manassas, VA) and cultured according to the supplier’s recommendations. Immortalized bone marrow macrophage cell lines derived from wild-type (WT), TLR2/TLR4-deficient, and MyD88/TRIF-deficient mice were a gift from Douglas Golenbock (University of Massachusetts Medical School, Worcester, MA). Immortalized macrophage cell lines were cultured and phenotypically characterized by analysis of macrophage surface marker expression and cytokine expression profile as described previously (24).

**Plasmids**

The lentiviral pHAGE vector containing CD82 fused at the C terminus to mRFP1 was described previously (25). The retroviral pMSCV vector containing murine TLR9 fused at the C terminus to GFP (pMSCV-TLR9-GFP) and plasmids encoding VSV-G and Gag-Pol were gifts from Douglas Golenbock (University of Massachusetts Medical School, Worcester, MA). Immortalized macrophage cell lines were cultured and phenotypically characterized by analysis of macrophage surface marker expression and cytokine expression profile as described previously (24).

**Viral transduction**

Lentivirus production and transduction were performed as described previously (25). Retroviral pMSCV vector containing murine TLR9 fused at the C terminus to GFP (pMSCV-TLR9-GFP) and plasmids encoding VSV-G and Gag-Pol were gifts from Douglas Golenbock (Whitehead Institute for Biomedical Research, Cambridge, MA). The TLR9 deletion mutant lacking residues 441–470 fused to the C terminus to GFP (pMSCV-TLR9Δ441–470-GFP) was generated from the pMSCV-TLR9-GFP vector by using QuikChange XL site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) using the following primers: 5’-AGC CTG CTA GAA GCC ACC CTT GAG TGT AAC TTC AAG TTC AAG TTC ACC ATG GAC-3’ (forward) and 5’-CCG AGA CAG GTC CAT GGT GAA CTT GAT CCT ACA CTC TTC AGG GGT GCC TTC-3’ (reverse). The final construct sequence was confirmed by sequencing.

**Image analysis**

The data are representative of at least three independent experiments in which a minimum of 100 cells were visualized. Raw image data files were processed using Adobe Photoshop in accordance to the ethical guidelines for scientific image manipulation as proposed by Rossner and O’Donnell (28). Quantitative colocalization analysis was performed in Velocity version 5.3.2.0 using the colocalization module that computes Pearson’s correlation and Manders’ coefficient for the region of interest. Pixels chosen for quantitative analysis were those that lie in a circumferential ring on the equatorial plane of the phagosome. Pixels that may be associated with vesicles were excluded.
Results

Phagosomes containing A. fumigatus conidia specifically recruit TLR9

We studied the effect of phagocytosis of A. fumigatus resting conidia on the subcellular localization of TLR9 using a stable RAW macrophage cell line expressing TLR9-GFP. As we have previously shown that the tetraspanin CD82 is recruited from the endolysosomal compartment to A. fumigatus and Cryptococcus neoformans phagosomes but not to polystyrene bead-containing phagosomes (K. Artavanis-Tsakonas, P.V. Kasperkovitz, E. Papa, M.L. Cardenas, A.G. Van der Veen, H.L. Ploegh, and J.M. Vyas, submitted for publication), we stably coexpressed CD82-mRFP1 in these cells for use as a marker of endolysosomal and A. fumigatus phagosomal compartments. We found that the presence of A. fumigatus phagosomes dramatically changed the subcellular distribution of TLR9 from its typical pattern of ER and endolysosomal distribution (5, 8–10, 29) to a bright, ring-shaped compartment around the A. fumigatus conidia that colocalized with CD82 (Fig. 1A). To demonstrate that the recruitment of TLR9 and CD82 was uniform throughout the phagosomal membrane, we imaged serially the entire volume of the phagosome and observed robust recruitment throughout (Supplemental Video 1). Additionally, RAW cells expressing either TLR9-GFP or CD82-mRFP1 alone demonstrated similar recruitment as cells expressing both fluorescent proteins (data not shown). To ensure that the A. fumigatus conidia were found within intracellular compartments, we used surface labeling of the fungal pathogen with Alexa Fluor 647 (Fig. 1A). DIC images and fluorescent images confirmed that A. fumigatus conidia resided in phagosomes (full Z-stack shown as a movie can be found in Supplemental Video 2). Addition of the fluorescent label to A. fumigatus did not affect its ability to recruit CD82 or TLR9 (data not shown). In sharp contrast to A. fumigatus conidia, phagocytosis of a polystyrene bead, regardless of size, did not result in enrichment of TLR9 or CD82 around the bead (Fig. 1B), indicating that recruitment of TLR9 is not a result of uptake itself but is specifically induced by the content of the phagosome. Uptake of A. fumigatus conidia by macrophages that had previously been exposed to polystyrene beads resulted in specific enrichment of TLR9 and CD82 only around the A. fumigatus conidia but not around the bead (Fig. 1C). The merged images of TLR9 and CD82 showed close apposition of the ring-shaped signals around the A. fumigatus phagosome, confirming that these two proteins are found on the phagosomal membrane. Using time-lapse imaging, the associations remained close throughout the time imaged (data not shown). To determine the extent of overlap of TLR9-GFP and CD82-mRFP1, isolated images of a phagosome were subjected to statistical analysis (Fig. 1D). Pixels

FIGURE 1. A. fumigatus phagosomes specifically induce recruitment of TLR9. A–D, Confocal microscopy of RAW macrophages expressing TLR9-GFP (green) and CD82-mRFP1 (red). One focal plane is shown. Scale bar, 5 μm. Original magnification ×100. A, Phagosomes containing A. fumigatus resting conidia acquire both TLR9 and CD82. RAW cells were incubated with A. fumigatus resting conidia labeled with Alexa Fluor 647 (blue) for 1 h. Colocalization of TLR9 and CD82 in the phagosomal membrane is apparent in the merged image (third panel) (see also Supplemental Video 1). The DIC image and the merged DIC/A. fumigatus (blue) image demonstrate the presence of two labeled A. fumigatus conidia within the cell (see also Supplemental Video 2). B, Bead-containing phagosomes fail to acquire TLR9 and CD82. RAW cells were incubated for 16 h with Flash far-red–labeled 5-mm beads (blue). C, TLR9 is specifically recruited to A. fumigatus-containing phagosomes in cells that have taken up both A. fumigatus resting conidia and polystyrene beads. RAW cells were incubated for 16 h with Flash far-red–labeled 5-mm beads (blue) and then exposed to unlabeled A. fumigatus conidia for 1 h. The DIC image demonstrates the presence of unlabeled A. fumigatus conidia within the cell. D, Colocalization of TLR9 and CD82 in an A. fumigatus-containing phagosomal membrane. A detail of a representative cell having taken up an A. fumigatus resting conidium was quantitatively analyzed. Pixels associated with large vesicle (10 o’clock position) and two smaller vesicles (4 o’clock position) were excluded from this analysis.
conidia and one extracellular 1-, recruitment of TLR9 and CD82 to having taken up five unlabeled merged image on the right shows a representative cell lysosomes is reached 1 h after Dynasore washout. The lapse images were acquired at time points in minutes of dynamin inhibition by Dynasore washout. Time-
A. fumigatus conidia was inhibited, although the conidia still adhered to the plasma membrane (Fig. 2A). Using time-lapse imaging, we studied the kinetics of conidial uptake and recruitment of TLR9 and CD82 after reversal of inhibition by washout (Fig. 2B). Phagocytic activity was restored 15 min after Dynasore washout, and recruitment of TLR9 and CD82 was seen after 45 min. Maximal levels of recruitment were reached 1 h after washout (Fig. 2C).

TLR9 recruitment is independent of A. fumigatus conidial viability, spore-germination stage, surface hydrophobin, and pigment Because our bead experiment demonstrated that TLR9 phagosomal recruitment is specific to A. fumigatus conidia, we next wanted to investigate whether the ability to induce TLR9 recruitment was related to conidial surface properties that change throughout the different A. fumigatus spore stages. During the spore germination process, A. fumigatus conidia undergo an initial period of isotropic expansion associated with the uptake of water, and upon the establishment of a polarity axis, a short germ tube emerges and grows into a hypha (31). Conidial swelling during germination increases surface exposure of fungal β-glucans that can trigger the induction of inflammatory responses through the β-glucan receptor Dectin-1 and allow macrophages to distinguish between A. fumigatus spore stages (18, 32–34). To prevent conidial swelling and increased β-glucan exposure from occurring during our experimental conditions, we heat-killed dormant conidia immediately at 100°C for 30 min. When we exposed cells to these heat-killed conidia, recruitment of TLR9 was not impaired (Fig. 3A) compared with what we had seen for conidia that were dormant but viable. Induction of conidial swelling in RPMI 1640 for 6 h prior to introduction to the cells had no effect on phagocytosis and TLR9 recruitment (Fig. 3B). To confirm that spore germination was induced by this swelling procedure, we microscopically examined the conidia and observed an increase in size (data not shown). Additionally, we stained the conidia for β1,3-glucan and, in agreement with previously described findings (33, 34), we detected low levels of β1,3-glucan on dormant conidia, whereas ~90% of swollen conidia displayed increased β1,3-glucan levels (Supplemental Fig. 1). Heat-killing did not affect β-glucan exposure (Supplemental Fig. 1). Thus, our results indicate that TLR9 recruitment by A. fumigatus conidia occurs independently of spore germination stage. Strikingly, after overnight incubation with conidia that were dormant but viable at the time of introduction to the cells, we occasionally observed intracellular hyphal forms that retained the ability to recruit TLR9 (Fig. 3C, Supplemental Video 3). Because a low proportion of phagocytosed A. fumigatus conidia can survive, and germinate, within cells (35), it is unclear whether germination occurred intracellularly after phagocytosis of the conidia or if the hyphae were phagocytosed after germination. We next examined whether removal of the hydrophobic surface layer on dormant conidia that masks their recognition by the immune system would augment TLR9 recruitment. When we exposed cells to dormant conidia from an A. fumigatus mutant rodA−/− strain lacking the hydrophobic RodA protein that is covalently bound to the conidial cell wall through GPI remnants (26), we did not see a change in the level of TLR9 recruitment (Fig. 3D). Additionally, the conidial pigment melanin protects conidia from oxidative damage by leukocytes and modulates the response to A. fumigatus conidia by shielding fungal pathogen-
FIGURE 3. TLR9 recruitment is independent of A. fumigatus spore viability, germination stage, surface hydrophobin, and pigment. Confocal microscopy of RAW macrophages expressing TLR9-GFP (green). One focal plane is shown. Scale bar, 5 μm. Original magnification ×100. WT A. fumigatus resting conidia were either heat-killed immediately or swollen for 6 h and heat-killed. Resting heat-killed A. fumigatus conidia (A), swollen heat-killed conidia (B), and A. fumigatus hyphae (C) retain the ability to recruit TLR9 (see also Supplemental Video 3). Absence of the hydrophobic surface protein RodA (D) or the conidial surface pigment melanin (E) does not interfere with phagosomal TLR9 recruitment by A. fumigatus resting conidia.

associated molecular patterns (36). To determine whether the absence of melanin would affect TLR9 recruitment, we exposed RAW cells to the A. fumigatus mutant alb1/2 strain (27) (Fig. 3E). Taken together, although our bead experiment demonstrated that recruitment of TLR9 to the phagosome is dependent on a signal specific to the A. fumigatus conidia, the signal required for TLR9 recruitment appears to be continuously present throughout the different spore stages and selected A. fumigatus mutants.

TLR2 and TLR4 expression and downstream TLR signaling are not required for TLR9 recruitment to A. fumigatus phagosomes

As TLR2 and TLR4 have been implicated to be critical in the immune response to A. fumigatus (reviewed in Ref. 37) and these receptors are readily recruited from the plasma membrane to microbial phagosomes (38), we hypothesized that the induction of recruitment of TLR9 by A. fumigatus was mediated by signals derived from surface-expressed TLR2 or TLR4 during phagocytosis. To test this hypothesis, we expressed TLR9-GFP and CD82-mRFP1 in immortalized bone marrow macrophage cell lines. As expected, WT cells behaved identically as RAW cells (Fig. 4A). Interestingly, when we used cells deficient in both TLR2 and TLR4, phagocytosis of A. fumigatus conidia and subsequent recruitment of TLR9 and CD82 were not affected (Fig. 4B), indicating that expression of TLR2 and TLR4 is not required for induction of recruitment. To investigate whether downstream signaling from any of the TLR family members was involved in relaying the signal that induces TLR9 recruitment, we next used cells deficient in both MyD88 and TRIF. In these cells, which are completely incapable of all TLR signaling, we still observed robust recruitment of TLR9 and CD82 (Fig. 4C). Thus, our results indicate that TLR9 redistribution to the A. fumigatus phagosome is independent of TLR signaling.

TLR9 accumulation in CpG- or A. fumigatus-containing compartments requires the TLR9 N-terminal proteolytic cleavage motif

Biochemical experiments have demonstrated that the full-length form of TLR9 must undergo proteolytic maturation in the endolysosomal compartment to become a functionally competent receptor to signal in response to CpG (5, 6). We hypothesized that proteolytic cleavage is a requirement for successful TLR9 intracellular trafficking and retention in subcellular compartments. To investigate the role of proteolytic cleavage in TLR9 trafficking to, and retention at, the A. fumigatus phagosome, we pretreated cells with a specific inhibitor of vacuolar-type H+-ATPase, BafA. Interestingly, when we exposed these cells to A. fumigatus resting conidia, TLR9 recruitment to the A. fumigatus phagosome was impaired although not completely absent (Fig. 5A). In agreement with our previous data (K. Artavanis-Tsakonas et al., submitted for publication), recruitment of CD82 was not affected by BafA.

FIGURE 4. Expression of TLR2 and TLR4 and downstream TLR-signaling are not required for TLR9 recruitment. A–C, Confocal microscopy of immortalized bone marrow-derived macrophages expressing TLR9-GFP (green) and CD82-mRFP1 (red). One focal plane is shown. Scale bar, 5 μm. Original magnification ×100. Cells were incubated for 1 h with A. fumigatus resting conidia. Representative cells showing phagosomal acquisition of TLR9 and CD82 are shown in WT macrophages (A), TLR2/TLR4-deficient macrophages (B), and TLR-signaling incompetent MyD88/TRIF-deficient macrophages (C).
CpG was found in CD82-positive compartments that costained with TLR9. When we incubated CD82-mRFP1–expressing RAW cells with fluorescent conidia. TLR9 trafficking pathway and marks the site where WT TLR9 is recruited. RAW cells expressing CD82-mRFP1 (red) and WT TLR9-GFP (green, second panels) were incubated with 1 μM CpG-Alexa Fluor 647 (blue) for 8 min and medium was replaced. The merged image shows colocalization of CpG (blue), OVA (green), and CD82 (red) after 30 min. B, TLR9Δ441–470-GFP fails to be recruited to CpG-containing compartments. CD82 intersects the TLR9 trafficking pathway and marks the site where WT TLR9 is recruited. RAW cells expressing CD82-mRFP1 (red) and WT TLR9-GFP (green, first panels) or TLR9Δ441–470-GFP (green, second panels) were incubated with 1 μM CpG-Alexa Fluor 647 (blue) for 8 min and medium was replaced. The merged image shows that 1 h after CpG addition WT TLR9 has accumulated in CpG/CD82-positive compartments, whereas TLR9Δ441–470 still displays an ER-like distribution. The merged image shows no overlap between TLR9Δ441–470 and the CpG/CD82 double-positive compartment.

Discussion

The importance of TLR9 trafficking to endolysosomal compartments for receptor functionality and ligand-induced signaling has been well documented (5–9), but exactly how these processes are...
integrated into the dynamic cellular infrastructure in which TLR9 operates after pathogen uptake has not been established. We have now demonstrated that TLR9 is specifically redistributed to the phagosomal membrane of *A. fumigatus* spore-containing phagosomes but not bead-containing phagosomes. The TLR9 N-terminal proteolytic cleavage site was a critical requirement for TLR9 recruitment to *A. fumigatus* phagosomes. Although TLR9 has been implicated in host defense against *A. fumigatus* (12, 14), the intracellular processes that enable *A. fumigatus* Ag recognition by TLR9 in innate immune cells remain unclear. To our knowledge, this report is the first to demonstrate that TLR9 specifically accumulates at the fungal phagosome. Selective compartmentalization of pattern recognition receptors to the phagosome may be a requirement for effective orchestration of antifungal innate immunity.

TLR9 recruitment to phagosomes was dependent on the content of the phagosome. Indeed, polystyrene beads failed to recruit TLR9 to the phagosomes to levels detectable by fluorescence microscopy. However, the presence of TLR9 in phagosomes has been shown in latex bead-containing RAW cells using biochemical methods (5, 39). Despite the presence of functional TLR9 in latex bead-containing phagosomes, conjugation of beads with CpG did not result in visible enrichment of the total amount of phagosomal TLR9 (5). In sharp contrast, *A. fumigatus* induced robust recruitment of TLR9 to its phagosome. Moreover, in cells where both *A. fumigatus* and polystyrene beads were taken up, a clear difference in the level of TLR9 could be visualized. By live cell imaging, we were able to determine that this recruitment occurred within minutes and remained present on the phagosome for hours.

Phagocytosis of *A. fumigatus* conidia has been shown to require actin polymerization and PI3K activity (40). By using Dynasore, a reversible inhibitor of endocytic pathways that blocks coated vesicle formation, we have now demonstrated that GTPase activity of dynamin is an essential step in *A. fumigatus* conidial phagocytosis. A similar requirement for dynamin activity has been demonstrated for cell entry of the intracellular parasite *Trypanosoma cruzi* (41) and active invasion by *Toxoplasma gondii* (42). While dynamin activity was essential for phagocytosis of both *A. fumigatus* conidia and beads, TLR9 recruitment after phagocytosis was specific to *A. fumigatus* conidia. We hypothesized that TLR9 redistribution to *A. fumigatus*-containing phagosomes involved signaling through another receptor, possibly a plasma membrane-localized receptor responding to a component on the *A. fumigatus* conidial surface. Multiple pattern recognition receptors besides TLR9 have been implicated in the innate immune response to *A. fumigatus* (12, 18–20, 43–45). A link between the TLR2-mediated recognition of *A. fumigatus* and the phagocytic response has been suggested (34), and internalization of TLR2 with the *A. fumigatus* phagosome was shown by microscopy (44). In our search for the receptor mediating the specific recruitment, we demonstrated that expression of TLR2 and TLR4 was not required for *A. fumigatus* phagosomal TLR9 recruitment. Additionally, TLR9 recruitment occurred normally in the absence of both TLR adaptors MyD88 and TRIF, thereby eliminating the involvement of all downstream TLR signaling in the recruitment process. Our data did not show that deficiency in any of the molecules we analyzed resulted in impaired phagocytosis.

Experiments demonstrating recognition of β1,3-glucan on the *A. fumigatus* cell wall have suggested that the immune response is tailored toward metabolically active conidia (18, 32) due to preferential recognition by dectin-1 of *A. fumigatus* swollen conidia that have increased β1,3-glucan surface exposure (33, 34). However, we observed robust TLR9 recruitment to dormant, heat-killed conidia and did not see a correlation between TLR9 recruitment levels and spore germination stage or increased β1,3-glucan surface exposure. Additionally, alterations of the fungal cell wall using mutants of RodA or melanin synthesis did not perturb the recruitment of TLR9 to the *A. fumigatus* phagosome. These data suggest that a cell wall component present throughout all developmental stages may mediate TLR9 recruitment. The fungal cell wall is a complex and dynamic structure that contains proteins, glycolipids, and polysaccharides that are endowed with distinct immunostimulatory capacities (46). Identification of the component responsible for induction of TLR9 recruitment may significantly enhance our understanding of the innate immune pathways that intracellularly orchestrate antifungal defense.

Maturation of *A. fumigatus* phagosomes results from fusion with compartments of the endocytic pathway, and killing of conidia depends on phagolysosome acidification in alveolar macrophages (40).

Our data show that the endolysosomal tetraspanin CD82 intersects the trafficking pathway of TLR9 and marks the compartment where CpG and TLR9 interact. Strikingly, while some TLR9 was already present in the CD82-positive compartment in unstimulated RAW cells, the drastic redistribution of TLR9 to the phagosomal membrane occurred rapidly after *A. fumigatus* phagocytosis and coincided with recruitment of CD82. It is unclear whether the TLR9 molecules recruited to *A. fumigatus*-containing phagosomes pass through the endolysosome or translocate to the phagosome directly from the ER. Occasionally we observed TLR9/CD82-positive vesicles around the phagosomal membrane that suggested codelivery of TLR9 and CD82 to the phagosome (Fig. 1D). However, it is possible that TLR9 can reach the *A. fumigatus* phagosome by both pathways.

Multiple factors have been implicated in TLR9 targeting to subcellular compartments. Delivery of TLR9 to endolysosomal compartments required its interaction with the ER-resident protein UNC93B1, mediated through the transmembrane segment of TLR9 (8). Cytoplasmic targeting motifs controlled TLR9 intracellular localization (47), and the addition of an N-terminal FLAG tag precluded TLR9 ER egress and proteolytic cleavage (5). The importance of the N-terminal cleavage site in TLR9 has been established for its role to generate the functional signaling-competent receptor (5, 6). We have now extended the requirement for the TLR9 N-terminal proteolytic cleavage site for TLR9 accumulation in the endolysosomal and phagosomal compartments. It is unclear whether the trafficking defect of the mutant is due to its failure to exit the ER or lack of retention in the endosome or phagosome after translocation. Because successful cleavage of TLR9 required an acidic compartment, it is tempting to speculate that uptake of *A. fumigatus* conidia and subsequent phagolysosomal acidity (40) may facilitate cleavage and retention in the phagosome. A recent report indicated that acidification induced by carbohydrates from encapsulated microbes facilitated TLR9 signaling (48). TLR9 recruitment, cleavage, and retention at the fungal phagosome may be prerequisite steps for successful signaling to TLR9 ligands that are released during phagosomal processing. Biochemical analyses on *A. fumigatus*-containing phagosomes are required to resolve the exact spatiotemporal relationship between phagosomal acidification, TLR9 recruitment, and TLR9 cleavage.

Our study provides evidence for a model in which *A. fumigatus* spore phagocytosis by macrophages induces TLR9 recruitment to *A. fumigatus* phagosomes and may thereby mediate TLR9-induced antifungal innate immune responses. As isolated *A. fumigatus* nucleic acids activated TLR9 (23), phagosomal TLR9 recruitment may function to enable an immediate response to microbial content released during processing in the phagosome. A similar mechanism
has been proposed for _F. caesi_ (49). Another possibility is that _A. fumigatus_ spores contain as yet unidentified ligands for TLR9, as the discovery that malarial hemoxan acts as a direct ligand for TLR9 demonstrated that TLR9 activation is not limited to nucleic acids (50). Given the frequent incidence of invasive pulmonary aspergillosis in immunocompromised patients and severe complications associated with ABPA, it is clear that understanding the molecular mechanisms responsible for triggering innate immune responses to _A. fumigatus_ has both clinical and therapeutic potential, particularly in terms of vaccine development.

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**Disclosures**

The authors have no financial conflicts of interest.

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