Receptor for advanced glycation end-products (RAGE) mediates phagocytosis in nonprofessional phagocytes

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In mammals, both professional phagocytes and nonprofessional phagocytes (NPPs) can perform phagocytosis. However, limited targets are phagocytosed by NPPs, and thus, the mechanism remains unclear. We find that spores of the yeast *Saccharomyces cerevisiae* are internalized efficiently by NPPs. Analyses of this phenomenon reveals that RNA fragments derived from cytosolic RNA species are attached to the spore wall, and these fragments serve as ligands to induce spore internalization. Furthermore, we show that a multiligand receptor, RAGE (receptor for advanced glycation end-products), mediates phagocytosis in NPPs. RAGE-mediated phagocytosis is not uniquely induced by spores but is an intrinsic mechanism by which NPPs internalize macromolecules containing RAGE ligands. In fact, artificial particles labeled with polynucleotides, HMGB1, or histone (but not bovine serum albumin) are internalized in NPPs. Our findings provide insight into the molecular basis of phagocytosis by NPPs, a process by which a variety of macromolecules are targeted for internalization.

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Diverse eukaryotic cells can engulf large particles (≥0.5 μm in diameter) and internalize them via an endocytic process called phagocytosis. In mammals, a class of cells have evolved to perform phagocytosis; these cells, including macrophages, neutrophils, and dendritic cells, are termed professional phagocytes. Apart from professional phagocytes, however, phagocytosis occurs in many other cells, such as epithelial cells, fibroblasts, and tumors; these cells are termed nonprofessional phagocytes (NPPs). Compared to professional phagocytes, the range of macromolecules internalized by NPPs is limited. Nevertheless, studies using macrophage-deficient mice have demonstrated the functional redundancy between NPPs and macrophages, at least for the removal of apoptotic cells. However, very few studies have been conducted to explore the physiological roles of phagocytosis by NPPs partly because the molecular mechanism is poorly understood.

Cells can internalize large particles via phagocytosis or macropinocytosis. Macropinocytosis is a process by which extracellular molecules are randomly internalized in cells. In contrast, phagocytosis is a receptor- and ligand-induced endocytic process. Thus, in this process, particles decollated with specific ligands are targeted for internalization. Various phagocytic receptors, such as Fcy receptors, integrins, and scavenger receptors, are found in professional phagocytes. In general, phagocytic receptors are activated by binding to their specific ligands, which leads to the reorganization of the actin cytoskeleton to deform the plasma membrane. When the plasma membrane is extended around the target particles, phagocytic receptors sequentially bind to their ligand presented on target particles. Eventually, the target particles are engulfed by the plasma membrane and internalized in phagocytic cells. Internalized particles are compartmentalized in a membrane-bound structure termed the phagosome, which matures into phagolysosomes by fusion with lysosomes.

One objective of our study is to use spores of the yeast Saccharomyces cerevisiae as microparticles. Yeast spores are a dormant and stress-resistant cell form that is generated when diploid cells are incubated under starvation conditions. Spore formation occurs inside of the mother cells, where four nuclei produced via meiosis are individually enclosed by the spore plasma membrane and spore wall. Through this process, the mother cells become asci, including four spores. Unlike vegetative cells, spores have dityrosine and chitosan layers on the exterior of their cell (spore) wall. Chitosan is often found in fungal cell walls, but the dityrosine layer is a unique structure found in the S. cerevisiae spore wall. The primary constituent of the dityrosine layer is LL-bisformyl dityrosine. While the detailed structure of this layer remains unclear, LL-bisformyl dityrosine molecules are presumably cross-linked to produce a macromolecule which is covalently attached to the chitosan layer. The dityrosine and chitosan layers make spores resistant to environmental stresses.

During the course of our study, we performed an experiment to incubate spores with cultured mammalian cells; in this experiment, we found that spores are phagocytosed in NPPs. Further analysis of this phenomenon revealed that internalization of spores by NPPs is mediated by receptors for advanced glycation end-products (RAGE). RAGE is a member of the immunoglobulin receptor superfamily and was originally identified as a receptor for advanced glycation end-products. However, RAGE is now known to recognize multiple ligands, including polynucleotides, phosphatidylserine (PS), and high mobility group box 1 (HMGB1). Most RAGE ligands are known as damage-associated molecular patterns (DAMPs), and activation of the RAGE signaling pathway is known to induce inflammation. RAGE can internalize its ligands via the endocytic pathway. In addition, RAGE can recognize PS and is involved in the phagocytosis of apoptotic cells (efferocytosis) in macrophages. However, its role in phagocytosis in NPPs is unknown. Spores are phagocytosed in NPPs because a RAGE ligand, RNA, is attached to the spore wall. Apart from spores, particles decorated with RAGE ligands are phagocytosed in NPPs. Our results demonstrate that various macromolecules can be internalized in NPPs via RAGE-mediated phagocytic processes.

**Results**

**Spores are internalized in NPPs.** Yeast spores are produced in the ascus (Supplementary Fig. 1a). By rupturing the ascical membrane and ascus wall, spores can be released from the ascus (Supplementary Fig. 1b). When spores were incubated with HEK293T cells, we found that spores, but not vegetative yeast cells, exhibited toxicity to HEK293T cells (Supplementary Fig. 1c). Unlike vegetative cells, spores have dityrosine and chitosan layers on the exterior of their cell (spore) wall (Supplementary Fig. 1a). Since vegetative cells were harmless (Supplementary Fig. 1c), we speculated that the toxicity of spores is attributable to the unique structure of the spore wall. Consistent with this hypothesis, dit1Δ spores, which lack the dityrosine layer, did not exhibit toxicity (Supplementary Fig. 1c). When HEK293T cells incubated with spores were observed under the microscope, we realized that spores were internalized in the mammalian cells. We confirmed that spores were incorporated into lysosomes by staining with an acidotropic probe LysoTracker (Fig. 1a, b). Spores were not stained by LysoTracker in the condition used in our internalization assay (Supplementary Fig. 1d). Compared to wild-type spores, the internalization levels of vegetative cells and dit1Δ spores by HEK293T cells were lower (Fig. 1c, d). We found that a fraction of spores was swollen in HEK293T cells when they were incubated for 12 h (Supplementary Fig. 1e), suggesting that spores could germinate in the cultured cells. To assess whether the viability of spores is related to their toxicity, heat-killed spores were incubated with HEK293T cells. The levels of spore internalization by HEK293T cells were not altered by heat treatment (Supplementary Fig. 1f); however, heat-killed spores did not exhibit toxicity (Supplementary Fig. 1g). These results suggest that the cytotoxicity of spores is attributable to their internalization and germination. Since S. cerevisiae is not a pathogenic organism, the physiological significance of the spore’s toxicity is unclear. Nevertheless, we are interested in the phenomenon that spores are internalized in cultured cells; thus, the basis of the internalization mechanism was further analyzed.

Spores can be internalized in various cell lines, including HEK293, HeLa, and human uroepithelial cells (Fig. 1c). Among the cell lines we tested, HEK293T cells internalized spores most efficiently (Fig. 1c); thus, this cell line was used for further studies. Given that spores are ~3 μm in diameter (Supplementary Fig. 1b), they are likely internalized via phagocytosis or macropinocytosis. Because spores are targeted for internalization as described above, we hypothesized that spores are internalized via phagocytosis. Since the cell lines used in the internalization assay were all derived from epithelial tissues, these results suggested that spores induce phagocytosis in NPPs. Similar to Fcy receptor-mediated phagocytosis in macrophages, internalization of spores in HEK293T cells was inhibited by inhibitors of actin polymerization, spleen tyrosine kinase (SYK), and phosphatidylinositol-3 kinase (PI3K) (Fig. 1e). In addition to the microscopy-based phagocytosis assay, inhibitory effect of the inhibitors in spore internalization by HEK293T cells was verified with fluorescence-activated cell sorting (FACS)-based phagocytosis assay (Supplementary Fig. 2). Expression of SYK in HEK293T cells was confirmed by western blotting.
Furthermore, we found that the levels of spore internalization by HEK293T cells were decreased by SYK knockout (Supplementary Fig. 3b). Phagocytosis assays were performed in the media supplemented with heat-inactivated serum in this study. In addition, HEK293T cells internalized spores in the absence of serum (Fig. 1f). Thus, internalization of spores in HEK293T cells occurs even in the absence of antibodies and the complement system.

RNA fragments are attached to the spore wall. Spores are efficiently internalized in NPPs, suggesting that the spore wall is decorated with a ligand to induce phagocytosis. Given that the internalization of spores was inhibited by washing with a high-salt (0.6 M NaCl) solution (Fig. 2a), the ligand is most likely noncovalently associated with the outermost layer (dityrosine layer) of the spore wall. Since spores are formed in the mother cell cytosol (Supplementary Fig. 1a), we speculated that cytosolic materials would attach to the spore wall. Strikingly, the levels of spore internalization by HEK293T cells were decreased by RNase treatment but not by DNase treatment (Fig. 2a). In line with the result, RNA was detected in the eluate of spores washed with high-salt solution (Fig. 2b). Internalization levels of spores were also decreased by protease treatment (Fig. 2a). Compared to the case with wild-type spores, washing of dit1Δ spores with high-salt solution released lower amounts of RNA from the spore wall (Fig. 2c), a result that correlated with the observation that

Fig. 1 Spores are internalized by NPPs. a Representative images of HEK293T cells with internalized spores expressing GFP was obtained with differential interference contrast (DIC) or fluorescence microscopy (spore and lysosome). HEK293T cells were incubated with spores at 3.6 × 10⁷ spores/4–6 × 10⁵ HEK293T cells/ml. Lysosomes were stained with LysoTracker Red. Scale bar, 10 μm. b Numbers of spores internalized per HEK293T cell (black circles) and percentages of HEK293T cells with internalized spores (red triangles). HEK293T cells were incubated for 1 h with various quantities of spores in DMEM. c Internalization of yeast cells in NPPs. Spores or vegetative (veg) cells were incubated with HEK293T cells at 1.2 × 10⁷ yeast cells/ml or with HEK293 cells, human uroepithelial cells (HUC), or HeLa cells at 1.2 × 10⁷ yeast cells/ml or 3.4–4.6 × 10⁵ mammalian cells/ml. d Internalization of wild-type (wt) or dit1Δ spores in HEK293T cells. e Internalization of spores in HEK293T cells treated with or without (control) actin polymerization inhibitor latrunculin A (LAT-A), spleen tyrosine kinase inhibitor piceatannol (PT), or phosphatidylinositol-3 kinase inhibitor wortmannin (WTM). f Effect of the presence (+) or absence (−) of 10% fetal calf serum (FCS) on spore internalization in HEK293T cells. Data were presented as the mean ± SEM (b–f). Statistical significance was determined by two-tailed unpaired Student’s t-tests. n = 3 (b–d, f), n = 4 (e). ***P < 0.001; ****P < 0.0001; ns, not significant (P ≥ 0.05).
Internalization levels of spores by HEK293T cells were decreased by dit1Δ mutation (Fig. 1d). The FACS-based phagocytosis assay also showed that HEK293T cells containing spores were decreased when spores were treated with RNase, protease or high-salt solution (Supplementary Fig. 4).

Internalization of spores may be induced by a unique RNA attached to the spore wall. Thus, spore wall-bound RNA was identified by RNA sequencing. The majority of the RNA fragments released from the spore wall ranged from 25 to 29 nucleotides (nt) (Supplementary Fig. 5a). The RNA sequencing result (deposited in the Sequence Read Archive database, accession number PRJNA748575) showed that more than half of the RNA fragments were derived from tRNA sequences (Supplementary Fig. 5b). Notably, among tRNA-derived fragments, 87% were fragments derived from tRNAAsp species (Supplementary Fig. 5c). Apart from tRNA fragments, rRNA-, viral RNA-, and mRNA-derived fragments were found (Supplementary Fig. 5b). Thus, the spore wall is decorated with a variety of cytosolic RNA sequences.

Polynucleotides serve as ligands to induce phagocytosis in NPPs. Since tRNA was most abundantly found in spore wall-bound RNA, we analyzed whether phagocytosis by HEK293T cells is induced solely by tRNA. For this purpose, we assessed the internalization of tRNA-bound latex beads by HEK293T cells. Latex beads that contain surface amine groups were shown to adsorb purified tRNA (Fig. 3a). The levels of latex bead internalization by HEK293T cells were increased by the binding of tRNA (Fig. 3b). Latex beads were not stained by LysoTracker in the internalization assay (Supplementary Fig. 1d). However, the levels of internalization of polynucleotide-bound latex beads were lower than spores; as shown in Fig. 2a (control) and 3b (tRNA), when 1.2 × 10^7 beads (incubated with tRNA at 300 µg/2 × 10^8 beads/ml) or spores were incubated with HEK293T cells, their internalization levels were 0.18 or 1.18 per HEK293T cells, respectively. We also examined the internalization of tRNA-bound latex beads (Fig. 3b) by HEK293T cells. Latex beads adsorbed tRNA-derived RNA more than tRNA for unknown reason (Fig. 3a), although no differences were found between the internalization levels of spore wall-derived RNA-bound latex beads and tRNA-bound latex beads (Fig. 3b).

Like latex beads that contain surface amine groups, dit1Δ spores have a positively charged chitosan layer at the spore wall surface (Supplementary Fig. 1a). Nevertheless, wild-type spores could hold more RNA than dit1Δ spores (Fig. 2c), suggesting the presence of machinery to accommodate RNA in the dityrosine layer. This machinery likely involves proteins, given that protease treatment resulted in a loss of spore wall-bound RNA (Supplementary Fig. 6a). The RNA-binding machinery persists on the spore wall during the high-salt wash. Indeed, high-salt-washed spores adsorbed spore wall-derived RNA (Fig. 4a). Internalization of high-salt-washed spores by HEK293T cells was improved by binding of spore wall-derived RNA (Fig. 4b). The levels of internalization of spore wall-derived RNA-bound high-salt-washed spores were higher than those of spore wall-derived RNA-bound latex beads; when the phagocytosis assay was performed with the 1.2 × 10^7 spore wall-derived RNA-bound latex beads (incubated with the RNA at 40 µg/2 × 10^8 beads/ml) or latex beads (incubated with the RNA at 300 µg/2 × 10^8 beads/ml), their internalization levels were 0.87 or 0.22 per HEK293T cells, respectively (Figs. 3b, 4b). Since high-salt-washed spores adsorbed more polynucleotides than latex beads (Supplementary Fig. 6b), the efficiency of internalization by HEK293T cells may be relative to the amount of polynucleotides attached to particles. As mentioned earlier, no differences were found between the internalization levels of spore wall-derived RNA- and tRNA-bound latex beads. This contradiction maybe attributable to the low levels of internalization levels of polynucleotide-bound latex beads (Fig. 3b).

Apart from spore wall-derived RNA, spores adsorbed purified tRNA and a single-stranded 22-nt DNA fragment named DNA1 (Fig. 4a). DNA1 is a mimic of a tRNA^Asp fragment that was most abundantly found in spore wall-bound RNA. The amount of polynucleotide bound to high-salt-washed spores was diminished by protease treatment (Supplementary Fig. 6b), which is consistent with the hypothesis that proteins are involved in the polynucleotide-binding machinery. Internalization of high-salt-washed spores by HEK293T cells was improved by binding of tRNA and DNA1 (Fig. 4b). As with latex beads, spore wall-derived RNA bound to spores more efficiently than other polynucleotides (Fig. 4a). In line with the hypothesis that the levels of particle internalization by HEK293T cells were relative to the amount of polynucleotides bound to the particles, spores that had adsorbed spore wall-derived RNA were internalized more efficiently than spores that had adsorbed other polynucleotides before their bindings were saturated (Fig. 4b). Then, we analyzed the binding of another single-stranded DNA fragment which
consisted of a sequence that was not related to tRNAs (its sequence is shown in Supplementary Table 1); the DNA fragment was named DNA2. DNA1 and DNA2 bound to spores at similar levels (Supplementary Fig. 6b). Notably, two DNA fragments with distinct sequences induced internalization of high-salt-washed spores at comparable levels (Fig. 4c). These results collectively suggest that polynucleotides serve as ligands to induce phagocytosis and the nucleotide sequence is not critical to the induction of internalization by cultured cells. Spores are internalized by HEK293T cells more efficiently than polynucleotide-bound latex beads maybe because spores can hold more ligand (RNA) than latex beads (Supplementary Fig. 6b). In further support of this hypothesis, a competitive inhibition assay (Fig. 4d) showed that the addition of free polynucleotides inhibited the internalization of spores by HEK293T cells.

RAGE is a phagocytic receptor in NPPs. Next, we sought to identify the receptor that mediates the internalization of spores in mammalian cells. Given that polynucleotides are most likely ligands, several receptors known to bind polynucleotides\(^\text{18,28–32}\) were overexpressed as red fluorescent protein (RFP) fusions in HEK293T cells (not HEK293T cells) and assessed for their effects on spore internalization (Fig. 5a). HEK293T cells were used in this experiment because this cell line exhibited lower phagocytic efficiency than HEK293T (Fig. 1c); we speculated that phagocytic levels in HEK293 cells could be enhanced by overexpression of the receptor. We found that the levels of spore internalization were elevated by more than twofold by the expression of RAGE-RFP (Fig. 5a). Expression levels of RAGE mRNA in HEK293T cells were approximately 1.5-fold higher than those in HEK293 cells (Supplementary Fig. 7a). Spore internalization in HEK293T cells was also enhanced by the overexpression of RAGE (Supplementary Fig. 7b). Consistent with a role for RAGE in spore internalization, this process was decreased 10-fold in RAGE knockout cells (Fig. 5b). The loss of RAGE expression in HEK293T RAGE KO cells was verified by western blotting analysis (Supplementary Fig. 7c). The result of FACS analysis also showed that phagocytosis of spores by HEK293T was decreased by RAGE KO (Supplementary Fig. 7d). Furthermore, the defect in spore internalization in HEK293T RAGE KO cells was rescued by the expression of RAGE-RFP (Fig. 5b). Internalization of polynucleotide-bound beads was also decreased in HEK293T RAGE KO cells (Fig. 5c). In addition to RAGE, we found that internalization of spores in HEK293T cells was also increased by overexpression of Toll-like receptor 3 (TLR3) (Fig. 5a). However, the levels of spore internalization in HEK293T cells were not altered by TLR3 knockout (Supplementary Fig. 7e), showing that this receptor is either not required for spore internalization or is redundant with another receptor providing the same function. Therefore, TLR3 is not considered further here.

RAGE has two polynucleotide-binding patches, which are termed Site 1 and Site 2.\(^\text{18}\) Spore internalization in HEK293T RAGE KO cells expressing RAGE-RFPs harboring mutations in either of the nucleotide-binding sites (termed RAGE\(^\text{mut1-LFP}\) and RAGE\(^\text{mut2-RFP}\), respectively) was lower than that seen in cells expressing RAGE-RFP (Fig. 5b). To further analyze the binding between RAGE and RNA, we performed in vitro binding assays. The extracellular region of RAGE (amino acids 1–341) fused to FLAG and His tags was expressed in HEK293 cells; the truncated RAGE was named RAGE\(^\text{1–341-His-FLAG}\). Purified RAGE\(^\text{1–341-His-FLAG}\) was attached to agarose beads conjugated with an anti-FLAG tag antibody and incubated with an RNA fragment (a mimic of DNA1) fused to cyanine 3 (Cy3). As shown in Fig. 5d, the RNA fragment is bound to the extracellular region of RAGE. Furthermore, we found that RAGE\(^\text{1–341-His-FLAG}\) was precipitated with tRNA-bound latex beads more than bare latex beads (Fig. 5e). These results demonstrate that RNA directly binds to RAGE. Purified molecular weights of RAGE\(^\text{1–341-His-FLAG}\) is 40.7 kDa. Purified RAGE\(^\text{1–341-His-FLAG}\) (Fig. 5d) detected by SDS-PAGE was larger than the predicted molecular weight (Fig. 5d), presumably because RAGE has two N-linked glycosylation sites.

To assess the localization of RAGE during the internalization of spores, we performed time-lapse microscopy. This analysis showed that the spore was engulfed by green fluorescent protein (GFP) fused to RAGE before its internalization (Fig. 5f and Supplementary Video 1). RAGE-GFP was internalized together...
with the spores, and later, the GFP fusion- and spore-containing compartments were stained with LysoTracker (Fig. 5f and Supplementary Video 1). Levels of fluorescent intensities of RFP in certain areas in phagocytic cups (plasma membrane engulfling spores) were higher than those of areas adjacent to the phagocytic cup in the plasma membrane in fixed cells (Supplementary Fig. 7f, g). This result suggests that RAGE-RFP is recruited to phagocytic cups. Taken together, our findings indicated that spores are internalized via RAGE-mediated phagocytosis.

RAGE is primarily expressed in the lung compared to other organs. To assess whether RAGE-mediated phagocytosis occurs in vivo, a spore internalization assay was performed with primary mouse alveolar type II (ATII) epithelial cells. Spores were internalized by primary ATII cells (Fig. 6a, b). Internalization of spores in ATII cells was inhibited by reagents or mutations that similarly compromised spore internalization in HEK293T cells (Fig. 6b–d). To determine whether Rage (the mouse homolog) is required for spore internalization in ATII cells, RNA interference (RNAi)-mediated Rage knockdown was performed. Quantitative real-time PCR (qRT-PCR) analysis showed that the levels of the mouse Rage transcript were decreased by transfection of ATII cells with small interfering RNAs (siRNAs) targeting Rage (Fig. 6e, left panel). Accordingly, the levels of spore internalization by the mouse cells were decreased in Rage knockdown cells relative to control ATII cells (Fig. 6e, right panel). This result suggests that RAGE-mediated phagocytosis occurs in various NPPs.

Various molecules are internalized via RAGE-mediated phagocytosis in NPPs. Since RAGE is a multiligand receptor, we examined whether RAGE-mediated phagocytosis is induced by another ligand, HMGB1. HMGB1-bound latex beads (HMGB1 beads) were prepared by crosslinking recombinant HMGB1 to latex beads. Internalization of latex beads was increased threefold by the binding of HMGB1 to the beads (Fig. 7a, b and Supplementary Fig. 8a, b). Compared to wild-type HEK293T cells, the internalization levels of HMGB1 beads were decreased in Rage KO cells (Fig. 7b). These results suggest that HMGB1 induces phagocytosis by NPPs.
Like polynucleotide-bound latex beads, the levels of internalization of HMGB1-bound beads were lower than those of spores (compare Fig. 2a (control) and Fig. 7b (DNA and HMGB1)). As described earlier, this difference may indicate that spores display greater densities of ligand (RNA) than latex beads (Supplementary Fig. 6b). Additionally, or alternatively, the spore wall may contain other substance(s) that can augment or actively induce internalization. Regarding the latter possibility, a previous study showed that the RAGE-mediated inflammatory response is activated synergistically by DNA and HMGB1. By analogy, we found that internalization of DNA- and HMGB1-bound (double-labeled) beads (DNA/HMGB1 beads) by HEK293T cells was
higher than that observed with DNA or HMGB1 beads (i.e., single-labeled particles) (Fig. 7a, b). For this assay, DNA/HMGB1 beads were prepared by adsorbing HMGB1 to DNA beads. In DNA/HMGB1 beads, HMGB1 appeared to bind to DNA on latex beads, given that HMGB1 was not adsorbed by bare beads (Supplementary Fig. 8c). The levels of DNA bead internalization by HEK293T cells were increased by the binding of HMGB1 (Supplementary Fig. 8c, d). The levels of DNA/HMGB1 bead internalization by HEK293T RAGE KO cells were lower (Fig. 7b). Thus, DNA and HMGB1 synergistically augment RAGE-mediated phagocytosis.

Then, we performed a similar experiment using another DNA-binding protein, histone. Extracellular histones are known to act as DAMPs, but previously no studies have been reported to not serve as ligands for RAGE. Thus, first, we utilized histones as a negative control. However, histone-labeled beads were internalized in HEK293T cells to levels approximately twofold higher than DNA/HMGB1 beads (Fig. 7a, b and Supplementary Fig. 9a, b). In contrast, bovine serum albumin (BSA)-labeled beads were not internalized in HEK293T cells (Fig. 7b), showing that RAGE-mediated phagocytosis is not induced by proteins in general. Notably, the internalization of histone beads was decreased by RAGE knockout (Fig. 7b). These results suggest that histones are another ligand for RAGE. In support of this hypothesis, RAGE-GFP was precipitated with histone beads, but not with BSA beads, from a cell lysate harboring the RAGE-GFP fusion protein (Supplementary Fig. 9c). As seen with HMGB1, internalization of DNA beads was improved by the additional binding of histones (Fig. 7a, b and Supplementary Fig. 9d, e). In HEK293T RAGE KO cells, internalization of the DNA/histone beads was decreased (Fig. 7b). Histone beads and DNA/histone beads were also internalized by mouse ATII cells (Fig. 7c). We note, however, that internalization of beads by ATII cells was not induced by HMGB1 (Fig. 7c). Furthermore, unlike HEK293T cells, primary mouse ATII cells were more efficient at internalizing spores than any of the modified artificial beads (compare Fig. 6c (control) and Fig. 7c). Nevertheless, internalization of DNA/histone beads or histone beads by ATII cells was diminished by knockdown of RAGE (Fig. 7d), showing that internalization of the beads by these primary mouse cells is mediated by RAGE-dependent phagocytosis.

Finally, we examined whether RAGE is required for HEK293T cells to internalize apoptotic cells, which are well-known targets for phagocytosis by NPPs in vivo. Jurkat cells were internalized in HEK293T cells when they were treated with the apoptosis inducer staurosporine (Fig. 8a, b). In HEK293T RAGE KO cells, however, the levels of internalization of staurosporine-treated Jurkat cells were approximately fourfold lower than those in wild-type cells (Fig. 8b). Furthermore, we found that the sizes of Jurkat cells (fragments) internalized in HEK293T RAGE KO cells were smaller than those internalized in wild-type cells (Fig. 8c). These results demonstrate that efferocytosis by NPPs is also mediated by a RAGE-dependent pathway.

Discussion

Phagocytosis occurs in NPPs; however, the mechanism is not clear. Here, we demonstrate that phagocytosis by NPPs is mediated by RAGE. Given that RAGE knockout cells exhibit residual activity to internalize microsized particles, NPPs may be equipped with multiple phagocytic pathways. Nevertheless, NPPs can phagocytose various molecules via the RAGE-mediated pathway due to the multiligand-recognition property of the receptor.

In vivo, NPPs are known to perform efferocytosis. We show that RAGE can mediate the clearance of apoptotic corpses; presumably, PS on apoptotic cells serves as a ligand for this process. Apart from cell corpses, targets for phagocytosis by NPPs in vivo were not clear. Our results suggest that various macromolecules containing RAGE ligands are phagocytosed by NPPs. Macromolecules containing RAGE ligands are known to be present in the extracellular space. For example, DNA fragments bound to histones or HMGB1 can be released from damaged cells. These molecules are potential targets for RAGE-mediated phagocytosis. Intriguingly, most, if not all, RAGE ligands are harmful molecules. However, the underlying mechanism for how RAGE can recognize multiple harmful ligands awaits further structural analysis.

Direct binding between RAGE and its ligands has been demonstrated in previous reports. In RAGE-mediated phagocytosis, their binding most likely induces the signaling pathway. We found that the internalization of polynucleotide-bound latex beads was augmented by the binding of other DNA-binding RAGE ligands. The synergistic effect may reflect the fact that the DNA/HMGB1 complex binds to RAGE with higher affinity than DNA or HMGB1 alone, as has been reported previously. Our pharmacological analysis suggests that SYK and PI3K are involved in the signaling pathway. These kinases are known as downstream signaling molecules in Fc receptor-mediated phagocytosis, indicating similarity between professional and nonprofessional phagocytic pathways. The Fc receptor has an immunoreceptor tyrosine-based activation (ITAM) motif in the cytosolic domain, SYK directly binds to the activated Fc receptor via the phosphorylated ITAM motif. However, unlike the Fc receptor, the ITAM motif is absent in the cytosolic domain of RAGE. Thus, activation of RAGE-mediated phagocytic signaling may involve an adapter protein or coreceptor. Notably, HEK293T and mouse primary ATII cells internalize particles with distinctive preferences; HEK293T cells phagocytose DNA/histone beads most efficiently, whereas ATII cells prefer spores. This result suggests that internalization efficiency is not determined.
solely by RAGE’s affinity for particles. Thus, internalization efficiency may be mediated by one or more additional coreceptors.

While we focus on the mechanism of phagocytosis by NPPs in the present report, our study also provides several intriguing findings from the perspective of microbiology. In particular, we found that RNA fragments bind to the spore wall. The spore wall is equipped with machinery to hold RNA, suggesting that RNA fragments may be beneficial for spores. Since *S. cerevisiae* is a nonpathogenic yeast, it is unlikely that spore wall-bound RNA is invasive machinery. Apart from the spore wall, the presence of extracellular RNA or DNA is reported in several biological structures, including neutrophil extracellular traps and biofilms. In these structures, extracellular polynucleotides are used as materials to protect cells or organisms. By analogy, we propose that spore wall-bound RNA has a protective function. Given that spores are formed in the cytosol, it would be reasonable to use otherwise useless RNA fragments as protective materials. Thus, it would be intriguing to assess whether RNA is included in the spore wall of other organisms. In the yeast spore wall, tRNA, especially tRNA<sub>Asp</sub>, fragments are concentrated in spore wall-bound RNA for unknown reasons. In mammalian cell cultures and biofluids, fragments of tRNA<sub>Gly</sub> and tRNA<sub>Glu</sub> are major nonvesicular extracellular RNAs because they adopt RNase-resistant forms. In yeast, therefore, tRNA<sub>Asp</sub> may be resistant to RNase digestion in the ascal cytosol. Alternatively, the bias may be attributable to the binding specificity of the RNA-binding machinery present in the spore wall.

**Fig. 6 Spores are internalized in primary mouse ATII cells.**

- **a** Representative images of ATII cells with internalized spores. Images were obtained with differential interference contrast (DIC) or fluorescence microscopy (spore and lysosome). Lysosomes were stained with LysoTracker Red. Scale bar, 20 µm.
- **b** Internalization of wild-type (wt) or dit1Δ spores in ATII cells. Wild-type (wt) or dit1Δ spores without any treatment (—), wild-type spores treated with protease or RNase A (RNase) were incubated with ATII cells.
- **c** Internalization of spores in ATII cells treated with or without (control) piceatannol (PT) or wortmannin (WTM).
- **d** Spore internalization in ATII cells in the presence or absence (control) of DNA1.
- **e** Left panel: Levels of the *Rage* mRNA in primary mouse ATII cells transfected with two *Rage*-targeting siRNAs (siRNA1 and siRNA2) or a nontargeting siRNA control (nc). The ratios of *Rage* mRNA levels in cells transfected with *Rage*-targeting siRNAs to those in cells transfected with nc are shown. Right panel: Internalization of spores in *Rage* knockdown- and nc-transfected ATII cells. Data were presented as the mean ± SEM. Statistical significance was determined by two-tailed unpaired Student’s t-tests. *n* = 3. **P < 0.01; ***P < 0.001; ****P < 0.0001.
Yeast spores exhibit unique properties in that they are internalized by NPPs far more efficiently than polynucleotide-bound latex beads. There are two possibilities to explain the efficient phagocytosis of spores. One is that spores could accommodate more polynucleotides than latex beads with surface amine groups. The other possibility is that additional molecules bound to the RNA on the spore wall could improve internalization efficiency, although currently, the identity of such a molecule remains elusive. The RNA-binding machinery in the spore wall would be intriguing not only from the point of view of microbiology but also for the development of delivery systems for mammalian cells.

Fig. 7 RAGE ligands induce phagocytosis in NPPs. a Representative images of cells with internalized latex beads. For HMGB1 or histone beads, 49.57 or 44.40 fg/bead (respectively) of the proteins were bound to latex beads. For DNA/HMGB1 or DNA/histone beads, 65.94 or 61.39 fg/bead (respectively) of the proteins were bound to DNA beads (amount of DNA1 bound was 1.27 fg/bead). Images were obtained with differential interference contrast (DIC) or fluorescence microscopy (spore and lysosome). Lysosomes were stained with LysoTracker Red. Scale bar, 10 µm. b Internalization of latex beads in HEK293T or HEK293T RAGE KO cells. Bare latex beads (control) or the indicated beads were incubated with HEK293T or HEK293T RAGE KO cells. For BSA beads, 48.02 fg/bead of the protein-bound latex beads. c Internalization of latex beads in ATII cells. The indicated types of latex beads were incubated with primary mouse ATII cells. d Internalization of latex beads in ATII cells transfected with Rage-targeting siRNAs. ATII cells were transfected with two Rage-targeting siRNAs (siRNA1 and siRNA2) or a nontargeting siRNA control (nc). These cells were incubated with the indicated beads as described in a. Data were presented as the mean ± SEM (b–d). Statistical significance was determined by two-tailed unpaired Student’s t-tests. n = 3 (b–d). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns not significant (P ≥ 0.05).
RAGE is known to be involved in a number of disorders\(^\text{44-46}.\) Activation of RAGE can induce inflammatory responses, and many disorders are reported to be linked to RAGE-mediated chronic inflammation. Nevertheless, some disorders, such as infectious diseases, may be related to RAGE’s function as a phagocytic receptor. RAGE is highly expressed in the lung, where epithelial cells encounter a variety of microorganisms\(^\text{47}.\) Given that yeast spores are phagocytosed in NPPs, other microorganisms may be internalized in epithelial tissues via RAGE-mediated phagocytosis. In this context, it is notable that various bacteria form biofilms containing polynucleotides. Implications for RAGE in infectious diseases have been reported in mouse models. Intriguingly, RAGE may cause either beneficial or detrimental effects depending on the pathological organisms and conditions\(^\text{44.}\) Variable results may be attributable to the pathogen’s tolerance of endocytic compartments in NPPs; for some pathogens, the phagocytic process would be helpful for host invasion. In support of this hypothesis, spores internalized in HEK293T cells are alive and commence germination, which leads to the death of the cultured cells.

Since NPPs are not equipped with microbicidal mechanisms, they are vulnerable to pathogens compared to professional phagocytes. Thus, professional phagocytes perform better for the clearance of pathogens. Notably, a previous study showed that phagocytic activity in NPPs is suppressed by IGF-1 released from professional phagocytes\(^\text{48}.\) This cross-regulation may be beneficial to prevent pathogen propagation. NPPs and professional phagocytes may interact in various ways and contribute to the development and maintenance of tissues and to the disease response. We hope that our findings will pave the way for further analyses and insights into the physiological roles of phagocytosis by NPPs.

**Materials and methods**

**Mammalian cells.** HEK293T, HEK293, HeLa, and HUC cells were obtained from American Type Culture Collection (ATCC). These cells were cultured in Dulbecco’s modified Eagle’s medium (Biological Industries) containing 10% (v/v) fetal calf serum (FCS) (Biological Industries). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO\(_2\). Mouse primary ATII cells were purchased from Procell and cultured in ATII cell special medium (Procell). Jurkat cells were obtained from ATCC and cultured in Roswell Park Memorial Institute (RPMI) 1640 (Biological Industries) containing 10% (v/v) FCS. FCS was inactivated by incubation at 56 °C for 45 min before addition to media. Jurkat cells stably expressing GFP were cultured in RPMI 1640 containing 10% (v/v) FCS and 5 μg/ml blasticidin (InvivoGen). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO\(_2\).
Yeast strains, culture, and storage preparation. The strains used in this study are AN120 (wild-type) (MATa/MATa ARG4/arg4-NspI his3A50 his3A50::LEU2//2-3′ M218 ura3//3′ M218) and SK/his3 ΔSK/his3::LEU2//2-3′ M218 ura3//3′ M218 (MATa/MATa ARG4/arg4-NspI his3A50 his3A50::LEU2//2-3′ M218 ura3//3′ M218) and H3S (15×) (MATa/MATa ARG4/arg4-NspI his3A50 his3A50::LEU2//2-3′ M218 ura3//3′ M218) and H3S (15×). These strains are SK-1 background strain that sporulates with high efficiency58. To express GFP in AN120, the haploid strains were transformed with pRS306TEF-GFP digested with EcoRV and the transformants were mated. Yeast cells were cultured in YPAD (10 g/l yeast extract, 20 g/l peptone, 30 mg/l adenine, and 20 g/l glucose). Agar (20 g/l) was added to prepare plates. Yeast cells mants were mated.

Transfection of mammalian cells. For transfection of mammalian cells stably expressing GFP. The GFP (mEGFP) was digested out of pME-mEGFP. Transfection of mammalian cells was performed with RNA interference (RNAi) with small interfering RNA (siRNA). One siRNA (siRNA1) was purchased from Santa Cruz Biotechnology and the other siRNA (siRNA2) (sense, 5'-GCACUUAGAUGGGAAACUTT-3') was produced by GenPharma. ATII cells (2.5×10⁵) were seeded in 24-well plates and were cultured for 20 h; then, 20 pmol of siRNA was transfected with GenMuteTM siRNA Transfection Reagent (SignaGen) according to the manufacturer’s instructions. The sequences of non-targeting siRNAs were as follows: sense 5'-UUUCGGAAGUGUCCAGUTT-3'; antisense 5'-ACUGAGAGUGUGGAGAAUUT-3'. After 6 h of incubation, the cells were subjected to qRT-PCR or siRNA or latex bead internalization assays.

Plasmids. All the oligonucleotides and plasmids used in this study are listed in Supplementary Tables 1 and 2, respectively. For the CRISPR-Cas9 system used to knockout target genes, guide RNA sequences were designed using the E-CRISPR website52 (RRID:SCR_019088), and the corresponding DNA fragments were ligated into the Bpi1-digested vector pX330EGFP-hu6-GRXN3Cas958. The RAGE and SYK cDNA fragments were amplified from cDNA derived from HEK293T cells and cloned into the pME-tagRFP or pME-mEGFP vectors to generate pME-RAGE-tagRFP, pME-RAGE-mEGFP, pME-SYK-mEGFP. DNA-binding site RAGE mutants were constructed with PCR-based site-directed mutagenesis. pME-Hyg-3FLAG was used to clone RAGE341-His-FLAG. The sequences of the primers used to introduce the mutations are listed in Supplementary Table 1. The TLR3 cDNA fragment was amplified from human cDNA derived from HEK293T cells. TLR7 and TLR8 cDNA fragments were amplified from human cDNA derived from THP1 cells. TLR9 was amplified from an Ultimate33 human ORF clone (clone # 10H2194). To construct the yeast GFP expression plasmid, pRS306TEF-GFP, GFP was amplified by PCR using pME-hyg-mEGFP (S65T)-His3MX655 as a template. The TEF2 promoter and CYC1 terminator were obtained from pRS316TEF6, pPIB-mEGFP-BSD was used to make Jurkat cells stably expressing GFP. The GFP (mEGFP) was digested out of pME-mEGFP.

Transfection of mammalian cells. For plasmid DNA transfection, cells were grown to 60% confluence in 24-well plates and transfected with plasmid DNA (0.8 μg/μl) using Lipofectamine® 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Viability analysis of cultured cells. A round glass coverslip (14 mm in diameter) was placed on the bottom of 24-well plates. Cells (1.5×10⁵well) were seeded in the plate and allowed to grow to a density of 2.5×10⁵well. The cells were incubated with 0.6×10⁵ cells for 12 h at 37°C in a humidified atmosphere with 5% CO2. After being washed twice with PBS (Sangon Biotech), the cells were stained with 10 μM propidium iodide for 15 min at room temperature. After being washed with PBS three times, the dead cells were counted under a fluorescence microscope.

Establishment of knockout cell lines. To generate the RAGE knockout cell line. HEK293T cells were transiently transfected with two plasmids, pX330-RAGE gRNAA1 and pX330-RAGE gRNAB2 (Supplementary Table 2), which carried the following gRNAs targeting the exon regions of the RAGE gene: pX330-RAGE gRNAA1, CAAGAAAAACCCCCCCAGGCGC; pX330-RAGE gRNAB2, TTCTACCAGTAGACGGCCGACT. After 3 days of incubation, the cells expressing EGFP were sorted using a cell sorter S3e (Bio-Rad). Then, the collected cells were cultured for 8 days and subjected to limited dilution to obtain RAGE-knockout clone candidates. Clones lacking the wild-type alleles of the target region were selected by PCR using the RAGE check F1 and RAGE check R1 primers (Supplementary Table 1), and positive clones were further verified with DNA sequences using the Sanger method. The TLR3-knock out cell line was similarly generated using pX330-TLR3 gRNA1 and pX330-TLR3 gRNA2. The sequences of the gRNAs ligated into pX330-TLR3 gRNA1 and pX330-TLR3 gRNA2 are GTACCTGAGCAGCCTCAGGG and GCTCTTGATCTACCTGGGG, respectively. PCR primers used to select clones lacking the wild-type alleles of the target region were TLR3 check F1 and TLR3 check R1 (Supplementary Table 1). The SYK-knockout cell line was similarly generated using pX330-SYK gRNA1 and pX330-SYK gRNA2. The sequences of the gRNAs ligated into pX330-SYK gRNA1 and pX330-SYK gRNA2 are GCCGAAAGATTCCGCTTGGCAGAGCA, GCCGAAAGATTCCGCTTGGCAGAGCA, respectively. PCR primers used to select clones lacking the wild-type alleles of the target region were SYK check F1 and SYK check R1 (Supplementary Table 1).

Gene knockdown experiment. Knockdown of RAGE in mouse ATII cells was performed with RNA interference (RNAi) with small interfering RNA (siRNA). One siRNA (siRNA1) was purchased from Santa Cruz Biotechnology and the other siRNA (siRNA2) (sense, 5'-GCACUUAGAUGGGAAACUTT-3') was produced by GenPharma. ATII cells (2.5×10⁵) were seeded in 24-well plates and were cultured for 20 h; then, 20 pmol of siRNA was transfected with GenMuteTM siRNA Transfection Reagent (SignaGen) according to the manufacturer’s instructions. The sequences of non-targeting siRNAs were as follows: sense 5'-UUUCGGAAGUGUCCAGUTT-3'; antisense 5'-ACUGAGAGUGUGGAGAAUUT-3'. After 6 h of incubation, the cells were subjected to qRT-PCR or siRNA or latex bead internalization assays.

Quantitative real-time PCR (qRT-PCR). RNA was isolated from each cell by using the CellAmpTM Direct RNA Prep Kit for RT-PCR (TaKaRa). First-strand cDNA was synthesized using PrimeScriptTM RT Master Mix (TaKaRa). The reaction was performed in a 20 μl reaction mixture; cDNAs were stored at −20 °C. For qRT-PCR, the reaction mixture was prepared with TB Green® Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa) according to the manufacturer’s instructions and PCR was performed with a Prism 7000 Sequence Detection System (Applied Biosystems). The primers used for PCR are listed in Supplementary Table 1. PCR was performed under the following conditions: 95°C for 30 s, 40 cycles for 5 s, and 60°C for 30 s. The expression levels of the amplicons. Using the 2-ddCt method, relative internal mRNA expression of target genes was normalized to GAPDH.

Preparation of heat-killed spores. About 5×10⁵ spores suspended in water were incubated at 62°C for 60 min. To assay the viability of spores, 3×10⁵ of spores were spotted on the YPAD plate and incubated at 30°C for 2 days.

Expression and purification of RAGE341-His-FLAG. For the expression and purification of RAGE341-His-FLAG, pME-Hyg-RAGE341-His-FLAG was transfected into HEK293 cells cultured in 15-cm dishes. After 12 h of incubation, the medium was exchanged and the cells were further cultured for 36 h. The medium was collected and centrifugation at 3000×g for 3 min to remove cells and debris. Then, the medium was passed through a high-performance His trap column (GE Healthcare). The column was washed with PBS and RAGE341-His-FLAG was eluted by elution buffer (200 mM imidazole). For the pulldown of RAGE341-His-FLAG, His-FLAG with tRNA-bound beads, the truncated RAGE was further purified with anti-FLAG M2 affinity agarose beads (Sigma-Aldrich). 1 ml of the RAGE341-His-FLAG eluted form high-performance His trap column was mixed with 50 μl of anti-FLAG M2 affinity agarose beads (Sigma-Aldrich) prewashed with PBS and rotated at 4°C for 2 h. The agarose beads were washed with PBS four times and the recombinant RAGE341-His-FLAG was eluted by PBS containing 500 μg/ml Flag peptide.

Analysis of the internalization of spores or latex beads by mammalian cells. A round glass coverslip (14 mm in diameter) was placed on the bottom of 24-well plates. Cells (1.5×10⁵well) were seeded in the plate and allowed to grow to a density 2.5×10⁵well. The assay was performed in 500 μl of culture media supplemented with 0.025 μl of LysoTracker (Beyotime). Mammalian cells and purified spores or latex beads (2 μm in diameter, Sigma-Aldrich) were incubated in a CO2 incubator at 37°C for 1 h. Unless otherwise noted, spores or latex beads were incubated with cultured cells at 1×10⁵ or 1×10⁶ cells/ml in DMEM supplemented with LysoTracker. Then, the cells were placed on ice and washed twice with PBS, and intracellular spores or beads were analyzed under a fluorescence microscope. For RFP or RF fusion expressing cells (Fig. 5a, b and Supplementary Fig. 7b), at least 50 cultured cells were analyzed. For the other assay, at least 50 cultured cells were analyzed. Spore or latex bead-positive compartments were defined as internalized particles. For the competitive inhibition assay with polynucleotides, RNA or DNA was added to the assay media at a
concentration of 90 µg/ml prior to the addition of spores. Wortmannin, piceatannol, or latrunculin A were added at the final concentration of 100 nM, 50 µM, or 10 µM, respectively, 30-60 min before the addition of DNA/histone beads, 10³ DNA-bound beads suspended in 55 µl water were incubated with HMGB1 (Sangon Biotech) or histone (Sangon Biotech) with rotation at 4 °C for 24 h. The beads were centrifuged at 21,500× g for 5 min at 4 °C and washed twice with water. The amount of protein bound to beads was determined by subtracting the residual amount of the protein from that of the original amount. Rca kit (Byoteme) was used to measure protein amounts. To generate HMGB1-, histone-, or BSA-bound latex beads, first, 2 × 10⁴ latex beads were incubated in 500 µl of water supplemented with 4% glutaraldehyde (Aladdin) at 30 °C for 2 h and washed twice with water. A total of 10³ of gelatin-modified beads were incubated with HMGB1, histone, or BSA (Sangon Biotech) in 55 µl of water at 4 °C for 24 h with rotation. Then, the beads were washed twice with water.

Pulldown of RAGE-341-His-FLAG with RNA-bound beads. For the preparation of RNA-bound beads, 2 × 10³ latex beads were incubated with 200 ng RNA in 1 ml water at 4 °C for 24 h and washed twice with water. Seven hundred micrograms of purified RAGE-341-His-FLAG (after purification with anti-FLAG M2 affinity agarose beads) was incubated with 1.5 × 10⁴ bare beads or RNA-bound beads in 50 µl PBS at 4 °C for 2 h with rotation. Then, the beads were washed three times with PBS. The protein attached to the beads was eluted by boiling in 50 µl of SDS sample buffer and 10 µl of the samples were analyzed by western blotting.

Assay for RNA binding to RAGE-341-His-FLAG. One milliliter of the RAGE-341-His-FLAG eluted from high-performance Hitrap column was incubated with 50 µl of prewashed 341-His-FLAG M2 affinity agarose beads (Sigma-Aldrich) to attach the truncated RAGE to the agarose beads. The beads were washed three times with PBS and suspended in 50 µl RNase-free water. Then, 20 µl of RAGE-341-His-FLAG-bound agarose beads was incubated with 1.5 µg RNA fused to cyanine 3 (Cy3) at the S′-end suspended in 60 µl of RNase-free water. Then, after incubation at 4 °C for 2 h. After washing twice with water, the beads were incubated with 10% BSA at 4 °C for 1 h to block free glutaraldehyde.

Pulldown of RAGE with histone-bound beads. A total of 6 × 10⁴ latex beads were washed twice with water and incubated with 4% glutaraldehyde at 30 °C for 2 h. After centrifugation at 21,500× g for 5 min at 4 °C, the beads were washed twice with water. Glutaraldehyde-modified beads (3 × 10⁹) were incubated with 80 µg of histone or BSA at 4 °C in 100 µl of water for 12 h with rotation. After washing twice with water, the beads were incubated with 10% BSA at 4 °C for 1 h to block free glutaraldehyde. RAGE-341/T7 cells cultured in 6 cm dishes were transfected with PM-eMGFP or PME-RAGE-mEGFP using Lipofectamine 2000 and incubated for 36 h. Then, the cells were washed with PBS and incubated with 500 µl of lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% NP40) supplemented with 5 µl of EDTA protease inhibitor cocktails (MCE) on ice for 30 min. After centrifugation at 21,500× g for 10 min at 4 °C. 100 µl of the supernatant was incubated with 1.5 × 10⁶ histone- or RNA-bound beads with rotation. Then, the beads were washed four times with lysis buffer. The proteins attached to the beads were eluted by boiling in 50 µl of SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% Brij 35, 1% sodium deoxycholate) and 10 µl of the samples were analyzed by western blotting. One percent of the lysate after centrifugation was used as the input sample for western blotting.

Western blotting analysis. To detect endogenous RAGE, HEK293T and HEK293T RAGE KO cells cultured in 10 cm dishes were suspended in 500 µl of homogenization buffer (10 mM HEPES, pH 7.4, 0.22 M mannitol, and 0.07 M sucrose) supplemented with 5 µl of EDTA-Free protease inhibitor cocktails (MCE). Cells were homogenized on ice 40 times. The homogenates were transferred to 1.5 ml tubes and centrifuged at 10000× g for 5 min at 4 °C. The supernatants were further centrifuged at 100000× g for 1 h at 4 °C. The pellets were resuspended in 100 µl of homogenization buffer. Then, 10 µl of samples mixed with SDS sample buffer were boiled and subjected to 10% SDS-PAGE. To detect a loading control, GLUT1, 10 µl of the samples without boiling were subjected to 10% SDS-PAGE. A polyclonal rabbit antiflag antibody was used to detect the fusion of tagRFP to RAGE, TLR1, TLR1, TLR8, and TLR9, HEK293 cells harboring the pME-TLR3-tagRFP, pME-TLR7-tagRFP, pME-TLR8-tagRFP, or pME-TLR9-tagRFP plasmids were suspended in 500 µl of lysis buffer supplemented with protease inhibitor cocktails on ice for 30 min. After centrifugation at 21,500× g for 10 min at 4 °C, 20 µg of these samples was suspended in an SDS sample buffer and boiled for 5 min. The samples were subjected to 6% SDS-PAGE. After SDS-PAGE, the proteins were transferred to PVDF membranes (Bio-Rad). The membranes were blocked in 5% milk (Sangon Biotech) in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween-20) and probed with the appropriate antibodies diluted in QuickBlock (Thermo Scientific) 1 h at room temperature. The membranes were incubated with primary antibody and horseradish peroxidase conjugated secondary antibody at room temperature. After washing 3 times, the membranes were incubated with HRP-conjugated secondary antibodies diluted in 5% milk in TBST buffer for 1 h at room temperature.
room temperature and washed three times in TBST buffer. The following primary antibodies were used: rabbit anti-RAGE (Abcam, 1: 2000); rabbit anti-SYK (Abcam, 1: 3000); rabbit anti-GLUT1 (Abcam, 1: 8000); mouse anti-actin (TransGen Biotech, 1:3000); mouse anti-His (TransGen Biotech, 1:5000). Primary antibodies were detected using the secondary anti-mouse IgG HRP-linked antibody (TransGen Biotech, 1:5000), or anti-rabbit IgG HRP-linked antibody (TransGen Biotech, 1:5000). Signals were detected with ECL Substrate (Bio-Rad). Images were captured using a Tanon 5200 Automatic Chemiluminescence Image Analysis System.

**Microscopy.** Microscopy images were obtained using a Nikon C2 Eclipse Ti-E inverted microscope with a DS-Ri camera (live imaging and yeast images) or a Nikon C2 Eclipse Ti-E inverted microscope with confocal laser scanning microscopy (the other images) equipped with NIS-Element AR software.

For live imaging, cells transiently transfected with RAGE-mEGFP were cultured on glass-bottom dishes with 1.5 ml of DMEM. When the membrane density reached ~80% confluence, 0.06 ml of LysoTracker Red was added to the culture media and incubated for 20 min. Then, the culture media was exchanged with prewarmed DMEM-supplemented spores (1.5 × 10^7/ml). After 20 min incubation, the dish was subjected to live imaging.

Quantitative analysis of RAGE-RFP in phagocytic cup and plasma membrane was performed as follows. HEK293T cells expressing RAGE-RFP were incubated with spores (1.2 × 10^5 spores/4 × 10^6 cultured cells/ml) for 40 min. After washing with PBS twice, the cells were fixed with 4% (vol/vol) paraformaldehyde for 20 min at room temperature. The fixed cells were washed with PBS twice. Fluorescent intensities were measured in certain selected areas (32 × 55 pixels) in the phagocytic cup (plasma membrane engulfing spores) and those adjacent to each phagocytic cup. The mean pixel intensities of the selected areas were obtained with NIS-Element AR software.

**Statistics and reproducibility.** Statistical analysis of the data was performed using GraphPad Prism 8 software (GraphPad Software). All values are presented as the means ± SEM (n as indicated in the figure legends). Statistical significance was determined with a two-tailed unpaired Student’s t-test. P < 0.05 was considered statistically significant.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The data supporting the findings of this study are available within the Article and its Supplementary Information. The RNA-sequencing data have been deposited in the NCBI Sequence Read Archive database (accession number PRJNA748575). Source data of the graphs presented in the figures are available in Supplementary Data 1. Unedited immunoblots and gels are provided as Supplementary Figs. 11–13 in the Supplementary Information file. Further relevant data were available from corresponding authors upon reasonable request.

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