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Phagocytosis and Phagosome Acidification Are Required for Pathogen Processing and MyD88-Dependent Responses to Staphylococcus aureus

W. K. Eddie Ip,*1,2 Anna Sokolovska,*,1 Guillaume M. Charriere,* Laurent Boyer,* Stephanie Dejardin,*, Michael P. Cappillino,*, L. Michael Yantosca,*, Kazue Takahashi,*, Kathryn J. Moore,† Adam Lacy-Hulbert,* and Lynda M. Stuart*

Innate immunity is vital for protection from microbes and is mediated by humoral effectors, such as cytokines, and cellular immune defenses, including phagocytic cells (e.g., macrophages). After internalization by phagocytes, microbes are delivered into a phagosome, a complex intracellular organelle with a well-established and important role in microbial killing. However, the role of this organelle in cytokine responses and microbial sensing is less well defined. In this study, we assess the role of the phagosome in innate immune sensing and demonstrate the critical interdependence of phagocytosis and pattern recognition receptor signaling during response to the Gram-positive bacteria Staphylococcus aureus. We show that phagocytosis is essential to initiate an optimal MyD88-dependent response to Staphylococcus aureus. Prior to TLR-dependent cytokine production, bacteria must be engulfed and delivered into acidic phagosomes where acid-activated host enzymes digest the internalized bacteria to liberate otherwise cryptic bacterial-derived ligands that initiate responses from the vacuole. Importantly, in macrophages in which phagosome acidification is perturbed, the impaired response to S. aureus can be rescued by the addition of lysostaphin, a bacterial endopeptidase active at neutral pH that can substitute for the acid-activated host enzymes. Together, these observations delineate the interdependence of phagocytosis with pattern recognition receptor signaling and suggest that therapeutics to augment functions and signaling from the vacuole may be useful strategies to increase host responses to S. aureus.

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Phagocytosis is an evolutionarily ancient and conserved component of defense against pathogen invasion (1, 2). Material engulfed by phagocytosis is delivered into an intracellular organelle, the phagosome (3), which is constantly remodeled by fusion and limited fission events with endosomes and lysosomes (4). These changes ultimately deliver the internalized particle into a highly hydrolytic and bactericidal compartment known as the phagolysosome. Recent proteomic analyses showed that >600 proteins potentially associate with these organelles (5, 6). Some of the phagosome proteins reside in distinct flotillin-rich membrane domains that are likely to be dedicated regions for the assembly of signaling complexes (7). Supporting this possibility, we recently identified components of numerous signaling pathways associated with these organelles (6); our data suggest that the signals that emanate from the phagosome are likely to include defense pathways that are able to signal via NF-κB and MAP kinases.

Professional phagocytes, such as macrophages and neutrophils, destroy engulfed material and, after pathogen encounter, are potent secretors of proinflammatory cytokines. The inflammatory response to pathogens is triggered by pattern recognition receptors (PRRs), such as the TLRs that initiate inflammatory-signaling cascades (8, 9). Although these receptors are highly expressed by phagocytic innate immune cells, it is clear that they are not bona fide phagocytic receptors that participate in the cytoskeletal changes required for particle internalization. Instead, TLRs function almost exclusively to sense microbes and regulate proinflammatory signaling cascades. TLRs are found on the cell surface and in intracellular compartments, such as endosomes (TLRs 3, 7 and 9) and phagosomes that form around internalized bacteria and other large particles (TLR2 and TLR4) (10–12). The observed recruitment of surface TLRs to phagosomes provides strong support for the proposition that these organelles might function to not only destroy internalized bacteria but also contribute to pathogen sensing (10, 11, 13). Although subject to some debate, it was also suggested that these phagosome-associated TLRs might regulate phagosome maturation in an organelle-autonomous manner (14, 15). However, despite nearly a decade passing since the original observation of the association of TLRs with phagosomes, the full contribution of these organelles and associated PRRs to innate immune signaling remains to be fully defined.

We set out to formally assess the relationship between phagocytosis and innate immune signaling. We demonstrate the critical...
role of the phagosome in sensing and responding to the Gram-positive *Staphylococcus aureus* but not the Gram-negative bacteria *Escherichia coli*. We show that phagocytosis is needed for digestion of the microbe and presentation of material derived from internalized *S. aureus* to trigger TLR-dependent responses. Importantly, TLR-dependent responses to *S. aureus* occur only after phagosome maturation is complete, because the vacuole must acidify to allow activation of pH-dependent host enzymes that liberate the bacterial-derived ligands required for the full immunogenicity of these microbes. Together, these observations emphasize the interdependence of phagocytosis and PRR signaling for optimal response to *S. aureus* and suggest that strategies to augment functions of the phagocytic vacuole might be potential adjunct therapies to increase host response to this important re-emerging pathogen.

### Materials and Methods

#### Mice and cell cultures

*C.57Bl/6* mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Myd88*−/− mice were from M. Freeman (Massachusetts General Hospital, and *Thr2*−/− and *Thr4r*−/− mice were from R. Medzhitov (Yale University School of Medicine, New Haven, CT). All mice were kept and handled under a protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. All experiments were performed on thiglycollate-elicted peritoneal macrophages, unless otherwise stated. Macrophages were collected from mice by peritoneal lavage 4 d after i.p. injection of 3% thiglycollate (Difco Laboratories, Detroit, MI) and maintained in DMEM (Life Technologies, Carlsbad, CA) containing 10% heat-inactivated (HIA) FCS (Life Technologies) and penicillin-streptomycin.

Macrophages were collected from mice by peritoneal lavage 4 d after i.p. injection of 3% thiglycollate (Difco Laboratories, Detroit, MI) and maintained in DMEM (Life Technologies, Carlsbad, CA) containing 10% heat-inactivated (HIA) FCS (Life Technologies) and penicillin-streptomycin (50 IU/ml and 50 µg/ml; Mediatech, Herndon, VA); HEK293T and J774.A.1 cell lines (American Type Culture Collection [ATCC], Manassas, VA) were maintained according to the ATCC’s recommendations.

#### Bacterial strains

The strains of *S. aureus* used were Reynolds capsular serotype 5 (CP5), capsule-negative mutant (provided by Dr. J.C. Lee, Brigham and Women’s Hospital, Boston, MA), and Newman (provided by Dr. Fred Ausubel, Massachusetts General Hospital) and were grown at 37°C in Columbia media supplemented with 2% NaCl. Group B Streptococcus (strain GBS type III COH1-1; provided by Dr. Michael Wessels, Children’s Hospital, Boston, MA), *E. coli* (strain K12; ATCC), and *Salmonella montevideo* (strain SH5770; provided by Dr. Helena Mäkelä, National Public Health Institute, Helsinki, Finland) were grown as described previously (12, 16). The bacteria grown to the midexponential phase (OD₆₀₀ = 0.6–0.8) were HIA (65˚C for 30 min) or used as live bacteria grown to the midexponential phase (OD₆₀₀ = 0.6–0.8) were HIA (65˚C for 30 min) or used as live bacteria (12, 16). Briefly, peritoneal macrophages in DMEM with 1% FCS were incubated with HIA bacteria, labeled with TAMRA (Molecular Probes, Eugene, OR), at MOI of 25, for 30 min on ice, allowing the labeling of several single bacterial particles.

#### Bacterial digestion in vitro

*H. influenzae* was preincubated with 5 µg/ml lysostaphin in the indicated pH. The digestion of the bacteria over the indicated time course was assessed by measuring OD₉₀₀ of the bacterial suspensions. To assess lipoteichoic acid (LTA) release, the supernatants from 2 h of digestion with lysostaphin were collected, filtered with a 2-µm filter membrane, and assayed for LTA concentrations by ELISA. In some experiments, the filtered digest was from bacteria treated for 2 h with 5 µg/ml lysostaphin in PBS (pH 7.5) or 5 µg/ml lysozyme in potassium phosphate buffer (pH 6) were used to stimulate macrophages.

#### Reagents and plasmids

LTA (derived from *S. aureus*), LPS (derived from *E. coli* 026:B6), lysozyme (from human neutrophils), and protease inhibitor panel were purchased from Sigma-Aldrich (St. Louis, MO). Peptidoglycan (PGN; derived from *S. aureus*) was from InvivoGen (San Diego, CA). Recombinant lysostaphin was from Abzyme (Needham, MA). Monoclonal anti-LTA Ab (clone 55) was used to detect LTA release was from Cell Science (Canton, MA). Yellow fluorescent protein-tagged TLR2 (pcDNA3.1-TLR2-YFP) and cyan fluorescent protein-tagged TLR6 (pcDNA3.1-TLR6-CFP) expression vectors were kindly provided by D. Golenbock (University of Massachusetts Medical School, Worcester, MA).

### Cell stimulations and treatments

Peritoneal macrophages in DMEM medium with 1% FCS were stimulated with HIA or live bacteria, at the indicated multiplicity of infections (MOIs), or bacterial ligands (i.e., LPS, LTA, or PGN), at the indicated concentrations, at 37°C in 5% CO₂ for 2 to 4 h. To assess the role of phagocytosis in the induction of cytokine responses, prior to the stimulation with bacteria, macrophages were pretreated with 6 µM cytochalasin D (Sigma-Aldrich) for 60 min to inhibit phagocytosis. Inhibition of phagosomal acidification was done by preloading the cells with 50 nM bafilomycin A (Calbiochem, San Diego, CA) 60 min before the stimulation. Cells pretreated with the same volume of vehicle (DMSO) were used as a negative control for both inhibitors. In experiments in which live bacteria were used to infect macrophages, viability at 2 h was assessed by trypan exclusion and showed no significant cell death (<5%) in control or treated macrophages. Additionally, survival of different *S. aureus* mutants was determined by gentamicin-protection assay. No significant differences in bacterial numbers and survival were noted over the first 2 h. Where indicated, the cells were preloaded with 15 µg/ml lysostaphin, 15 µg/ml lysozyme, or protease inhibitors (Protease Inhibitor Panel; Sigma-Aldrich) at the indicated concentrations, 15 min before the stimulation. NF-κB activity was measured using a dual luciferase reporter assay for NF-κB activation in HEK293T cells transfected with TLR2/6, as previously described (17).

### ELISA

Cytokine secretion in cell culture supernatants was assayed for mouse IL-1β and -6 and TNF-α (Duoset ELISA Development System, R&D Systems, Minneapolis, MN), in accordance with manufacturer’s protocol.

#### Quantitative real-time PCR

Total RNA was extracted from stimulated macrophages using TRIzol reagents (Invitrogen, Carlsbad, CA), cDNA was synthesized from total RNA by reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and gene expression of *tnf-α* and *Il-6* was measured by quantitative real-time PCR (qPCR) using SYBR Green PCR core reagents (Applied Biosystems). The level of expression of each gene was determined by normalizing its mRNA quantity to the quantity of * GAPDH* in the same samples. The primer sequences used for qPCR were as follows: *tnf-α* forward: 5′-GCCGACAGAAAGCATGATCCG-3′; *tnf-α* reverse: 5‘-GCCGGGATCTTITGGG-3′; *il-6* forward: 5‘-TGTTCCTC-TGGGAAATCTGTGGA-3′; *il-6* reverse: 5‘-AAGTGCATACCTGTTG CTACA-3′; *gapdh* forward: 5‘-TGTCTCCTACCCCAATGTTG-3′; and *gapdh* reverse: 5‘-TGTAGGGAGATGCTAGTGG-3′.

### Phagocytosis and intracellular TNF-α

Phagocytosis and simultaneous cytokine response was measured as described previously (12, 16). Briefly, peritoneal macrophages in DMEM with 1% FCS were incubated with HIA bacteria, labeled with TAMRA (Molecular Probes, Eugene, OR), at MOI of 25, for 30 min on ice, allowing the synchrony of bacteria binding onto the cell. In all cases, before the incubation with macrophages, bacterial clusters were disrupted by passing them through a 30-gauge needle. After 30 min on ice, the cells were further incubated for the indicated times at 37°C in the presence of GobiStop (BD Biosciences, San Jose, CA) to accumulate intracellular TNF-α. The cells were washed twice with ice-cold PBS containing 5 mM EDTA (PBS/EDTA), detached with scrapers, and fixed in 3% paraformaldehyde. The cells were permeabilized and stained with allopheocyanin-conjugated anti-mouse TNF-α Ab (BD Biosciences) diluted in PBS with 0.2% saponin. After washing, the cells were analyzed by flow cytometry performed on FACS Calibur (BD Biosciences); analysis was performed with CellQuest Pro software (BD Biosciences) to determine phagocytosis and intracellular TNF-α production at the single-cell level. To estimate the number of bacteria engulfed by a single cell, TAMRA-labeled bacteria used in the same experiment were also analyzed by flow cytometry to quantify the mean fluorescence intensity (MFI) of a single bacterial particle. The amount of TAMRA fluorescence internalized by macrophages was quantified by FACS. Because the TAMRA dye was stable and knowing the MFI of an individual bacterium, estimates of the number of bacteria internalized could be extrapolated from the total internalized fluorescence. This allowed us to determine, using the mean intracellular TNF-α production in cells with the same bacterial loads (Supplemental Fig. 2), cytokine production normalized for bacterial load. In some cases, TNF-α induction at the transcriptional level was also determined in cells with different bacterial loads. To do that, phagocytosis of TAMRA-labeled bacteria was monitored simultaneously by flow cytometry, and cells that had engulfed different amounts of bacteria were sorted for total RNA extraction and subsequent measurement of TNF-α gene expression by qPCR, as described above.
Phagosomal pH

Peritoneal macrophages in DMEM with 1% FCS were incubated with S. aureus labeled with FITC (pH sensitive) and Alexa Fluor 647 (pH insensitive) fluorescent dyes (Fig. 4) at low MOI (≤10) for 30 min on ice, allowing the synchronization of bacteria binding onto the cell. In all cases, before the incubation with macrophages, bacterial clusters were disrupted by passing them through a 30-gauge needle. After 30 min on ice, the cells were further incubated for the indicated times at 37°C. The cells were washed twice with ice-cold PBS/EDTA, detached, and immediately analyzed by flow cytometry to determine the MFI emission between FITC and Alexa Fluor 647. To calculate pH using the ratiometric assay, values were compared to a standard curve obtained by resuspending and permeabilizing the cells that had phagocytosed bacteria for 2 h in buffers at a fixed pH (ranging from 3.5–8) and containing 0.05% Triton X-100. The cells were immediately analyzed by flow cytometry to determine the emission ratio of the two fluorescent dyes at each pH (Fig. 4C). In some cases, before the incubation with the bacteria, macrophages were treated with bafilomycin A (Calbiochem). Cytokine secretion and gene expression in the cells arrested at different phagosomal pHs were also determined by ELISA in the culture supernatants and RT-qPCR of the total RNA extracted from the same samples, as described above.

S. aureus infection in vivo

C57BL/6 mice 6–12 wk old were used for all in vivo experiments. In vivo S. aureus infections were performed as described previously (18). Previous studies reported in vivo blockade of the vacular adenosine triphosphatase (v-ATPase) with single injections of 25 ng/g body weight or ~0.5 μg/mouse bafilomycin A (19, 20). To determine the role of phagosome acidification in vivo, mice were injected i.p. with 0.5 μg bafilomycin A or with the same volume of vehicle (DMSO) at 5, 1, 4, and 8 h to decrease vacuolar acidification. S. aureus (5 × 10^7 CFU) was inoculated i.v. at 0 h, and mice were sacrificed at 4 h to measure cytokines and at 18 h to measure bacterial load.

Results

Response to S. aureus requires phagocytosis

TLRs were reported to associate with phagosomes containing a variety of particles, suggesting a relationship between engulfment and sensing of certain microbes. To determine the role of microbial uptake in initiating the innate immune response to different pathogens, we first tested whether it was required for cytokine production by blocking bacterial internalization with cytochalasin D. To obviate the problem of different rates of replication altering the effective MOIs during the course of the assay and, hence, confounding the interpretation of results, we first tested a number of HIA bacteria. Inhibiting phagocytosis did not affect macrophage response to heat-killed S. montevideo or E. coli or purified TLR ligands LPS and LTA (Fig. 1A). In contrast, inhibition of phagocytosis completely blocked the production of TNF-α (Fig. 1A) and IL-6 (Supplemental Fig. 1) in response to heat-killed Gram-positive Group B Streptococcus and S. aureus. These data suggested that the response to Gram-positive bacteria was intimately associated with internalization; to further explore this possibility, we chose to focus subsequent experiments on S. aureus. To ensure that the observed decreased stimulatory capacity in the absence of internalization was not a consequence of loss of immunogenicity during heat inactivation, we performed similar experiments and decreased internalization of live S. aureus using cytochalasin D. Similar to observations using HIA bacteria, decreasing the phagocytosis of live S. aureus decreased TNF-α and IL-6 (Fig. 1B) production, indicating that the majority of inflammatory signaling in response to S. aureus occurred after internalization of the bacteria.

To determine the relationship between phagosome formation and innate immune activation, a FACS-based assay, in which phagosome number and cytokine production could be simultaneously measured at a single-cell level, was used (Fig. 1C) (12, 16). To allow accurate measurement of internalized bacteria we used HIA bacteria labeled with TAMRA, a fluorescent dye stable within the phagosome for >4 h (data not shown), to permanently mark macrophages that had internalized bacteria. Single-cell analysis indicated that following incubation with E. coli, TNF-α was detected in the phagocytosing macrophages (top right quadrant of each plot) and macrophages that had been cultured with, but not internalized, bacteria (top left quadrant of each plot). In contrast, cytokines were produced only by macrophages that had internalized S. aureus (top right quadrant in each plot). As an example, 43.5% of macrophages that had been in contact with E. coli for 60 min, but had not internalized them, produced TNF-α, whereas only 0.3% of macrophages that had contacted, but not internalized, S. aureus had detectable TNF-α expression at this time point (Fig. 1C, top left quadrant of the 60-min plots). To further analyze the contribution of phagocytosis to the innate immune response, this assay was used to estimate the number of phagosomes (Materials and Methods, Supplemental Fig. 2) and to determine the correlation with cytokine production. Unlike E. coli, cytokine response was proportional to the estimated number of S. aureus-containing phagosomes, reaching a maximum when ~16–32 phagosomes per cell had formed (Fig. 1D). Furthermore, when macrophages were sorted into those without S. aureus-containing phagosomes or those containing low or high numbers of phagosomes, cytokine expression (as determined by qPCR) correlated with phagosome number (Fig. 1E). These data indicated that E. coli was able to trigger cytokine production from the cell surface, whereas engulfment was required for response to S. aureus.

The TLR2-dependent and -independent component of the response to S. aureus occurs from the phagosome

To determine the relative contributions of the TLR2/MyD88 signals to the phagocytosis-dependent cytokine response to S. aureus, macrophages unable to signal through these pathways were analyzed using our FACS-based assay. Because it was suggested that TLR signaling is involved in the rate of phagocytosis and phagosome maturation (14), we first tested the kinetics of S. aureus uptake in MyD88−/− macrophages. No defect in bacterial internalization was detected in the absence of this adaptor (Fig. 2A), indicating that these MyD88-dependent signals did not participate in the initial uptake process and, hence, would not confound our interpretation of subsequent results. Consistent with a critical role of MyD88-dependent signals for response to S. aureus, phagosome- associated cytokine production was abolished in macrophages lacking MyD88 (Fig. 2A, 2B). In contrast, Tlr2−/− cells demonstrated ~50% reduction in the production of TNF-α (Fig. 2B, 2C), confirming the role of this receptor in S. aureus response, as well as suggesting that other receptors that use the MyD88 adaptor are involved in sensing these bacteria, potentially by responding to nucleic acids liberated after bacterial destruction in the vacuole.

To formally test whether phagocytosis was required for the TLR2-dependent or -independent component of the response to S. aureus, we used Tlr2−/− macrophages and determined the consequence of blocking internalization on their ability to respond. Consistent with our FACS assay, Tlr2−/− macrophages demonstrated a 50% reduction in TNF-α secretion, confirming a TLR2-independent component of this response to S. aureus (Fig. 2C). Cytochalasin D completely blocked cytokine production by S. aureus in wild-type (WT) and Tlr2−/− macrophages, indicating that TLR2-dependent and -independent components of the response to this Gram-positive bacteria were sensitive to inhibition of internalization (Fig. 2C). When similar experiments were performed using Tlr4−/− macrophages stimulated with E. coli, we also observed a residual, TLR4-independent component of the response to this Gram-negative bacterium. However, in contrast to what was observed for S. aureus, but in keeping with our other observations, cytochalasin D had no effect on the TLR4-dependent or -independent response to E. coli (Supplemental Fig. 3).
FIGURE 1. Bacterial internalization is required for macrophages cytokine response to *S. aureus* and Group B Streptococcus but not to *E. coli* or *S. montevideo*. 

**A** and **B**, TNF-α production by peritoneal macrophages pretreated with DMSO (control) or 6 µM CytoD for 30 min to block internalization and incubated with HIA *E. coli*, *S. montevideo*, *S. aureus* (Reynolds CP5 strain), or Group B Streptococcus at MOI 50 or bacterial ligand LPS (10 ng/ml) or LTA (2 µg/ml) (A) or exposed to live *S. aureus* strain Reynolds CP5 or Newman at MOI 10 (B). Induction of cytokine responses at 2 h (TNF-α) or 4 h (IL-6) was measured by ELISA in culture supernatants. Data represent mean ± SD of triplicates. 

**C**, Single-cell analysis by FACS determined bacterial engulfment and intracellular TNF-α production. Peritoneal macrophages were preincubated with TAMRA-labeled HIA *E. coli* or HIA *S. aureus* at MOI 25 for 30 min at 4°C to synchronize phagocytosis and for the rest of the indicated time course at 37°C. Phagocytosis and intracellular TNF-α production were measured simultaneously by FACS. Contour plots show the percentages of TNF-α-producing (top right quadrant) or -nonproducing (bottom right quadrant) cells that had phagocytosed bacteria or TNF-α-producing cells without bacterial internalization (top left quadrant). 

**D**, Correlation of intracellular TNF-α with the number of internalized bacteria for which the FACS analysis of the 120-min time point from **C** was used to estimate the number of internalized *E. coli* or *S. aureus* and to determine the correlation with intracellular TNF-α production (Supplemental Fig. 2). 

**E**, TNF-α gene expression in macrophages with low or high numbers of the phagosome containing *S. aureus*. Macrophages exposed to HIA *S. aureus* for 90 min were sorted into cells with no internalized bacteria (R0) and cells with low (R1) or high (R2) numbers of internalized bacterial (inset). TNF-α gene expression in sorted cells was determined by qPCR. Data represent TNF-α gene expression levels normalized to GAPDH. Data are representative of three (A, B) or two (C, D) independent experiments. *p ≤ 0.05; **p ≤ 0.01. CytoD, cytochalasin D.
Our observation that the TLR2-dependent and -independent component of response was dependent on phagocytosis suggested that the nature of the particulate ligand, and not the receptor it engages for signaling, dictated the need for engulfment. To test this, we used zymosan, a yeast cell wall-derived particle that, similar to *S. aureus*, recruits TLR2 to its phagosomes and signals via this PRR (10). In contrast to what was observed for *S. aureus*, blocking internalization of zymosan increased cytokine production (Fig. 2D). These data indicate that signaling from the cell surface was more efficient than from the phagosome at triggering response to this particular TLR2 ligand. Thus, despite the fact that zymosan and *S. aureus* stimulated TLR2, they demonstrated opposite requirements for phagocytosis, indicating that the cargo, and not the PRR involved in response, determines the need for internalization.

**Phagosome acidification is required for cytokine response to *S. aureus***

The nascent phagosome undergoes a process termed “maturation” by fusion and limited fission events with endosomes and lysosomes to generate an acidic and highly hydrolytic mature phagolysosome. To determine whether formation of a phagosome was sufficient for response to *S. aureus* or whether phagosome acidification was also required, we neutralized phagosomes using a weak base NH₄Cl. NH₄Cl blocked *S. aureus*- but not *E. coli*- induced TNF-α production (Fig. 3A), suggesting that phagocytosis and phagosome acidification are required for response to *S. aureus*. To further test this, we perturbed phagosome acidification using bafilomycin A, a specific inhibitor of v-ATPase. Similar to NH₄Cl, bafilomycin A had no effect on TNF-α produced by *E. coli*, *S. montevideo*, or purified TLR ligands (LTA and LPS) (Fig. 3B). In contrast, bafilomycin A

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**FIGURE 2.** The relative contributions of TLR2 and MyD88 to phagosome-dependent response to *S. aureus*. A and B. Phagocytosis and cytokine response in Myd88- or Tlr2-deficient macrophages. Peritoneal macrophages from WT, Myd88<sup>−/−</sup>, or Tlr2<sup>−/−</sup> mice on C57BL6 background were preincubated with TAMRA-labeled HIA *S. aureus* at MOI 25 for 30 min at 4˚C and incubated for the rest of the indicated time course (A) or for 4 h (B) at 37˚C. Phagocytosis and intracellular TNF-α were measured simultaneously by FACS. Contour plots show the percentages of TNF-α-producing (top right quadrant) or -nonproducing cells (bottom right quadrant) after internalization of the bacteria. Graphs in the lower row in B show TNF-α production in macrophages of different genotypes that had engulfed bacteria in the different genotypes (WT in blue; Myd88<sup>−/−</sup> or Tlr2<sup>−/−</sup> in red). The number of internalized *S. aureus* in the macrophages was also estimated and correlated with intracellular TNF-α production as in Fig. 1D. C and D. The requirement for internalization on TLR2-dependent and -independent responses to *S. aureus*. Peritoneal macrophages from WT (C, D) or Tlr2<sup>−/−</sup> mice (C) were pretreated with DMSO (control) or 6 μM CytoD and incubated with HIA *S. aureus* at MOI 50 (C) or zymosan (100 μg/ml) (D). TNF-α production at 2 h was measured by ELISA in culture supernatants. Data represent mean ± SD of triplicates. Data are representative of two (A, B) or three (C, D) independent experiments. *p ≤ 0.05; **p < 0.01. CytoD, cytochalasin D.
decreased cytokine response to \textit{S. aureus}, as measured by ELISA (Fig. 3B), intracellular cytokine staining (Fig. 3C), and qPCR (Fig. 3D). Importantly, bafilomycin A treatment decreased TNF-\(\alpha\) and IL-6 secretion in response to HIA and live \textit{S. aureus} (Fig. 3E).

To determine whether vacuolar acidification played a role in regulating cytokine responses in vivo, mice were injected at 2, 4, and 16 h with bafilomycin \(\alpha\) i.p. to reduce the efficiency of the v-ATPase in vivo (20). These mice were then challenged with \(5 \times 10^7\) \textit{S. aureus} i.v., and cytokines were measured at 4 h. Although i.p. bafilomycin \(\alpha\) is unlikely to completely block acidification in vivo, bafilomycin \(\alpha\) treatment blunted the early IL-6 response (Fig. 3F), consistent with our in vitro observations. This decreased early cytokine response was also associated with higher bacterial numbers at 24 h (Fig. 3F). Together, these data indicate that \textit{S. aureus} must be internalized and delivered into an acidic phagolysosome to trigger an inflammatory response.
FIGURE 4. Phagosome acidification is required, but is not sufficient, for response to S. aureus. A–D. S. aureus ratioimetric assay to determine phagosome pH. A, HIA S. aureus was labeled with FITC (pH sensitive) and Alexa Fluor 647 (pH insensitive) (upper panels) (original magnification ×10). Quantification of FITC (green) and Alexa Fluor 647 (red) fluorescence intensity of the dual-labeled bacteria incubated for 1–2 min in a series of known pH buffers was assessed by FACS to confirm pH sensitivity of the dyes (lower panels). pH sensitivity of FITC was also confirmed within acidic compartment in macrophages. After 30 min, FITC-labeled S. aureus were internalized into acidic compartments by macrophages preloaded with acidophilic LysoTracker (red). B, The FITC signal from these compartments (arrowheads) was significantly reduced compared with noninternalized or surface-bound FITC-labeled S. aureus (original magnification ×60, oil). C, Phagosome pH standard curves were obtained as described in Materials and Methods. D, Kinetics of phagosome pH (left panel) were measured in peritoneal macrophages using the dual-labeled S. aureus ratioimetric assay, and examples of phagosome FITC signals from the cells at 5 and 60 min are shown in the graphs (right panels). E and F, Cytokine response in macrophages with arrested phagosome pH. Peritoneal macrophages pretreated with BafA at the indicated concentrations were incubated with the dual-labeled S. aureus at low MOI (≤10). After 30 min at 4°C to synchronize uptake, macrophages were incubated for an additional 90 min (E) or 6 h (F) at 37°C, and phagosome pH was determined. Cytokine gene expression in the cells (E) and IL-6 secretion in the supernatants (F) were measured by qPCR and ELISA, respectively, and correlated with the phagosome pH. G and H, Low pH is insufficient to rescue the impaired cytokine response to S. aureus in the absence of phagocytosis. G, Peritoneal macrophages in a neutral or acidic extracellular pH were stimulated with bacterial ligand LTA (2 μg/ml), PGN (10 μg/ml), or LPS (10 ng/ml LPS). H, Peritoneal macrophages were incubated with DMSO (control) or CytoD, as in Fig. 1A, were stimulated with HIA S. aureus at MOI 50 in a neutral or acidic extracellular pH. IL-6 secretion was measured by ELISA in culture supernatants at 4 h. Data represent mean ± SD of triplicate experiments. Data are representative of three independent experiments. BafA, bafilomycin A; CytoD, cytochalasin D.
Acidification is required but not sufficient for response to *S. aureus*

To establish the exact pH required for induction of the inflammatory response, we developed a FACS-based ratiometric assay in which Alexa Fluor 647/FITC-labeled *S. aureus* acts as a pH-sensitive probe (21). Low pH quenches the fluorescence of FITC-*S. aureus* but not that of pH-stable dyes, such as Alexa Fluor 647. During phagocytosis, this pH-dependent loss of fluorescence could be visualized by microscopy as reduced intensity of FITC-*S. aureus* when localized in LysoTracker*®* (and hence acidic) phagolysosomes (Fig. 4B). The ratio of FITC/Alexa Fluor 647 correlated with the pH of the phagosome (Fig. 4C) and, using this to monitor vacuolar pH, we determined that phagosomes acidified to pH < 5.0 by 1 h (Fig. 4D). Notably, the kinetics of this acidification preceded the onset of cytokine production after *S. aureus* encounter, as determined by intracellular staining (Figs. 1C, 2A). We next used different concentrations of bafilomycin A to clamp phagosomes at different pHs (as measured using Alexa Fluor 647/FITC-labeled *S. aureus*) and simultaneously measured cytokine production. Arresting *S. aureus* phagosomes at a pH > 5 blocked cytokine production as determined by qPCR (Fig. 4E) or by ELISA (Fig. 4F).

Ligand binding to certain TLRs (such as TLR9) occurs preferentially in acidic environments (22, 23). Therefore, we determined whether the sole role of the phagosome was to provide an acidic milieu that allowed *S. aureus* to encounter, as determined by intracellular staining (Figs. 1C, 2A). We next used different concentrations of bafilomycin A to clamp phagosomes at different pHs (as measured using Alexa Fluor 647/FITC-labeled *S. aureus*) and simultaneously measured cytokine production. Arresting *S. aureus* phagosomes at a pH > 5 blocked cytokine production as determined by qPCR (Fig. 4E) or by ELISA (Fig. 4F).

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ligands within the vacuole. To test whether this was a possibility, we first compared the macrophage response to capsulated and uncapsulated *S. aureus* strains. CPS *S. aureus* induced more robust cytokine production than their capsulated counterparts (Fig. 5), suggesting that one function of the capsule might be to limit access of the host PRR to its agonistic ligands in the bacterial cell wall.

We next set out to identify host enzymes that might digest *S. aureus* and to test whether they contributed to liberate bacterial ligands and increase bacterial sensing. Lysozyme, a prominent component of the mature phagolysosome of activated macrophages, is a 1,4-N-acetylmuramidase, which catalyzes hydrolysis of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in PGN (25). It is likely that lysozyme digestion helps to open the PGN backbone and to release LTA and lipopeptides buried in this matrix. Consistent with this possibility, preloading macrophages with excess lysozyme increased the ability of *S. aureus* to induce proinflammatory cytokines, indicating that lysozyme-mediated digestion facilitated the sensing of *S. aureus* (Fig. 6A). However, lysozyme alone would not be predicted to be efficient for generation of a full repertoire of PRR ligands from *S. aureus*, especially because many strains are somewhat resistant to this enzyme (26). To identify other enzymes that might be involved in the liberation of bacterial ligands, we tested a collection of protease inhibitors for the ability to selectively block IL-6 response to *S. aureus* but not LPS. Response to *S. aureus* was inhibited by ~50% by leupeptin and pepstatin and 25% by phosphoramidon and E-64 (Fig. 6B), suggesting that serine, cysteine, and acid proteases (Supplemental Table I) may also contribute in bacterial digestion and ligand release.

**Increasing digestion of *S. aureus* can rescue cytokine responses in the absence of phagosome acidification.**

The above observations suggested that bacterial digestion might be a critical step required for response to *S. aureus*. We hypothesized

**FIGURE 7.** Lysostaphin-mediated digestion of *S. aureus* rescues cytokine responses in the absence of phagosome acidification. A and B, Lysostaphin efficiently digests *S. aureus* at neutral pH. HIA *S. aureus* were incubated without (control) or with 5 μg/ml lysostaphin at the indicated pH. The digestion of the bacteria was assessed by measuring OD of the bacterial suspensions at 600 nm over 60 min (A), and LTA release was measured by ELISA in the bacteria-free filtered supernatant at 2 h (B). C, Cryptic ligands released from lysostaphin- or lysozyme-treated *S. aureus* induce TNF-α response. HIA *S. aureus* were incubated in PBS (pH 7.5) without (−) or with (+) 5 μg/ml lysostaphin or in potassium phosphate buffer (pH 6) without (−) or with (+) 5 μg/ml lysozyme for 2 h. Soluble digests (filtered bacteria-free supernatants) were used to stimulate peritoneal macrophages, and TNF-α secretion at 4 h was measured by ELISA of culture supernatant. D, Lysostaphin-mediated digestion of *S. aureus* increases immunostimulatory capacity of *S. aureus* by release of TLR2/6 ligands. HEK 293 cells stably expressing TLR2, cotransfected with NF-κB reporter system and TLR6, were incubated with HIA *S. aureus* in the absence (control) or presence of 15 μg/ml lysostaphin. Reporter gene activity at 4 h was measured by a luciferase assay. E, Preloading of lysostaphin in macrophages rescues cytokine response to *S. aureus* in the absence of phagosome acidification. Peritoneal macrophages pretreated with BafA were preloaded with 15 μg/ml lysostaphin for 15 min and incubated with HIA *S. aureus*. TNF-α secretion at 2 h was measured as in C. Data are representative of three independent experiments. **p < 0.01. BafA, bafilomycin A.
that using an alternative pH-independent means of digesting the bacterial cell wall might increase the stimulatory capacity of \textit{S. aureus} and rescue the block seen after bafilomycin A treatment. Lysostaphin, a bacteria-derived glycolglycine endopeptidase, which cleaves the pentaglycine cross bridges of PGN found in the bacterial cell wall (27), more efficiently digested \textit{S. aureus} at neutral pH than at acidic pH, as measured by the kinetic loss of OD (Fig. 7A). Therefore, we used lysostaphin to test our hypothesis that \textit{S. aureus} digestion liberates cryptic TLR ligands. As determined by ELISA, addition of lysostaphin to bacteria caused a 5–10-fold increase in the stimulatory capacity of TLR2 ligands in \textit{S. aureus}-treated macrophages (Fig. 7B). Confirming that immunostimulatory ligands were released after bacterial digestion, filtered supernatants from lysostaphin predigested \textit{S. aureus} were more efficiently activating macrophage production of TNF-α than filtered supernatants from undigested \textit{S. aureus} (Fig. 7C). Compared with lysozyme, lysostaphin digestion was three to five times more efficient at releasing ligands from \textit{S. aureus} that stimulated macrophages. This was due, in part, to the release of TLR2/6 ligands, because \textit{S. aureus} showed increased capacity to induce NF-kB in HEK 293T cells transfected with TLR2/6 and an NF-kB reporter construct in the presence of lysostaphin (Fig. 7D). Because lysostaphin was optimally active at a neutral pH, we hypothesized that it would efficiently digest bacteria and release PRR ligands, even in bafilomycin A-treated cells in which acid-activated endogenous phagolysosomal enzymes could not function. To test this, macrophages treated or not with bafilomycin A were preloaded with lysostaphin and stimulated with \textit{S. aureus}. Consistent with our model, lysostaphin rescued the cytokine response in bafilomycin A-treated macrophages (Fig. 7E). These observations demonstrated that signaling can occur from a neutral phagosome once the pathogen has been digested and were consistent with our hypothesis that a critical role of phagosome acidification is to facilitate bacterial digestion and liberation of cryptic PRR ligands.

**Discussion**

Phagocytosis is an evolutionarily conserved and central component of host defense of many organisms (3). Critical to the process of bacterial killing is the maturation of phagosomes that ultimately fuse with lysosomal compartments that contain numerous lytic enzymes. In this study, we expanded our understanding of the role of the phagolysosome by showing that it is required not only to destroy the internalized bacteria, but also to liberate cryptic PRR ligands through digestion of the Gram-positive cell wall. Without phagocytosis and phagosome maturation, the immunostimulatory ligands of \textit{S. aureus} remain inaccessible and, hence, are unable to fully activate TLR-dependent responses. Consistent with recent work, our observations are also compatible with the proposal that phagosomes are a source of ligands for intracellular nucleotide-binding oligomerization domain-like receptors (28), which are also liberated during this pathogen processing. Thus, phagosome digestion of \textit{S. aureus} regulates two important arms of the innate immune response: TLRs and nucleotide-binding oligomerization domain-like receptors.

The demonstrated relationship between the TLRs and the phagosome links these two arms of the innate immune system and confirms their critical interdependence for optimal host defense against certain pathogens. However, not all pathogens display a similar requirement for phagocytosis to trigger a proinflammatory response as we demonstrated for \textit{S. aureus}. Gram-negative microbes, such as \textit{E. coli} or \textit{S. montevideo}, do not require internalization, presumably because LPS and the other immunostimulatory components of their bacterial cell wall are readily accessible and able to stimulate TLRs at the cell surface or within the vacuole without additional processing. Intriguingly, a different scenario exists for the yeast cell-wall extract zymosan, in which inhibition of phagocytosis increases response (29, 30). Recent work showed that Dectin-1, an important receptor that cooperates with TLR2 to mediate response to zymosan, rapidly dissociates from the phagosome, suggesting that, in cases such as Dectin-1, internalization can also be a mechanism of receptor desensitization (31). This contrasts with the situation for \textit{S. aureus}, for which we previously showed that two of the key TLR2 coreceptors (CD36 and mannose-binding lectin) need to be internalized along with the ligand to functionally cooperate with TLR2/6 in the phagosome (12, 17).

Our data indicate that phagosome acidification is an essential step for optimal MyD88-dependent responses to \textit{S. aureus} and raise the question of how the rates of acidification of \textit{S. aureus}-containing vacuoles are regulated. Although a subject of debate, TLR signals were suggested to regulate phagosome maturation in an organelle-autonomous manner (14). Although we did not set out to formally test this possibility, our data do not support a model of TLR-dependent phagosome maturation for \textit{S. aureus}. Notably, we found that phagocytosis and phagosome acidification must precede TLR signaling to \textit{S. aureus} but not purified TLR ligands, such as LTA or LPS. Instead, our observations are more consistent with those of Yates and Russell (15), who failed to observe a role for TLR signaling in phagosome maturation. Nonetheless, our data indicating the critical role of phagosome acidification in regulating the host response to \textit{S. aureus} emphasizes the importance of identifying mechanisms that regulate this process after microbial engulfment. Additionally, because perturbations in phagosome maturation underlie a number of clinical syndromes associated with increased susceptibility to \textit{S. aureus}, such as chronic granulomatous disease (32) and cystic fibrosis (33), our observations suggest that augmenting functions of this organelle to increase the rate of acidification or microbial digestion may be important adjunct therapies in such conditions. Supporting this notion, we showed that lysostaphin, a bacterial-derived endopeptidase able to substitute for host enzymes and digest \textit{S. aureus} at a neutral pH, increases host response to these bacteria; therefore, it may be a particularly useful adjunct antimicrobial in situations associated with impaired vacuolar function.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


