Phagosomal Degradation Increases TLR Access to Bacterial Ligands and Enhances Macrophage Sensitivity to Bacteria

Andrea J. Wolf, Andrea Arruda, Christopher N. Reyes, Amber T. Kaplan, Takahiro Shimada, Kenichi Shimada, Moshe Arditi, George Liu and David M. Underhill

*J Immunol* 2011; 187:6002-6010; Prepublished online 26 October 2011;
doi: 10.4049/jimmunol.1100232
http://www.jimmunol.org/content/187/11/6002

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/10/26/jimmunol.1100232.DC1

**References**
This article cites 25 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/187/11/6002.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Phagosomal Degradation Increases TLR Access to Bacterial Ligands and Enhances Macrophage Sensitivity to Bacteria

Andrea J. Wolf,* Andrea Arruda,*,† Christopher N. Reyes,* Amber T. Kaplan,*‡ Takahiro Shimada,§ Kenichi Shimada,‡ Moshe Arditi,‡ George Liu,† and David M. Underhill*§

Signaling by innate immune receptors initiates and orchestrates the overall immune responses to infection. Macrophage receptors recognizing pathogens can be broadly grouped into surface receptors and receptors restricted to intracellular compartments, such as phagosomes and the cytoplasm. There is an expectation that ingestion and degradation of microorganisms by phagocytes contributes to activation of intracellular innate receptors, although direct demonstrations of this are rare, and many model ligands are studied in soluble form, outside of their microbial context. By comparing a wild-type strain of *Staphylococcus aureus* and a lysozyme-sensitive mutant, we have been able directly to address the role of degradation of live bacteria by mouse macrophages in determining the overall innate cellular inflammatory response. Our investigations revealed a biphasic response to *S. aureus* that consisted of an initial signal resulting from the engagement of surface TLR2, followed by a later, second wave on inflammatory gene induction. This second wave of inflammatory signaling was dependent on and correlated with the timing of bacterial degradation in phagosomes. We found that TLR2 signaling followed by TLR2/TLR9 signaling enhanced sensitivity to small numbers of bacteria. We further found that treating wild-type bacteria with the peptidoglycan synthesis-inhibiting antibiotic vancomycin made *S. aureus* more susceptible to degradation and resulted in increased inflammatory responses, similar to those observed for mutant degradation-sensitive bacteria. *The Journal of Immunology*, 2011, 187: 6002–6010.

Phagocytosis is the process by which myeloid cells such as macrophages and dendritic cells internalize and kill microorganisms (1, 2). The process is intimately linked to the activation of inflammatory signals that help to coordinate broader tissue and systemic immune responses to potential pathogens. Because many soluble microbial components can stimulate strong inflammatory responses, it is clear that such responses do not always require phagocytosis. However, recent studies are beginning to define the processes by which phagosomes influence the nature and magnitude of an inflammatory response.

TLRs are key innate immune receptors that detect microorganisms and are responsible for a large fraction of the inflammatory response elicited from macrophages during bacterial, fungal, and viral infection. Some TLRs including TLR3, TLR7, and TLR9 are found exclusively in intracellular compartments where they detect nucleic acids (3). As such, the expectation that ingestion and degradation of microorganisms should contribute to activation of these receptors is implicit in their biology, although direct demonstrations of this are rare, and many model ligands are studied in soluble form. Other TLRs including TLR1, TLR2, TLR4, and TLR6 are expressed at the cell surface and can detect astonishingly small amounts of soluble ligands including LPSs and lipoproteins shed from or embedded in the bacterial cell walls (4). In these cases, signaling occurs primarily at the cell surface, and no intracellular processing of the ligands is necessary. Nevertheless, these surface TLRs are recruited to phagosomes during ingestion of microbes, although the significance of their recruitment to phagosomes has remained uncertain (5).

Whereas TLRs sense stimuli that are outside of the cell or in the lumen of intracellular compartments, another class of innate immune receptors, the Nod-like receptors, is poised to detect microbial components in the cell cytoplasm (6). Nod1 and Nod2 are specifically important for detecting fragments of peptidoglycan (PGN) from bacterial cell walls (7, 8) and are implicated in host defense against a variety of intracellular and extracellular pathogens. In the case of pathogenic bacteria that penetrate the cytoplasm, Nod1 and Nod2 would have direct access to PGN and potential ligands. However, in the case of extracellular pathogens, the mechanism by which PGNs are detected is not clear. It can be hypothesized that degradation of PGNs within phagosomes releases fragments that are transported into the cytosol for detection by Nods. Indeed, Marina-Garcı´ a et al. (9) have demonstrated that soluble muramyl dipeptide (MDP; a ligand sensed through Nod2) must be endocytosed to signal effectively.

*Staphylococcus aureus* is a Gram-positive bacterium that is both a commensal organism colonizing human mucosal surfaces and a serious pathogen that can cause life-threatening mucosal and systemic infections. Mouse studies have demonstrated that both...
TLR2 (10) and Nod2 (11, 12) are important for controlling infection by *S. aureus*. Recently, Ip et al. (13) have demonstrated that phagocytosis of *S. aureus* by macrophages contributes to the activation of MyD88-dependent signaling. In the current study, we have made use of a variant of *S. aureus* that is especially sensitive to degradation by lysozyme inside macrophage phagosomes to explore the role of phagocytosis and phagosomal degradation in the activation of inflammatory responses through TLRs and Nod2. We recently made use of these bacteria to document an absolute requirement for phagosomal degradation of PGN in activation of the inflammasome and IL-1β secretion by macrophages (14). In the current study, we have determined that phagosomal degradation can be additionally important for determining the sensitivity of macrophages to live bacteria through TLRs. In contrast, Nod2 plays little or no role in detection of infection with live bacteria. Our results suggest that *S. aureus* cell walls contain TLR2 ligands that are directly accessible to macrophage surface receptors. At reasonably low multiplicities of infection (MOIs), this accessible pool is insufficient fully to activate cytokine production by macrophages. *S. aureus* mutants that are susceptible to phagosomal degradation allow for additional activation of TLR9 that enhances and prolongs inflammatory responses to even small numbers of organisms.

**Materials and Methods**

**Bacterial strains**

All experiments were done using wild-type *S. aureus* SA113 (WT-SA) and peptidoglycan O-acetyl transferase-deficient *S. aureus* mutant (ΔoatA-SA) kindly provided by Friedrich Götz (15). *S. aureus* was grown in Luria broth or Bacto Todd Hewitt broth (THB) at 37°C to stationary phase. Bacteria were diluted to OD<sub>600</sub> = 0.4, determined to be 1 × 10<sup>8</sup> CFU/ml.

**Mice and cell culture**

TLR2<sup>−/−</sup>, NOD2<sup>−/−</sup>, and MyD88<sup>−/−</sup> mice were bred and housed under specific pathogen-free conditions in the Cedar-Sinai Medical Center animal facility. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR9<sup>−/−</sup> bone marrow macrophages were derived from bones kindly provided by Dr. Miao (Institute for Systems Biology). Bone marrow-derived macrophages (BMDM) were generated from femurs and tibias by culturing for 6–7 d in complete RPMI 1640 (10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine; Mediatech) supplemented with 10% L-cell conditioned media. BMDM were plated the night before infection. Infections were done by adding indicated MOIs of bacteria onto cells and spinning at 450 g for 5 min. The infection was allowed to progress for 30 min, and the cells were washed and put in new media containing 100 μg/ml gentamicin to kill extracellular bacteria. Lysates were harvested at designated times, and cytokine levels were measured by ELISA (BioLegend and BD Biosciences).

**Lysozyme digestion and treatment**

Bacteria were grown overnight to stationary phase as described earlier. Bacteria were washed once, resuspended in PBS, and diluted to yield OD<sub>600</sub> = 0.3 in a 200-μl volume in a 96-well plate reader. WT-SA and ΔoatA-SA cultures were split, and 300 μg/ml fresh hen egg lysozyme (Sigma) was added to one culture of each. Cultures with or without lysozyme were incubated at 37°C with shaking, and a 200-μl aliquot was removed each hour to measure the OD<sub>600</sub>. Serial dilutions of the starting cultures and the 4-h time point were plated to determine the CFUs in the cultures. Percent survival was determined to be the percentage of CFUs in the lysozyme-treated compared with untreated cultures at 4 h. Each culture was passed through an 0.2-μm syringe filter to remove whole bacteria.
BMDM were treated with 5, 10, 25, or 50 μl filtered lysate for 8–12 h. DNA was removed by incubating a portion of the lysate with RNase-free DNase I (Qiagen) at 2.5 U/ml for 1 h at 37°C.

**Antibiotic treatment**

Wild-type SA113 bacteria were grown in THB for 18 h at 37°C with shaking to reach stationary phase. The overnight culture was diluted in THB media to obtain 10^5 CFU/ml by measuring 0.4 at OD_600. The antibiotics vancomycin hydrochloride (Acros Organics) or linezolid (Pfizer) were prepared in ddH2O, filtered, and added to the bacteria at concentrations of 32, 4, and 0.5 μg/ml in RPMI 1640 plus 10% FBS media for 2 h at 37°C with shaking (minimum inhibitory concentration for both antibiotics = 4 μg/ml). After treatment, the OD was readjusted, and bacteria were plated on THB-agar plates to measure bacterial concentration. Dilutions of treated bacteria were made to obtain MOIs of 6, 12, 25, and 50 in RPMI 1640 plus 10% FBS media and added to 1 × 10^5 BMDM in 100 μl/well. Media containing vancomycin or linezolid or media containing bacteria only were used as controls. The plates were centrifuged to settle bacteria at 500 × g for 5 min at room temperature and incubated for 30 min at 37°C and 5% CO2. Postinfection, cells were washed four times in 150 μl prewarmed RPMI 1640 (no additions) to remove nonadherent extracellular bacteria, and 200 μl/well of freshly made gentamicin (200 μg/ml) was added. BMDM and intracellular bacteria were incubated at 37°C and 5% CO2 for a total of 24 h. After specific time points, the plates were centrifuged at 500 × g for 5 min at room temperature to remove cellular debris, and supernatants were collected for analysis by ELISA. The cells were lysed using autoclaved distilled water, and intracellular survival was calculated by counting CFUs on THB plates. Degradation sensitivity was assessed by centrifuging the antibiotic-treated bacteria and resuspending in sterile PBS with or without 250 ng/ml lysostaphin (Sigma). Bacteria were incubated at 37°C, and the OD_600 was measured at different time points.

**Internal bacterial pH determination**

Bacteria were grown as above, and 1 ml overnight culture was washed once with sterile PBS. Bacteria were resuspended in 5 ml sterile PBS containing 1 μM CFDA-SE (CFSE) (Invitrogen) and incubated at 37°C for 15 min (16). Bacteria were washed with PBS plus 20% FCS once followed by PBS once. Bacteria were resuspended in 1 ml PBS containing 20 μg/ml tetramethylrhodamine B isothiocyanate (TRITC; Fluka) and incubated at 37°C for 15 min. Labeled bacteria were washed 3× with PBS plus 10% FCS. The toxicity of the labeling was checked by determining the CFUs with and without labeling, which we found to have a negligible effect. The bacteria were then diluted to OD_600 = 0.4 and infected at MOI = 25 as described earlier. At different times postinfection, several TRITC and CFSE images were taken of both WT-SA and ΔoatA-SA infected cells under identical magnification and exposure settings using a Leica SPS confocal microscope. A standard curve of the ratio of CFSE/TRITC versus pH was established for each strain of bacteria by incubating each strain in buffers at different pHs containing 10 μM nigericin (Calbiochem) to permeabilize the bacteria. These images were collected with the same settings as the cell images. The fluorescence intensity of CFSE and TRITC was determined using the ImageJ software particle analysis tool and converted to pH using the standard curves.

**Quantitative PCR**

BMDM were plated and infected with each strain of bacteria as described earlier. At designated time points cells were lysed, and RNA was isolated using Qiagen RNeasy kit following the manufacturer’s protocol. cDNA was converted to pH using the standard curves.
synthesis and TaqMan real-time PCR reactions were done as previously described (17).

Results
The lysozyme resistance of *S. aureus* PGN results in significantly reduced inflammatory responses by macrophages

In our previous investigations into the mechanisms by which *S. aureus* activates the NLRP3 inflammasome in macrophages and dendritic cells, we used a bacterial strain deficient for the enzyme necessary for O-acetylation of PGN, a modification that normally renders *S. aureus* PGN highly resistant to lysozyme (15). We observed that live *S. aureus* and *S. aureus* PGN are poor activators of the NLRP3 inflammasome even at very high doses and that the lysozyme resistance conferred by PGN O-acetylation was a key feature of *S. aureus*’s reduced inflammasome activation (14).

We realized that directly comparing degradation-resistant or degradation-sensitive bacterial strains could provide a unique way to define the role of phagosomal degradation in contributing to general innate inflammatory signaling. Specifically, we hypothesized that degradation could release ligands that activate macrophages and that as a result, macrophages would be more sensitive to bacteria that are degradable. We infected BMDDM with various doses of WT-SA or ΔoatA-SA and measured TNF-α production. Consistent with our previous report, infection with high doses (MOI ≥ 25) of wild-type or mutant *S. aureus* stimulated similar amounts of TNF-α production (Fig. 1A). However, low-dose infection with ΔoatA *S. aureus* (MOI ≤ 12) produced significantly more TNF-α than that of cells infected with the same dose of wild-type bacteria (Fig. 1A). The enhanced sensitivity of macrophages to ΔoatA-SA was apparent within 4 h of infection and persisted for at least 18 h (Fig. 1B).

We next analyzed whether other common inflammatory responses to *S. aureus* exhibited similarly enhanced sensitivity to degradable bacteria. Like TNF-α, IL-6 was induced more potently by the mutant bacteria, and this difference was greatest at lower levels of infection, although unlike TNF-α there was still a significant (2- to 3-fold) difference at high levels of infection (Fig. 1C). Like TNF-α, the induction of IL-6 by the wild-type and mutant bacteria was similar (and very low) at early time points and diverged strongly after 4 h of infection (Fig. 1D). In addition to cytokines, we also looked at the induction of NO, which has been shown to play a role in killing of *S. aureus* in mice (18, 19). Previous work has indicated that for optimal NO production, BMDDM need to be primed with IFN-γ (20), so we assessed NO in BMDDM that were either unprimed or primed with IFN-γ for 24 h. In both primed and unprimed BMDDM, the ΔoatA-SA induced on average 2- to 3-fold more NO than WT-SA (Fig. 1E, 1F). The combination of these results demonstrates that the lysozyme resistance of *S. aureus* PGN significantly influences the level of proinflammatory responses mounted against the microbe and that this effect is most prominent at lower levels of microbial exposure.

**TLR2 and TLR9 are both involved in sensing internalized *S. aureus***

*S. aureus* has been shown to possess TLR2 activating lipoproteins and lipoteichoic acid, and the degradation products of *S. aureus* PGN are able to activate responses through the cytoplasmic pattern recognition receptor Nod2. Therefore, we compared the proinflammatory cytokine responses of Nod2- and TLR2-deficient BMDDM to determine the relative contributions of each receptor to the proinflammatory responses induced by the WT-SA compared with the ΔoatA-SA. Considering the ΔoatA-SA mutation results in degradation-sensitive PGN, we expected the increased inflammation would be mediated through the detection of PGN degradation products by Nod2. However, we observed little requirement for Nod2 in macrophage responses to infection with live *S. aureus* (Fig. 2). When we examined TNF-α induction at 18 h postinfection, we found that the BMDDM response to low MOI = 6 of WT-SA was largely dependent on TLR2 (85%), whereas the enhanced response to ΔoatA-SA was only 50% dependent on TLR2 (Fig. 2A). This suggests that the ΔoatA-SA releases a component that stimulates an additional inflammatory response through a mechanism other than TLR2. This additional component was unlikely to be MDP derived from PGN degradation, as the absence of Nod2 had little or no effect on TNF-α production in response to infection with wild-type or mutant bacteria. When cells were exposed to a high MOI of ΔoatA-SA (≥25), TNF-α production was no longer dependent on TLR2 (Fig. 2B). This suggests that at high MOIs, the other signal is sufficient to induce the cytokine maximally, whereas at lower MOIs, both TLR2 and the other signal are required to drive maximal signal.

Similar results were obtained when examining IL-6 production. During low-dose infection (MOI = 6), 76% of the IL-6 induced by ΔoatA-SA was TLR2 dependent (Fig. 2D). Although the induction of IL-6 by WT-SA at MOI = 6 was dramatically lower than that of ΔoatA-SA, it was similarly TLR2 dependent (61%) (Fig. 2C). However, when the dose of ΔoatA-SA was increased to MOI = 25, there was no longer any dependence on TLR2 (Fig. 2F), whereas
WT-SA induction of IL-6 was still 44% TLR2 dependent (Fig. 2E). Nod2 was not required for these responses.

To identify principle mediators of this non–TLR2-dependent response, we infected MyD88-deficient cells with bacteria. TNF-α production in response to wild-type and mutant bacteria was entirely MyD88-dependent at MOI = 6 (Fig. 3A, 3B). Even at a high MOI = 25, the response was still largely MyD88 dependent (data not shown). The lack of response in the MyD88−/− macrophages also supports our previous observation that Nod2 contributes very little to the response to S. aureus in macrophages (Fig. 2A, 2B). We therefore examined whether the additional response could come from TLR9 activated by DNA. TLR9-deficient cells respond similarly to wild-type and mutant bacteria at an early time point (Fig. 3C). However, later during infection, optimal TNF-α production is dependent on TLR9, with TLR9 deficiency reducing cytokine production induced by ΔoatA-SA to levels identical to those induced by wild-type bacteria (Fig. 3D).

**Phagocytosis and phagosomal maturation are essential for the induction of maximal inflammatory responses to S. aureus**

Taken together, the above data suggest that low-dose infection of macrophages with ΔoatA-SA induces more cytokine because, unlike wild-type S. aureus, it is more susceptible to degradation and release of DNA within phagosomes. However, it is equally possible that ΔoatA-SA are simply more fragile than wild-type bacteria and that preparations of these bacteria contain more free DNA. To address this possibility, we first examined the requirement for phagocytosis and phagosomal maturation. Macrophage internalization of wild-type and ΔoatA S. aureus was blocked by cytochalasin D, an inhibitor of the actin remodeling necessary for phagocytosis (Fig. 4A). Cytochalasin D strongly inhibited both basal TNF-α gene expression induced in response to low-dose infection with wild-type bacteria and the enhanced response to mutant bacteria (Fig. 4B). Blockade of phagosome maturation with inhibitors of acidification including bafilomycin A, ammonium chloride, or chloroquine had no effect on TNF-α production in response to infection with wild-type bacteria, but completely inhibited the enhanced response to mutant bacteria reducing TNF-α to levels identical to wild-type (Fig. 4C–E). These data are consistent with internalization being important for both the initial TLR2-dependent signal (through efficient binding and capture of bacteria and concentration of TLR2 to new phagosomes) and a later phagosome maturation-dependent TLR9-dependent enhanced response to degradable mutant bacteria.

To determine formally whether significant amounts of free DNA contribute to the enhanced response to mutant bacteria, we compared activation of macrophages with culture supernatants of wild-type and mutant bacteria before and after exposure to lysozyme. As reported previously for these bacteria, wild-type S. aureus are highly resistant to lysozyme (15), whereas ΔoatA-SA treated with lysozyme lost integrity as measured by a decrease in the OD of the culture over time (Fig. 5A). As indicated by the drop in OD, the lysozyme-treated ΔoatA-SA showed a 90% reduction in viability, whereas wild-type bacteria actually grew (Fig. 5B). These cultures were then filtered to remove any intact bacteria, and increasing amounts of the supernatants were used to stimulate BMDM. After 4 h, only the ΔoatA-SA supernatants induced TNF-α, and this induction was completely dependent on lysozyme digestion (Fig. 5C). Treating TLR9−/− cells with lysozyme-degraded bacterial supernatants demonstrated a significant release of DNA from only

**FIGURE 4.** Phagocytosis is required for optimal detection of live S. aureus, and phagosomal maturation is specifically required for enhanced response to mutant bacteria. A, BMDM were pretreated for 30 min with 5 μM cytochalasin D and infected with FITC-labeled WT-SA and ΔoatA-SA at MOI = 25 for 30 min. Phagocytosis was assessed by flow cytometry. Light-gray histogram represents phagocytosis by untreated BMDM, and black histogram is in the presence of cytochalasin D. B, BMDM were pretreated for 30 min with 5 μM cytochalasin D (CytoD) or DMSO control and infected with WT-SA or ΔoatA-SA at MOI = 6 for 4 h. TNF-α gene expression was measured by quantitative PCR. C–E, BMDM were pretreated for 1 h with either 100 nM bafilomycin A, 40 μM NH₄Cl, or 200 μM chloroquine to block phagosome acidification. Control samples were treated with DMSO for the case of bafilomycin A or left untreated for treatment with the aqueous soluble NH₄Cl and chloroquine. Cells were then infected with WT-SA or ΔoatA-SA at MOI = 6 for 4 h. TNF-α was assayed by ELISA. All data represent measurements done in triplicate ± SD, and statistics were done by Student t test. **p ≤ 0.001.
The survival of each bacterial strain was tracked by measuring the OD$_{595}$ over 4 h. After 4 h, cultures of WT-SA and oatA-SA were treated with DNase I, which resulted in a decrease in TNF-α-inducing capacity (Fig. 5E). We also assessed the lysozyme supernatants for TLR2 stimulating activity and surprisingly found an almost complete dependence on TLR2 (Fig. 5F). The data shown represent the relative roles of TLR2 and TLR9 respectively, although the absolute concentrations of cytokines measured varied with different batches of murine TLR2 and TLR9 receptor agonists. The data are displayed as the percentage CFUs in the untreated of each strain at 4 h. The survival of each bacterial strain was confirmed by plating serial dilutions of each culture at 0 h and 4 h to determine the number of CFUs. The data are displayed as the percentage CFUs in the untreated of each strain at 4 h.

Inflammatory responses to bacteria occur in two phases

We next explored whether we could directly visualize breakdown of the bacterial cell wall within phagosomes and determine whether the timing of this would be consistent with when the enhanced response to mutant bacteria develops. When macrophages are infected in vitro with S. aureus, the bacteria fail to grow, but they are poorly killed by the macrophages with about a 1 log reduction in bacterial numbers over 24 h (Fig. 6A). oatA-SA, however, suffer a greater than 100-fold reduction in cell numbers after just 1 h. To measure bacterial breakdown in real time, we opted for a strategy in which we loaded bacteria with the cytosolic fluorescent dye CFSE. CFSE, being a fluorescein-based probe, exhibits pH-dependent fluorescence. We also labeled the cell walls with the pH-insensitive probe TRITC. When these bacteria were exposed to buffers at different pH values, the bacteria maintained the cytosolic pH near neutral in buffers as low as pH 4 as illustrated by the visible CFSE fluorescence (Fig. 6B). When the bacterial membrane becomes compromised as demonstrated by adding pore-forming toxin, nigericin, CFSE fluorescence is lost as the pH of the buffer decreases (Supplemental Fig. 1A). When macrophages were infected with labeled live wild-type bacteria, the average CFSE fluorescence remained high for at least 2 h (Fig. 6C), indicating a neutral internal bacterial pH, consistent with the observation that they are killed poorly. In contrast, CFSE fluorescence in oatA bacteria remained high for up to 40 min but then started to decrease as it became exposed to the acidic environment of the phagolysosome from 60 min (Supplemental Fig. 1B), indicating breakdown of the bacterial cell wall (Fig. 6C). The fact that the observed pH drops to just 6.7 in the first 2 h does not indicate that the phagosomal pH is 6.7 but instead reflects the fact that during this interval, only a subset of bacteria completely lose cell wall integrity. To examine directly whether this timing coincides with the enhanced sensing of bacterial products, we examined TNF-α mRNA production by RT-PCR. For the first hour of infection with live bacteria, BMDM make identical amounts of TNF-α mRNA in response to wild-type and oatA bacteria (Fig. 6D). However, consistent with the release of additional stimuli by cell wall breakdown, oatA bacteria begin to trigger more TNF-α beyond 1 h.
Antibiotics targeting cell wall PGN enhance macrophage sensitivity to bacteria

Comparison of degradation-resistant and degradation-sensitive bacteria provided a useful opportunity directly to test the hypothesis that phagolysosomal degradation of bacteria enhances the sensitivity of innate immune detection mechanisms used by macrophages. ΔoatA-SA are sensitive to phagolysosomal degradation due to an alteration in the cell wall PGN. This prompted us to investigate whether other perturbations of normal bacterial cell wall architecture might make bacteria more inflammatory. We hypothesized that antibiotics that specifically disrupt cell wall architecture in *S. aureus* might be significantly more inflammatory than antibiotics that inhibit other cellular processes, as cell wall destabilization might facilitate bacterial degradation in phagolysosomes. To test this hypothesis, we treated *S. aureus* with vancomycin, an antibiotic that inhibits PGN synthesis (21), or linezolid, an antibiotic that inhibits protein synthesis (22), for 2 h. These bacteria were allowed to infect BMDM, and secretion of TNF-α was measured. Consistent with our hypothesis, we observed that vancomycin-treated bacteria stimulated significantly more TNF-α secretion than either linezolid-treated bacteria or WT-SA alone (Fig. 7A). The decreased responsiveness to linezolid-treated bacteria was not due to adverse affects of the antibiotic on the BMDM as there was no significant cell death (Fig. 7B) or change in macrophage responses to purified PGN (Fig. 7C).

Although at the highest dose both vancomycin and linezolid did kill some bacteria, when similar numbers of live bacteria were added to BMDM, vancomycin-sensitized bacteria were more susceptible to killing by macrophages (Fig. 7E). Linezolid-sensitized bacteria remained highly resistant to killing by macrophages. The enhanced sensitivity of macrophages to vancomycin-treated bacteria was partly dependent on TLR9 (Fig. 7F), consistent with the idea that the enhanced sensitivity arises from phagosomal breakdown of the bacterial cell wall. To support further this conclusion, we exposed the antibiotic-treated bacteria with lysozyme and observed that vancomycin specifically makes the bacteria more susceptible to degradation, whereas linezolid made the cells more resistant to degradation (Fig. 7G). These differences in degradation may explain the observed alterations in TNF-α induction by antibiotic-treated bacteria.

**Discussion**

Innate inflammatory signals are the culmination of the activation of several surface and intracellular receptors. By using degradation-sensitive mutants of *S. aureus*, this study provides evidence for the importance of macrophage lysosomal degradation in the induction of maximal inflammatory responses to live bacteria including the induction of TNF-α, IL-6, and NO. Our examination of the responses to a degradation-sensitive mutant of *S. aureus* has revealed that macrophage inflammatory responses occur in two phases. The initial response to *S. aureus* is a TLR2-driven response to ligands exposed on the surface of the bacteria, occurring within the first hour of infection, which our experiments found were equivalent between wild-type and the degradation-sensitive ΔoatA *S. aureus*. In addition, although these initial responses required phagocytosis, they were independent of phagosomal maturation. After phagocytosis, the lysozyme-mediated degradation of the cell wall PGN of ΔoatA-SA mutant but not the wild-type *S. aureus* releases additional TLR2 as well as TLR9 ligands and leads to a second burst of inflammatory cytokines. Further, we demonstrated the secondary induction of TNF-α transcription by ΔoatA-SA occurred concurrent with the death/cell wall disruption of the bacteria inside the macrophage phagosome. We were also able to mimic enhanced inflammatory responses by treating wild-type *S. aureus* with PGN synthesis-disrupting antibiotics.
Our data are in agreement with recent work by Ip et al. (13) showing that for bacterial pathogens, the availability of TLR ligands varies considerably between different bacterial species and correlates with whether the bacterium is Gram-positive or -negative. Whereas the work by Ip et al. (13) suggests that the responses to S. aureus are completely degradation dependent, we have shown using a specific degradation-sensitive mutant that only the second wave of responses are dependent on degradation. This difference is likely due to the fact that the Ip et al. (13) findings are based on treatment with heat-killed wild-type bacteria, whereas we have exclusively used live bacteria. Heat killing might denature the cell wall in a way that buries TLR2 ligands such that they are harder for the receptor to see without proteolytic processing of the cell wall or without concentrating TLR2 in phagosomal membranes. It is widely appreciated that S. aureus stimulates TLR2, but what is additionally revealed by our data is that being resistant to degradation significantly limits the access of TLRs to S. aureus ligands. Contrary to our results, previous work had found no role for TLR9 in sensing heat-killed S. aureus (23). But by using degradation-sensitive mutants, we have characterized an additional role for TLR9 in sensing degradation of live bacteria that is specifically subverted by resistance to phagosomal degradation. Studies with bacterial lysates hint at synergy between TLR2 and TLR9 signaling such that TLR9 signaling might enhance a predominantly TLR2-dependent signaling. However, data using whole live bacteria do not necessarily suggest synergistic signaling and suggest a simpler model in which both receptors simply add to the net inflammatory response.

Considering the bacteria we use in our experiments have a mutation specifically affecting the degradability of their PGN, we...
had anticipated that the degradation of S. aureus PGN would be integral for the importance of Nod2 signaling in S. aureus infections. However, when comparing wild-type and a degradation-sensitive mutant of S. aureus, Nod2 played very little role in the difference in inflammatory responses that we measured. Reports in the literature regarding an in vivo role for Nod2 in the sensing of S. aureus have led to varying conclusions regarding the significance of the role Nod2 plays in survival after infection (11, 12). The most consistent finding suggests Nod2 contributes to overall cytokine induction are in line with currently published work. We consider the role of Nod2 on a cellular level, specifically in macrophages, our data demonstrating a minimal role for Nod2 in cytokine levels in the bronchial lavage fluid and blood, which is affected by the overall cellularity of the tissue. However, when we consider the role of Nod2 on a cellular level, specifically in macrophages, our data demonstrating a minimal role for Nod2 in cytokine induction are in line with currently published work. Typically, artificial activation of Nod2 with pure MDP only shows an effect when synergizing with another TLR ligand resulting in a modest increase in inflammatory cytokine production (9, 24). But there is little evidence for a significant contribution for Nod2 in macrophage responses to live S. aureus. In the case of our degradation-sensitive mutant, it is likely that the release of additional TLR2 and TLR9 ligands during degradation minimizes the overall impact of losing Nod2’s synergistic effect in the Nod2−/− macrophages, accounting for the lack of a significant impact on cytokine production. It has become clear from our research that when a macrophage encounters small numbers of bacteria, the exposure of surface TLR ligands is limited. However, through lysosomal degradation and response to secondary ligands, the macrophage is able to extend and maximize the inflammatory response. The importance degradation plays in the immune response is supported by the numerous mechanisms pathogens have evolved to inhibit lysosomal fusion, acidification, and lysosomal enzymes necessary to release additional ligands and enhance innate signaling (25). Notably, we were also able to mimic our mutant bacteria by treating wild-type bacteria with the PGN synthesis-inhibiting antibiotic vancomycin. Our ability to modulate the bacteria inflammatory capacity suggests that targeting the degradability of pathogens may be a useful means of modulating the inflammatory outcome of an infection, just as it plays an important role in immune evasion by bacteria.

Disclosures

The authors have no financial conflicts of interest.

References