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Tuning Innate Immune Activation by Surface Texturing of Polymer Microparticles: The Role of Shape in Inflammasome Activation

Christine A. Vaine,*† Milan K. Patel,*† Jintao Zhu,† Eunji Lee,† Robert W. Finberg,* Ryan C. Hayward,† and Evelyn A. Kurt-Jones*

Polymeric microparticles have been widely investigated as platforms for delivery of drugs, vaccines, and imaging contrast agents and are increasingly used in a variety of clinical applications. Microparticles activate the inflammasome complex and induce the processing and secretion of IL-1β, a key innate immune cytokine. Recent work suggests that although receptors are clearly important for particle phagocytosis, other physical characteristics, especially shape, play an important role in the way microparticles activate cells. We examined the role of particle surface texturing not only on uptake efficiency but also on the subsequent immune cell activation of the inflammasome. Using a method based on emulsion processing of amphiphilic block copolymers, we prepared microparticles with similar overall sizes and surface chemistries but having either smooth or highly microtextured surfaces. In vivo, textured (budding) particles induced more rapid neutrophil recruitment to the injection site. In vitro, budding particles were more readily phagocytosed than smooth particles and induced more lipid raft recruitment to the phagosome. Remarkably, budding particles also induced stronger IL-1β secretion than smooth particles through activation of the NLRP3 inflammasome. These findings demonstrate a pronounced role of particle surface topography in immune cell activation, suggesting that shape is a major determinant of inflammasome activation. The Journal of Immunology, 2013, 190: 3525–3532.

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Abbreviations used in this article: ASC, apoptosis-associated speck-like protein; CASP1, caspase-1; CTxB, cholera toxin subunit B; Cyt. D, cytochalasin D; dA:dT, poly(deoxyadenyllic-deoxythymidylic) acid; EDC, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; KO, knockout; Lat. A, latrunculin A; NLRP3, nucleotide-binding oligomerization domain 3, leucine-rich repeat and pyrin domain containing protein 3 (NLRP3), a NOD-like receptor protein located in the cytosol of macrophages (22–29). The process of NLRP3 inflammasome activation involves a conformational change in NLRP3 into its active form, which then associates with its adapter protein apoptosis-associated speck-like protein (ASC) (30) through Pyrin domain interactions. This complex leads to the recruitment of pro–caspase-1 (CASP1) through caspase activation and recruitment domain (also known as CARD) interactions (31–33). Pro-CASP1 is then cleaved into its active form, CASP1. Active CASP1 cleaves pro–IL-1β into its active secreted form, IL-1β.
IL-1 is a potent inflammatory cytokine, important for functions including macrophage and neutrophil recruitment, as well as T cell activation (34, 35).

Although a striking correlation between geometry and engulfment rate has been established for “simple” particle shapes (i.e., spheres, ellipsoids, and discs) (15–19, 28), the potential to use more complex particle shapes to engineer the phagocytic response remains largely untapped. Furthermore, questions of how particle geometry dictates the immune response following phagocytosis, such as IL-1β release, have not been addressed. Our understanding of these effects remains fairly limited, primarily because until recently, methods did not exist to produce uniform microparticles with controlled surface chemistry and systematically varying shapes. In this study, we take advantage of a recently developed route to prepare polymeric microparticles with complex but well-controlled surface topographies, based on emulsion processing of amphiphilic block copolymers (36, 37). This provides a simple platform to compare the response of phagocytes to particles of similar overall size and surface chemistry but where the particle surfaces are either smooth or densely covered with microscale protrusions (which we refer to as textured or “budding” particles). In this way, we can assess the role of shape independent of receptor interaction with particles based on their surface chemistry. Because phagocytes respond to local surface curvature (15–19, 28), we anticipated that the regions of high curvature on the budding particles should substantially alter the immune response. We examined the acute inflammatory response to polymeric microparticles via neutrophil recruitment in an in vivo mouse peritonitis model. We further analyzed the mechanism of this response using mouse macrophages to compare the ability of smooth and budding particles to be phagocytosed, activate immune cells, activate the inflamma-
some, and induce IL-1β cytokine release.

Materials and Methods

Microparticle preparation

Generation of solvent-in-water emulsion droplets of well-controlled sizes via flow-focusing and conversion to particle suspensions were conducted as described previously (37). The resulting suspensions of polystyrene-block-poly(ethylene oxide) (PS-PEO) microparticles were dialyzed against deionized water for 2–3 d to remove glycerol and residual chloroform and then centrifuged and resuspended in fresh deionized water five to eight times to remove excess and weakly adsorbed poly(vinyl alcohol) surfac-
tant. Budding and spherical particles were ~7–8 μm in diameter. Stock solution concentrations of particles were ~1.45 × 10^7 particles/ml. PEO functionalization of small particles

PEO-coated particles with diameters of 0.5 and 1.0 μm were prepared by modifying carbonyl-functionalized PS particles (Polysciences, Warrington, PA) with amino-PEO (α-aminoethyl, α-methoxy PEO, 10 kDa; Nanocs, New York, NY). Briefly, PS-PEO particles (0.86 μmol COOH groups, in 500 μl) were allowed to react with 6 μmol N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), and 9.5 μmol N-hydroxysuccinimide in an aqueous 100 mM MES buffer (pH 6) at 4°C. Solutions of EDC and N-hydroxysuccinimide were freshly prepared. After 1 h at room temperature, activated PS particles were washed twice with an MES solution (pH 6) via centrifugation and redispersion. Next, an excess of amino-PEO (1.29 μmol) in 2.58 ml PBS (pH 7.2) was added, followed by incubation for 1 h at room temperature and then washing twice with PBS via centrifugation and redispersion. Successful functionalization was confirmed by X-ray photoelectron spectroscopy on a sample of particles de-
posited on a silicon wafer. The particles were stored in aqueous suspension at 4°C until use. EDC, MES, and PBS were obtained from Sigma-Aldrich (St. Louis, MO). The stock solution concentrations of the small spherical particles were ~1 × 10^11 particles/ml.

Electron microscopy

Microparticle morphologies were observed by scanning electron micro-
scopy (ScEM) and transmission electron microscopy (TEM). For ScEM, a droplet of aqueous dispersion of particles was allowed to dry on a clean silicon wafer, followed by coating with a thin layer of gold. Samples were imaged using a JEOL 6320 FX ScEM at an accelerating voltage of 10 kV. For TEM, a droplet of particle dispersion was allowed to dry on a copper grid coated with a carbon film (Electron Microscopy Sciences) and imaged with a JEOL 2000 FX electron microscope operated at 200 kV.

Cell culture

Immortalized mouse macrophages from wild-type (WT), NLRP3-deficient, ASC-deficient, and CASP1-deficient mice were provided by K. Fitzgerald and E. Latz (University of Massachusetts Medical School, Worcester, MA) and were generated as previously described (22) using a 22 recombiant retroviruses carrying v-myc and v-raf/m mur oncogenes. Cells were grown in DMEM supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin-
streptomycin at 37°C with 5% CO2. Cells were plated in GM-CSF (1 ng/ml; eBioscience)-containing medium for 18 h prior to stimulations.

Cell stimulations

Mouse macrophages (4–5 × 10^5) were primed for 3 h with LPS (100 ng/ml; Sigma-Aldrich) to upregulate pro–IL-1β expression or left unprimed (me-
dia) and then stimulated with microparticles (budding, spherical, or small particles) at given particle-to-cell ratios (particle number:cell number), 130 μg/ml alum (Thermo Scientific), 5 μM nigericin (Sigma-Aldrich), or transfored with 400 ng poly(deoxyadenylenc-deoxithymidylid acid (dA:dT) (Sigma-Aldrich) using GeneJuice (EMD Chemicals) for an addi-
tional 6 or 18 h. Where indicated, cells were treated with 50 μM ca-
thepsin B inhibitor, CA-074-Me (EMD Millipore), 250 nM latrunculin A (Lat. A) (Sigma-Aldrich), or 1 μM cytochalasin D (Cyt. D) (Sigma-
Aldrich). Secreted IL-1β was measured using ELISA (R&D Systems), according to the manufacturer’s instructions.

Confocal microscopy

Cells were cultured on glass-bottom 35-mm tissue-culture dishes (MatTek) in complete medium. Where indicated, cells were stained with Lysotracker Green, Hoechst 34580, and Alexa 488–chola toxin subunit B (CTxB) from Molecular Probes (Invitrogen), according to the manufacturer’s instructions. Images were taken on a Leica SP2 Acousto Optical Beam Splitter confocal laser-scanning microscope with a ×63 objective, using Leica Confocal Software. Multicolor images were acquired by sequential scanning with only one laser active per scan to avoid cross-excitation. Overall brightness and contrast of images were optimized using Adobe Photoshop CS3.

Mice injections

C57BL/6 (WT), IL-1R-knockout (IL-1R KO), and CASP1KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME). NLRP3/KO mice were provided by K. Fitzgerald (University of Massachusetts Medical School). Mice were injected i.p. with sterile PBS (400 μl), 4% thioglycollate (1 ml), or 2 × 10^8 microparticles (~450 μg). Mice were sacrificed by isoflurane inhalation, followed by cervical dislocation. Peritoneal exudate cells (PECs) were isolated 6 or 16 h after injections as described previously (29). All mouse strains, age- and sex-matched with appropriate controls, were bred and maintained at the University of Massachusetts Medical School animal facility. Experiments involving live animals were in accordance with guidelines set forth by the University of Massachusetts Medical School Department of Animal Medicine and the Institutional Animal Care and Use Committee.

Flow cytometric analysis

Neutrophils in PECs were enumerated as described previously (29). Data were acquired by DIVA (BD Biosciences) and were analyzed with FlowJo 8.8.6 software (Tree Star).

Statistical analysis

An unpaired, two-tailed Student t test was used to determine statistical significance of independent experiments where two groups were com-
pared. When more than two groups were compared, ANOVA followed by Bonferroni’s correction for posttest comparisons was used. Values of p < 0.05 were considered significant with 95% confidence intervals. Statistics were performed using GraphPad (Prism version 5.0d) software.

Results

Preparation of budding particles

PS-PEO particles are an excellent model system as PS-PEO has been well studied in the context of generation of textured particles (37). There have also been several studies examining the use of PS
and PS-PEO particles as therapeutic agents (15, 38, 39). Therefore, to study the role of surface texturing on the immune response, we prepared polymer microparticles consisting of PS-PEO diblock copolymer (38 and 11 kg/mol number-average molecular weights \( M_n \), respectively) blended with PS homopolymer (\( M_n = 12.4 \) kg/mol). Initially, the polymers are dissolved in chloroform and then emulsified in water using a microfluidic flow-focusing device to provide droplets of uniform size (37). Removal of the organic solvent by evaporation subsequently yields solid polymer microparticles of phagocytosable size (7–8 \( \mu \)m diameter). As shown in Fig. 1, by adjusting the mass ratio of PS-PEO:PS, the morphology of the particles can be changed from budding particles (100:0; Fig. 1A), which are densely coated with vesicular protrusions of 1–2 \( \mu \)m diameter, to smooth spherical particles (20:80; Fig. 1B). Because of the interfacial activity of PS-PEO, the surfaces of both types of particles are coated with a similar “brush” layer of PEO as well as residual poly(vinyl alcohol) used to stabilize the emulsion droplets.

**Budding particles stimulate a more robust neutrophil response at early time points in vivo**

We first compared the in vivo immune response to budding particles versus spherical particles. Previous studies have shown that i.p. injections of particulates (e.g., monosodium urate crystals or titanium particles) lead to an increase in neutrophil recruitment (23, 29). To determine whether our polymer microparticles induced a similar increase in neutrophil recruitment, WT mice were first injected i.p. with budding or spherical particles at three different doses: \( \sim 6.7 \times 10^2 \), 2 \( \times 10^3 \), and 2.7 \( \times 10^3 \) particles (150, 450, and 600 \( \mu \)g, respectively), and lavage of the peritoneal cavity was analyzed for neutrophil influx 6 h later. We found that the 450-\( \mu \)g dose of budding particles induced significantly higher neutrophil recruitment (Ly6G\(^+\), 7/4 \(+\) cells) over spherical particles (\( p < 0.01 \), budding versus spherical; Fig. 2A). In fact, 450-\( \mu \)g budding particles induced neutrophil recruitment at levels similar to injection with the positive control, thioglycollate (\( p < 0.001 \), PBS versus thioglycollate; Fig. 2A). Next, we compared the neutrophil recruitment to 450-\( \mu \)g particles at 6 or 16 h after injection. Both budding and spherical particles induced a significant neutrophil response over PBS-carrier only at 6 h (\( p < 0.0001 \), both) and 16 h (\( p < 0.01 \), budding; \( p < 0.0001 \), spherical) (Fig. 2B). Budding particles exhibited significantly higher levels of neutrophil recruitment than spherical particles at 6 h but only slightly higher levels at 16 h when compared with spherical particles (Fig. 2A, 2B).

**Microparticle-induced neutrophil recruitment involves IL-1–associated signaling**

The IL-1R is required for neutrophil recruitment following exposure to stimulants/particulates in mice (22, 23, 29, 40). To determine whether the IL-1R was required for microparticle-induced neutrophil influx, IL-1R KO mice and WT mice were injected with budding or spherical particles for 16 h and compared with PBS alone injections. As predicted, IL-1R KO mice did not recruit neutrophils in response to particles over PBS alone, whereas WT mice exhibited a significant increase over PBS (\( p < 0.05 \), budding; \( p < 0.001 \), spherical; Fig. 2C).

**NLRP3 inflammasome is critical for microparticle-induced neutrophil recruitment**

The NLRP3 inflammasome plays a role in the response to particulate materials (22–29) through IL-1R and IL-1\( \beta \) secretion. To determine whether components of the NLRP3 inflammasome were

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**FIGURE 1.** Images of budding and spherical microparticles. ScEM and TEM (inset images) images of budding (A) and spherical (B) particles generated. Scale bars, 10 and 5 \( \mu \)m (inset).

**FIGURE 2.** Particle-induced neutrophil recruitment depends on surface curvature and requires IL-1R and NLRP3 inflammasome–associated signaling. Flow cytometric analysis on peritoneal neutrophil (Ly6G\(^+\), 7/4 \(+\) cells) recruitment at 6 h (A, D, E), 16 h (C), or at given times (B) after microparticle injections at varying doses as indicated (A) or at a fixed dose of 2 \( \times 10^3 \) particles; \( \sim 450 \mu \)g (B, C, D, E) in WT (C57BL/6), IL-1RKO, NLRP3KO, or CASP1KO mice. Graphs show mean \( \pm \) SEM of total number of mice indicated below, performed in two to three independent experiments. Y-axis scales are \((\times 10^5)\) [(A)–(D)] or \((\times 10^7)\) (E). Number of mice: (A) PBS (0), \( n = 4 \); 150 \( \mu \)g, \( n = 2 \); 450-\( \mu \)g, \( n = 6 \) (spherical) and 8 (budding); 600 \( \mu \)g, \( n = 2 \) (spherical) and 3 (budding); thioglycollate (Thio), \( n = 3 \). (B) 0, \( n = 9 \); 6B, \( n = 8 \); 6S, \( n = 6 \); 16B, \( n = 5 \); 16S, \( n = 5 \). (C) PBS, \( n = 5 \); WT, \( n = 5 \); KO, \( n = 3 \). (D) \( n = 2 \). Significance values are shown as budding versus spherical or Thio versus PBS (A), particle versus PBS injections (B), PBS versus particle injections (C), or KO versus WT (D). *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), ****\( p < 0.0001 \).
required for particle-induced neutrophil recruitment, we examined parieternal neutrophil recruitment to budding particles in NLRP3 KO and CASP1 KO mice and to spherical particles in NLRP3 KO mice 6 h following particle injections. When budding particles were injected, we found that the NLRP3 KO and CASP1 KO mice exhibited significantly blunted neutrophil recruitment, compared with WT controls in response to budding particles (p < 0.05, WT versus all KOs; Fig. 2D). Again, spherical particles induced substantially less neutrophil recruitment than budding particles (Fig. 2E). The neutrophil response to spherical particles was also inhibited in NLRP3 KO compared with WT mice, although trending did not reach statistical significance with a p value of 0.0674.

**Budding particles stimulate more IL-1β than spherical particles**

To determine whether the observed IL-1R- and NLRP3 inflammasome–dependent neutrophil influx in vivo exhibited similar characteristics in vitro, we analyzed the IL-1β inflammasome response of murine macrophages to microparticles. Immortalized macrophages from WT mice were primed for 3 h with LPS to induce upregulation of pro–IL-1β transcription and then incubated with budding or spherical microparticles for 6 h to induce pro–IL-1β processing to mature IL-1β. Levels of mature, secreted IL-1β were measured via ELISA using cell supernatants. As predicted by the neutrophil recruitment studies, budding particles were able to induce significantly higher levels of IL-1β secretion (p < 0.0001, 0.5:1 and 1:1; Fig. 3A, □) compared with spherical particles (Fig. 3A, ▪). WT macrophages responded to both types of microparticles in a dose-dependent manner with significantly higher levels of secreted IL-1β compared with priming alone at the 1:1 particle:cell ratio (4.5 × 10^5 particles) for both types of particles as well as the 0.5:1 particle:cell ratio (2.25 × 10^5 particles) for budding particles (Fig. 3A). As a positive control, macrophages were transfected with synthetic dsDNA, dA:dT. WT macrophages produced a significant amount of IL-1β in response to dA:dT stimulation (p < 0.01; Fig. 3B). As negative controls, supernatants from macrophages that received media or an LPS prime alone did not exhibit an increase in IL-1β production. Levels of NLRP3–independent cytokine IL-6 were equivalent for all samples (Supplemental Fig. 1A). Because the neutrophil response seen with budding particles was similar to spherical particles at 16 h in vivo, we also examined the in vitro kinetics of the IL-1β response to microparticles. Unlike neutrophil recruitment, budding particles stimulated higher levels of IL-1β at all time points compared with spherical particles (p < 0.01, 6 h; p < 0.05, 16 h; Fig. 3C).

**Microparticle-induced IL-1β production requires the NLRP3 inflammasome**

To further determine whether the NLRP3 inflammasome signaling complex was involved in the IL-1β response to microparticles in macrophages, immortalized macrophages generated from NLRP3 KO, ASC KO, and CASP1 KO mice were analyzed for IL-1β production in response to microparticle stimulation. Supernatants from NLRP3 KO, ASC KO, and CASP1 KO macrophages each exhibited undetectable levels of secreted IL-β in response to budding and spherical microparticles, whereas IL-1β secretion from WT macrophages was readily detected (p < 0.0001, WT versus all KOs; Fig. 4A). As expected, NLRP3 KO macrophages could respond to dA:dT similar to WT cells, whereas ASC KO and CASP1 KO macrophages could not (p < 0.01, NLRP3 versus WT; p < 0.0001, ASC/CASP1 KO versus WT; Fig. 4B). WT and deficient cells also produced equivalent levels of the inflammasome–dependent cytokine, IL-6 (Supplemental Fig. 1B), following LPS stimulation.

**Small, spherical particles do not induce significant IL-1β secretion**

Studies have shown that particles within the range of 1–3 μm in diameter exhibit the highest phagocytic rates when incubated with macrophages (19–21). The “buds” on the budding particles are ~1–2 μm in diameter. To determine whether the presence of these small buds is responsible for the increased IL-1β secretion exhibited by budding particles when compared with spherical particles, we also tested the response to smaller spherical particles with diameters of 0.5 and 1 μm, the surfaces of which were also coated with PEO. These smaller spherical particles were incubated with WT immortalized macrophages and assayed for IL-1β secretion. Unlike the budding particles, these small (bud-sized) spherical particles did not induce a significant amount of IL-1β secretion above prime-only background values (Fig. 5), even at high particle-to-cell ratios. All samples exhibited similar levels of NLRP3–independent cytokine IL-6 (Supplemental Fig. 1C) following LPS prime. Of note, uncoated 0.5- and 1-μm PS particles were also unable to induce significant IL-1β secretion over prime-only levels (data not shown).

**Budding particles are more likely to be associated with and internalized in mouse macrophages than spherical particles**

Because inflammasome activation of IL-1β release generally involves phagocytosis of the stimulant, we examined the ability of macrophages to attach to and internalize spherical and budding particles. To visualize phagocytosis of microparticles, immortalized macrophages from WT mice were incubated for 6 h with budding or spherical microparticles containing a fluorescent dye, Vibrant DiI. Using confocal microscopy, we found that a significantly higher percentage of budding particles (>85%) bound to or internalized in macrophages (Fig. 6A, 6C, 6D) when compared with spherical particles, where only ~20% were bound or inter-
We also found that budding particles were associated with more macrophages on a per particle basis, with the majority of budding particles associated with two to three macrophages each. In other words, a single budding particle was often associated with more than one macrophage at a time (Fig. 6C, 6E). In contrast, a single spherical particle only associated with a single macrophage (Fig. 6B, 6E). These data are trending toward significance with a p value of 0.0632. As internal controls, the total number of cells per field of view and number of particles per field of view were very similar between spherical and budding particles, whereas the absolute number of bound budding particles was still significantly higher than bound spherical particles (p < 0.01, budding versus spherical; Supplemental Fig. 2A–C).

Optimal inflammasome activation in response to silica crystals, alum, amyloid-b, and titanium requires uptake through actin polymerization and release of cathepsin B following lysosomal destabilization (22, 29, 41). To determine whether actin polymerization and cathepsin B are required for inflammasome activation and subsequent IL-1β production in response to microparticles, WT immortalized mouse macrophages were treated with the cathepsin B inhibitor CA-074-Me or actin inhibitors Lat. A and Cyt. D. Supernatants from cells pretreated with CA-074-Me had substantially lower levels of IL-1β following a 6-h microparticle stimulation compared with untreated cells (p < 0.0001, untreated versus treated; Fig. 6F). In addition, cells pretreated with Lat. A or Cyt. D exhibited a complete loss of IL-1β following an 18-h microparticle stimulation (p < 0.0001, untreated versus treated; Fig. 6G). As expected,
alum-induced IL-1β requires both cathepsin B and actin (p < 0.0001, untreated versus treated, all; Fig. 6F, 6G), whereas nigericin, a potassium ionophore known to induce IL-1β through potassium efflux, lysosomal destabilization, and cathepsin B release (42), requires cathepsin B but does not require actin polymerization (p < 0.0001, CA-074-Me treatment and p < 0.01, Lat. A and Cyt. D treatments; Fig. 6F, 6G). Furthermore, macrophages that were transfected with dsDNA, dA:dT, which induces mature IL-1β production in an NLRP3-independent manner (43), were unaffected by cathepsin B inhibition (data not shown).

**Budding particles localize with lipid raft components**

Recruitment of lipid rafts plays an important role in phagocytosis and inflammatory cytokine secretion by anchoring scavenger receptors, innate immune receptors (such as CD14 and Dectin-1) during activation of downstream kinases (44–46). In addition, we have previously shown that uptake of Ab-bound membrane proteins can internalize via clathrin through the endosomal pathway (47). To further elucidate the mechanisms involved in particle internalization, WT macrophages were stained with fluorescent CTxB, which binds to GM1 gangliosides on the cell surface, as a marker for lipid rafts. Cells were then incubated with fluorescent particles for 6 h. We found that the surface of budding particles highly localized with CTxB (arrows), whereas spherical particles did not (Fig. 7). Overall, our studies identify an important role for surface texturing in microparticle phagocytosis and NLRP3 activation, ultimately leading to neutrophil recruitment (illustrated in Fig. 8).

**Discussion**

This study illustrates a significant role for surface texturing in polymer microparticle-induced inflammasome stimulation, both in vivo and in vitro independent of the chemical composition of the particle. The use of macrophage cell lines allowed us to interrogate the potential pathways triggered by particles and revealed roles for NLRP3, ASC, CASP1, and IL-1R and suggest that budding particles induce a significantly greater inflammasome response than spherical particles. These observations have been validated in vivo using neutrophil recruitment, confirming the importance of these pathways in vivo and supporting our hypothesis that surface texture is an important determinant of inflammasome activation by particles.

We and others have demonstrated a critical role for IL-1-associated signaling in neutrophil responses following injections of particulate stimuli (22, 23, 29, 40). Here we show that IL-1R KO mice exhibit a diminished neutrophil response following microparticle injections, further implicating IL-1 signaling in the innate immune response to budding and spherical polymer microparticles. Furthermore, we verify that this response also requires a functional NLRP3 inflammasome, as NLRP3 KO and CASP1 KO mice were unable to recruit a significant amount of neutrophils following particle injections. It is possible that sensors in addition to NLRP3 may play a role in particle induced neutrophil recruitment and this may account for the lack of a complete abolition of neutrophil recruitment in KO mice. However, regardless of whether NLRP3 is the only inflammasome receptor or one of several inflammasome receptors that are triggered by particles, these studies clearly demonstrate that the downstream ASC and CASP1 pathways and the IL-1R pathway are very important for neutrophil responses to budding and spherical particles.

We determined that although there is some variation in the amount of IL-1β detected in macrophage supernatants, spherical particles consistently and reproducibly induced significantly less IL-1β than budding particles in every experiment in side-by-side comparisons. Particle IL-1β induction also occurs through activation of the NLRP3 inflammasome–signaling complex, similar to that seen with other particulate stimuli (22–29). Our kinetic studies in vitro also indicate that budding particles induced an early peak and continued high IL-1β secretion over time, whereas spherical particles induced lower IL-1β secretion levels that remained relatively constant. In vivo, it is unclear why spherical particles appear to “catch up” with budding particles for neutrophil recruitment at later time points. There is likely a complex interplay between a variety of signals in vivo, including the magnitude of inflammasome activation for IL-1β secretion, levels of IL-1β produced, IL-1β–driven neutrophil recruitment, and adherence of activated neutrophils to peritoneal tissues and/or pyroptosis/necrosis of highly activated neutrophils.

Budding particles also induced a more rapid phagocytic response in vitro and were more readily taken up by macrophages than spherical particles, again suggesting that the shape of the particle

**FIGURE 7.** Internalized budding particles localize with lipid raft components. Confocal microscopy images of macrophage-associated spherical (A) and budding (B) particles. Cells were incubated with particles for 6 h and then fixed with 4% paraformaldehyde for 20 min prior to visualization. Lipid rafts were visualized with CTxB–Alexa 488 (green). Nuclei were visualized with Hoechst 34580 (blue). Arrows indicate localization of lipid rafts with particle. Images are representative of two independent experiments. Scale bars, 5 μm. Images taken with a ×63 objective.

**FIGURE 8.** Particle-induced inflammasome activation and neutrophil recruitment. Particle internalization, via actin polymerization, triggers the release of cathepsin B from lysosomes, which together activate NLRP3. Activated NLRP3 recruits ASC through PYD domain interactions. This complex triggers recruitment and cleavage of activated caspase-1 through caspase activation and recruitment domain (also known as CARD) interactions. This inflammasome complex then cleaves pro–IL-1β into its active, secreted form IL-1β, which can trigger downstream IL-1–associated signaling, including neutrophil recruitment, through activation of the IL-1R.
affects the kinetics of the innate immune response. Our studies revealed that large (7–8 μm) polymer particles are efficiently associated with and phagocytosed by macrophages. We also noted higher concentrations of lysosomes surrounding the engulfed budding particles, suggesting that the budding particles trigger a stronger cellular response. Our findings corroborate recent studies indicating that macrophages are more likely to internalize particles if they contain regions of high positive surface curvature (15–19). Budding particles are presumably phagocytosed more efficiently because they contain higher local surface curvature compared with spherical particles of the same overall dimensions.

Although particle uptake is an important parameter for drug delivery, triggering of inflammatory responses may not require complete uptake of the particle. In fact, pathogenic crystals of uric acid, silica, β-amylloid, or cholesterol have all been shown to trigger inflammasome activation and IL-1β release by a “frustrated phagocytosis” mechanism (22, 41). The current view is that macrophages attempting to engulf large crystals form a phagolysosome around the crystal. However, in the process of engulfing very large crystals, the lysosomal membranes are ruptured, thus releasing enzymes into the cytosol that trigger cytosolic inflammasomes as a result. Our data showing multiple cells associated with a single budding particle are consistent with this proposed mechanism. However, whether budding particles are completely phagocytosed or partially phagocytosed by several cells, it is still clear that actin polymerization and cathepsin B release is necessary for IL-1β induction, because inhibitors to either completely abolish the particle induced IL-1β response.

In addition to triggering a stronger cellular response, it is possible that budding particles internalize through a different mechanism of phagocytosis than spherical particles. The process of phagocytosis can occur through one of several different cell surface proteins, including complement receptors, FcRs, pathogen-specific receptors, and scavenger receptors (2, 3). Studies have indicated that scavenger receptors and caveolae/ lipid rafts are involved in the internalization of a variety of therapeutic agents (48), bacteria (8, 49), and artificial particles such as latex, TiO₂, silica, and polystyrene particles (9–11, 50). Using CTxB as a marker for lipid rafts, our findings indicate that particles with high surface curvature (budding particles) recruit lipid rafts during internalization, whereas particles with lower surface curvature (spherical particles) do not. These findings suggest that particles can potentially be tailored to internalize through a specific phagocytosis pathway, based on surface curvature.

Several studies have reported that small particles induce the highest amount of IL-1β from immune cells (19–21, 28). One possibility for the increased phagocytosis and increased IL-1β production seen with budding particles compared with spherical particles is that the 1- to 2-μm diameter buds mimic smaller particles. However, our findings indicate that small, spherical particles in the 0.5- to 1-μm diameter range do not induce significant IL-1β secretion (whether PEO-derivatized or not). It is clear that the immune response to particles with textured surfaces is more complicated than predicted by models based on size alone. The combination of regions of high positive and negative surface curvatures to form the more complex surfaces of the budding particles is apparently responsible for induction of high levels of IL-1β.

These findings also clearly demonstrate that not all phagocytes are created equal, a concept that becomes clear when comparing studies on particle stimulation. For example, Sharp et al. (28) have suggested that particles in the range of 0.5–1 μm in diameter induce the highest amount of IL-1β in dendritic cells. Other studies have shown that particles of 1–3 μm in diameter exhibited the highest phagocytic rates when incubated with 1774 mouse macrophages (19), rat alveolar macrophages (20), and peritoneal mouse macrophages (21). We have demonstrated that immobilized mouse macrophages, which have been used for inflammasome activation studies to a variety of particulate material (22, 29), appear to respond differently (and very weakly) to small, spherical particles when compared with the response from murine dendritic cells, peritoneal macrophages, and rat alveolar macrophages.

Overall, the data presented in this report have broad implications for the future development of vaccine adjuvants and therapeutic delivery agents, because variation in surface curvature will modulate the resultant immune response. We suggest that larger biodegradable particles with low surface curvature and no complex surface structures would be ideal for use as delivery vehicles, because they exhibit a low level of uptake and induce a slow immune response and, thus, may exhibit a broader biodistribution, ideal for delivery of therapeutic agents. In contrast, we suggest that particles with high surface curvature and complex surface structure would be ideal for use as vaccine adjuvants, as they are more efficiently phagocytosed in cells and induce a more robust immune response. Thus, both the chemical composition-dependent receptor engagement and surface texture–dependent inflammasome activation are key elements determining the uptake and immune activation potential of particles.

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Disclosures

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