Fine Particles That Adsorb Lipopolysaccharide *Via* Bridging Calcium Cations May Mimic Bacterial Pathogenicity Towards Cells

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Fine particles (10^2- to 10^3-nm diameter) are potentially potent adjuvants in acquired immune responses but little is known about their interaction with pathogen-associated molecular patterns (PAMPs) and impact upon innate immunity. Here we show that 200-nm–sized, food-grade titanium dioxide avidly binds lipopolysaccharide (LPS) with bridging calcium cations, and the complex induces marked proinflammatory signalling in primary human mononuclear phagocytes. In particular, caspase 1-dependent interleukin-1β (IL-1β) secretion was induced at levels far greater than for the sum of the individual components, and without concomitant secretion of modulatory cytokines such as interleukin-1 receptor antagonist or transforming growth factor-β1 (TGF-β1). Secondly, the conjugate induced apoptotic-like cell death. These responses were inhibited by blockade of both phagocytosis and scavenger receptor uptake. Specific caspase 1–facilitated IL-1β secretion and apoptosis following phagocytosis are features of cellular responses to certain invasive, enteric pathogens, and hence induction of these events may be mimicked by fine particle–LPS conjugates. The inadvertent adsorption of PAMPs to ingested, inhaled, or "wear" fine particulate matter provides a further potential mechanism for the proinflammatory nature of fine particles.
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Introduction

Exposure to respirable particles is associated with increased morbidity and mortality, especially for cardiovascular and respiratory events (1), although mechanisms are not understood. Substantial particle uptake also occurs in lymphoid-rich areas of the human gastrointestinal tract and could be involved in the etiology of inflammatory bowel disease (2–4). Ultrafine particles (<100-nm diameter) are potent nucleation sites for induction of reactive oxygen species (ROS) (5) but cellular uptake is either largely nonactive or mediated through clathrin-coated pit endocytosis (6), neither of which appears to substantially activate further cytoskeletal events. In contrast, fine particles (100-nm to 1-μm diameter) and small coarse particles (1- to 3-μm diameter) are actively phagocytosed or macropinocytosed by phagocytic cells (7–13), which triggers membrane ruffling and cytoskeletal rearrangement that can profoundly influence cell survival (7–9), cytokine secretion (9, 10) and antigen presentation (11–13).

Environmental particles generally have large, negatively charged surfaces and favor the adsorption of cations, which further facilitates the adsorption of macromolecules such as exogenous antigens and bacterial proteins, as well as self proteins (14–18). Conjugation of proteins to particle surfaces, and the subsequent effects on cellular processing and presentation of antigen, have been well studied *in vitro*. Antigen-specific T cell proliferative responses are amplified for particulate versus soluble antigen, and major histocompatibility complex class switching may also occur (11–13). Hence, fine particles can promote immune responsiveness, which may have pathological implications (19, 20); for example, fine particles from diesel fumes can adsorb environmental antigens, such as the grass pollen major allergen, Lol p1 (21), and then act as mucosal adjuvants, skewing antibody and cytokine responses *in vivo* (22). Similarly, immune response to wear particles from titanium

implants may have important consequence in failure of these implants.

In contrast to such work with protein antigens, the conjugation of particles and major bacterial components (commonly now termed pathogen-associated molecular patterns [PAMPs]) and their influences on innate cellular responsiveness have been less well investigated. Yet this may be an especially important process in the gastrointestinal tract (23). In the Western world, about 10^{12} particles of titanium dioxide (TiO_2) are ingested per person per day (23), and probably about 10^{10} are absorbed. The interactions of dietary particles with endogenous bacterial products and mucosal secretions of the gastrointestinal tract have not been studied, but lipopolysaccharide (LPS), for example, is abundant, and it avidly binds to particle surfaces such as TiO_2 with calcium-bridging cations. Previously, we showed that particles of food additive–grade TiO_2 (mean diameter 200 nm) amplify calcium LPS-induced interleukin-1β (IL-1β) secretion of intestinal mononuclear cells (24, 25). This is especially marked in cells from patients with active inflammatory bowel disease, but the mechanisms involved and the specificity of action are unclear. Hence, in addition to ROS induction (5, 26) and adjuvant activity for antigen (11–13), this may be a further mechanism for the proinflammatory effects of environmental particles and it may be particularly relevant to the gastrointestinal tract. Indeed, it should be noted that under steady-state conditions, resident commensal gut bacteria do not cross the gut barrier to any signifcantly identifiable extent. Barrier functions provided by IgA, the mucus layer, the glycocalyx, and endless cellular factors prevent such unsolicited entry. However, through surface adsorption, particles could provide access of bacterial components into the mucosa, acting as Trojan horses and evading the normal host defenses. Certainly large numbers of these microparticles are observed in human intestinal tissue (3). Thus if, prior to tissue uptake, these microparticles become surface-modified through the adsorption of bacterial luminal components, then they could mimic simple invasive pathogens *via* induction of phagocytosis and the carriage of surface PAMPs such as LPS. With enteric infections such as *Shigella flexneri, Salmonella typhimurium*, and *Campylobacter jejuni*, cell death and caspase 1–dependent IL-1β release are hallmarks of the phagocyte response (27–29). Here, we investigated the adjunct effect of particulate titanium dioxide on LPS-induced proinflammatory signalling; we investigated the specificity of IL-1β secretion, cleavage of caspase 1, apoptosis, and the pathway of receptor-mediated uptake.

Materials and Methods

LPS Adsorption to Dietary Titanium Dioxide. Preliminary experiments were performed to assess optimal LPS adsorption to particulate TiO_2 using flow cytometry. This technique has been previously described for the detection of fluorescent latex particles in macrophages, following phagocytosis, through changes in fluorescence and right-angled light scatter (30–31). The presence of particles is identified by fluorochromes bound to the particle surface, whereas light scatter measurements indicate the degree of particle aggregation/accumulation within the cell. In this study, the technique was adapted to analyze fluorescent-labeled molecules binding to the surface of particles in the absence of cells.

Dietary grade titanium dioxide (Anatase, Special AHR; Tioxide UK Ltd, Cleveland, UK), fluorescein isothiocyanate (FITC)-labeled LPS derived from *Escherichia coli* type O55:B5 (Sigma, Poole, UK), and calcium chloride ($CaCl_2$; BDH Ltd, Poole, UK) were prepared in ultra–high-purity water (UHP; Elga Systems, Elga, UK) to limit the background caused by contaminating particles. Solutions of FITC-LPS (0–2500 ng/ml), TiO_2 (0–100 μg/ml), and $CaCl_2$ (0–4000 μg/ml of Ca^{2+}) were freshly prepared by vortexing and ultrasonication (Ultrawave, Cardiff, UK) for 5 mins to avoid particle aggregation, and the conjugates formed by mixing these species were immediately analyzed by flow cytometry using total volumes of 1 ml in 3-ml polypropylene tubes (Falcon; Sigma). The flow cytometer (FACScan; Becton Dickinson, Oxford, UK) was equipped with a 488-nm argon laser and fluorescence was read at 530 nm. Particle distribution and associated fluorescence were acquired and quantified using LYSIS II software (Becton Dickinson) and WIN MDI version 2.1.3 flow cytometry software (Scripps, San Diego, CA). Analysis of nonparticulate FITC-LPS (500 ng/ml) and particulate TiO_2 (25 μg/ml) then allowed quadrants to be established for particulate nonfluorescent matter, particulate fluorescent matter, and nonparticulate fluorescent matter. In all experiments data were collected from 10,000 events and the mean fluorescent intensity and percentage of events within each quadrant were recorded.

Cell Isolation and Culture. Following informed consent, heparinized blood (20 ml) was obtained from 34 healthy volunteers (21 male), aged 30.4 ± 6.1 years (mean ± SD), who were not taking any medication, had no history of inflammatory disease or atopy, and showed no sign of acute illness (e.g., common cold). Peripheral blood mononuclear cells (PBMNCs) were isolated by density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The cells were harvested at the gradient interface and washed and resuspended at a concentration of 5×10^5 cells/ml in tissue culture medium (TCM) consisting of RPMI 1640 medium containing 5×10^{-2} m*M* 2-mercaptoethanol (Sigma), 10 μg/ml gentamicin (Gibco, Paisley, UK), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 2 m*M* L-glutamine (Sigma), and supplemented with 10% heat-inactivated fetal calf serum (Gibco). Viability and enumeration were performed using 0.4% trypan blue (Sigma) and cell viability was 98% ± 2% (mean ± SD).

Stock solutions of the test components were vortexed and sonicated as above and then immediately added to the

cell cultures (1:5 dilution) and incubated for 24 hrs at 37°C in an atmosphere of 5% CO_2/95% air. The final concentrations of the test components in cell culture were: 1 ng/ml LPS from *E. coli* (type O55:B5; Sigma), 5 μg/ml TiO_2 (Tioxide UK) or 160 μg/ml calcium as calcium chloride ($CaCl_2$; equivalent to 4 m*M* Ca^{2+} ions; BDH). The LPS-Ca^{2+}-TiO_2 conjugate, with final cell culture concentrations of 1 ng/ml LPS plus 5 μg/ml TiO_2 plus 160 μg/ml calcium, was prepared by mixing freshly vortexed and sonicated stock solutions to yield a solution with a 5-fold final concentration of each component. This was then immediately added to the cell cultures (1:5 dilution). All test solutions were prepared in sterile, deionized, ultrapure water. Cell cultures were performed in 3-ml polypropylene Falcon tubes. Following culture of the cells, the supernatants were collected for cytokine analysis and the cells prepared for flow cytometry.

Flow Cytometry. Detection of the surface antigen CD14 and assessment of particle phagocytosis and cell death were performed by flow cytometry. Following culture, cells were washed in TCM and dispensed into polypropylene Falcon tubes where 10 μl of fluorochrome-conjugated monoclonal antibody was added, directed against human cell surface CD14 (FITC) (Leu-M3; Becton Dickinson). The tubes were incubated for 30 mins in the dark at 4°C. Isotype-matched, fluorochrome-conjugated irrelevant immunoglobulin was used at the same concentration for assessment of nonspecific binding. The cells were washed twice in TCM before fixation in 1% paraformaldehyde prior to analysis. A region of interest that included monocytes but excluded lymphocytes and debris was generated based on physical characteristics and confirmed by high expression of CD14 in this "monocyte gate." Ten thousand events occurring in this region were collected and analyzed. Forward-scatter and side-scatter measurements were also used to assess phagocytic cells in cultures challenged with TiO_2. This population of cells was assessed by increased side-scatter measurements with little change in forward scatter, indicating increased cytoplasmic internal reflection without an increase in size (30, 31).

Samples were prepared for assessment of cell death by a method that is not directly affected by changes in extracellular calcium levels, namely propidium iodide (PI) incorporation into the sub-G0/G1 peak. Cells were stained with CD14-FITC antibody or isotype control as above but, following the final wash in TCM, the cells were vigorously fixed in 2 ml 70% ice cold ethanol/30% deionized water, and stored overnight at 4°C in the dark before addition of PI (Sigma). Overnight incubation in 70% ethanol facilitates the leakage of small fragments of DNA from the cell, which are generated during apoptosis, and the reduced DNA content can be visualized as an increase in PI incorporation in the distinctive sub-G0/G1 peak (32, 33). Immediately prior to analysis samples were washed and the cell pellet resuspended in 800 μl phosphate-buffered saline (Unipath, Basingstoke, UK), 100 μl PI (400 μg/ml), and 100 μl RNAse (1 mg/ml; Pharmacia-Biotech, St. Albans, UK) and incubated at 37°C for 30 mins.

Assessment of Chemokine/Cytokine Secretion. Following incubation with test stimulants, cell culture supernatants were collected and assayed for the chemokines macrophage inflammatory protein-1 alpha (MIP-1α, also termed CCL3) and monocyte chemoattractant protein 1 (MCP-1, also termed CCL2) and for the cytokines IL-1β, interleukin-1 receptor antagonist (IL-1ra), interleukin-6 (IL-6), transforming growth factor-β1 (TGF-β1), and tumor necrosis factor-α (TNF-α). Enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's guidelines (R&D, Abingdon, UK). Interleukin-12 release from monocytes was also assessed by ELISA, using a high sensitivity kit for the heterodimer (IL-12p70; R&D). All ELISA plates were read using a Dynatec MR5000 plate reader (Dynatec, El Paso, TX). Concentrations (pg/ml) of cytokines/chemokines in each supernatant were computed by interpolation from the standard curve run with each plate.

Inhibition of Particle Uptake. The effect of cytochalasin D on phagocytosis was investigated in PBMNC cultures. Cell cultures were preincubated for 30 mins at 37°C in 5% CO_2/95% air with 0.5–8 μg/ml cytochalasin D (from *Zygosporium mansonii*; Sigma) before addition of the particle conjugate (LPS-Ca^{2+}-TiO_2). The cells were then incubated for a further 24 hrs at 37°C in 5% CO_2/95% air, supernatants were collected and assayed for IL-1β, and cells were analyzed for CD14 expression, phagocytosis, and PI incorporation.

The role of scavenger receptors in the uptake of particulates was investigated using the competitive polyribonucleotide inhibitor, polyinosinic acid (poly I; Sigma) at 250-1000 μg/ml. The control polyribonucleotide, polycytidylic acid (poly C; Sigma), which does not block scavenger receptor–mediated uptake, was also used. These were added to cell cultures for 30 mins at 37°C in 5% CO_2/95% air before addition of the LPS-Ca^{2+}-TiO_2 conjugate. Cell cultures were incubated for a further 24 hrs and again supernatants were analyzed for IL-1β, and cells were studied for CD14 expression, phagocytosis, and PI incorporation.

Effect of Inhibition of Interleukin-1β Converting Enzyme (ICE). An ICE (caspase I) inhibitor was examined in PBMNCs stimulated with the LPS-Ca^{2+}-TiO_2 conjugate. Dilutions of 0–60 μ*M* of the ICE inhibitor (Z-Val-Ala-Asp(OMe)-CH_2F; Enzyme Systems Products, Livermore, CA) were prepared in dimethylsulfoxide (DMSO; Sigma). Cell cultures were preincubated either alone, with DMSO, or with ICE inhibitor for 30 mins at 37°C in 5% CO_2/95% air. Following preincubation, the LPS-Ca^{2+}-TiO_2 conjugate was then added to the same cultures and cells were incubated for a further 24 hrs. IL-1β release and PI incorporation were assessed as above.

Statistics. The hypothesis examined in this study was that the LPS-Ca^{2+}-TiO_2 conjugate alters the balance between pro- and anti-inflammatory signals by preferen-

tially stimulating the release of proinflammatory cytokines. Furthermore, the separate contributions of the conjugate components to the overall activity of the LPS-Ca^{2+}-TiO_2 conjugate were investigated. Components were analyzed to ascertain whether the activity of the conjugate was primarily because of uptake of particles and whether the presence of LPS dictated the direction and magnitude of the response. Data were expressed as mean ± SEM, and the effect of the LPS-Ca^{2+}-TiO_2 conjugate on cytokine/chemokine production was analyzed using a paired Student's *t* test with Bonferroni correction for multiple testing, where a value of $P < 0.01$ was considered significant. The effects on particle uptake, blockade of scavenger receptors, and ICE inhibitor were analyzed by Student's *t* test for paired samples at the maximal effective dose, where a value of $P < 0.02$ was considered significant.

Results

Particle-LPS Conjugation Requires Bridging Calcium Cations. Dietary-grade TiO_2 is predominantly negatively charged at neutral pH and provides a large surface area for adsorption (pKa = 4 and mean particle diameter of 150 nm), allowing ionic binding of metal cations (14) and, in turn, the adsorption of negatively charged groups contained within larger biomolecules (34). Here, flow cytometric analysis showed that fluorescent LPS was avidly adsorbed to particulate TiO_2 in the presence of calcium ions (Ca^{2+}; as $CaCl_2$) but not in their absence (Fig. 1A). These data are consistent with previous studies that have demonstrated the adsorption of serum proteins, immunoglobulins, or extracellular matrix components to titanium oxide surfaces at physiological pH, a process that is reversible after treatment with the metal-chelating agent EDTA, indicating a requirement for bridging cations (14, 34, 35). Magnesium ions, and especially sodium and potassium ions, were markedly less effective in facilitating particle-LPS conjugation (data not shown).

Dose ranging studies indicated that [Ca^{2+}] was optimal for conjugation at 160 μg/ml (4 m*M*) whereas TiO_2 was effective across a broad range of concentrations (Fig. 1B). In further cellular studies, and consistent with previous work (24, 25), TiO_2 was used at 5 μg/ml and Ca^{2+} at 160 μg/ml, whereas LPS was used at 1 ng/ml, as titration studies indicated that this was a suboptimal dose for monocyte stimulation with the LPS in its native, soluble form.

Uptake of the Particle-Ca-LPS Conjugate Induces Cell Death. Cell culture with TiO_2, Ca^{2+}, or LPS reduced expression of CD14 (part of the LPS response complex; Ref. 36) by up to half in the monocyte-gated region. However, the LPS-Ca^{2+}-TiO_2 conjugate dramatically reduced expression from >60% in controls to <5% in treated cells (data not shown). Loss or internalization of cell surface CD14 may precede apoptosis (37), which has been shown previously by us in intestinal mononuclear cells exposed to calcium-bearing particles (24). Here, in control cultures, few cells were seen in the flow cytometric–determined "phagocyte gate" (Fig. 2), which was established independently with particle uptake studies in the presence and absence of an inhibitor of phagocytosis, namely cytochalasin D (see below, and data not shown). Culture with TiO_2 induced marked cellular uptake and appearance of cells in the phagocyte gate. This was similarly observed in the presence of the conjugate, except that cell size was also reduced, indicating cell shrinkage (Fig. 2), again consistent with cell death. No effects were seen with LPS alone.

PI enters dead cells and in apoptotic cells is observed in the subdiploid peak (G0/G1) area. The assay is especially useful for monocyte/macrophage cultures in calcium-rich media, in which the annexin V binding assay is less discriminatory. PI incorporation in the subdiploid peak was evident in cells challenged with the conjugate but significantly less so in cells challenged with LPS, Ca^{2+} or particulate TiO_2 alone (Fig. 2B).

Caspase 1–Dependent IL-1β Secretion and a Proinflammatory Cytokine Profile Are Associated with Phagocytosis of the Particle-Ca-LPS Conjugate. Elsewhere, particle-induced cell death has been associated with cellular secretion of proinflammatory cytokines (9, 24). Here, the chemokine MIP-1α was secreted in response to the conjugate mainly as an effect of exposure to [Ca^{2+}] and LPS (Fig. 3). In contrast, MCP-1 release was markedly reduced in response to the conjugate, driven only in part by increased [Ca^{2+}] (Fig. 3). Overall the ratio of MIP-1 α:MCP-1 was increased from 0.5 in control cells to 15 in conjugate-challenged cells (i.e., 30-fold), suggesting a significant skewing in proinflammatory and T_H1 signalling (37).

Release of the proinflammatory cytokines (IL-1β, TNF-α and IL-6) was increased in response to cellular incubation with the conjugate (Fig. 4). However, release of TNF-α and IL-6 was largely attributable to LPS and Ca^{2+} effects (Fig. 4), whereas IL-1β secretion, which was increased by the presence of additional LPS and Ca^{2+}, was dramatically increased in response to the conjugate (Fig. 4). A corresponding increase in IL-1ra and TGF-β1 (i.e., anti-inflammatory, IL-1β–antagonist cytokines; Ref. 38) was not observed and a reduction was even observed for TGF-β1. Some studies suggest that for assessment of proinflammatory IL-1β–mediated activity, the ratio of IL-1ra:IL-1β is a more discerning measure than IL-1β alone (38). Although this is probably an oversimplification of the *in vivo* process (38), the conjugate dramatically reduced this ratio by about 25-fold compared with control cells (Fig. 4). This effect was caused almost solely by changes in secretion of IL-1β and not IL-1ra, and was not explained by a combined effect of the individual components ($P < 0.005$; IL-1β secretion with the conjugate vs. the sum of IL-1β secretions for LPS + Ca^{2+} + TiO_2). Similar experiments indicated that, unlike the LPS-Ca^{2+}-TiO_2 conjugate, Ca^{2+} + LPS, Ca^{2+} + TiO_2 and

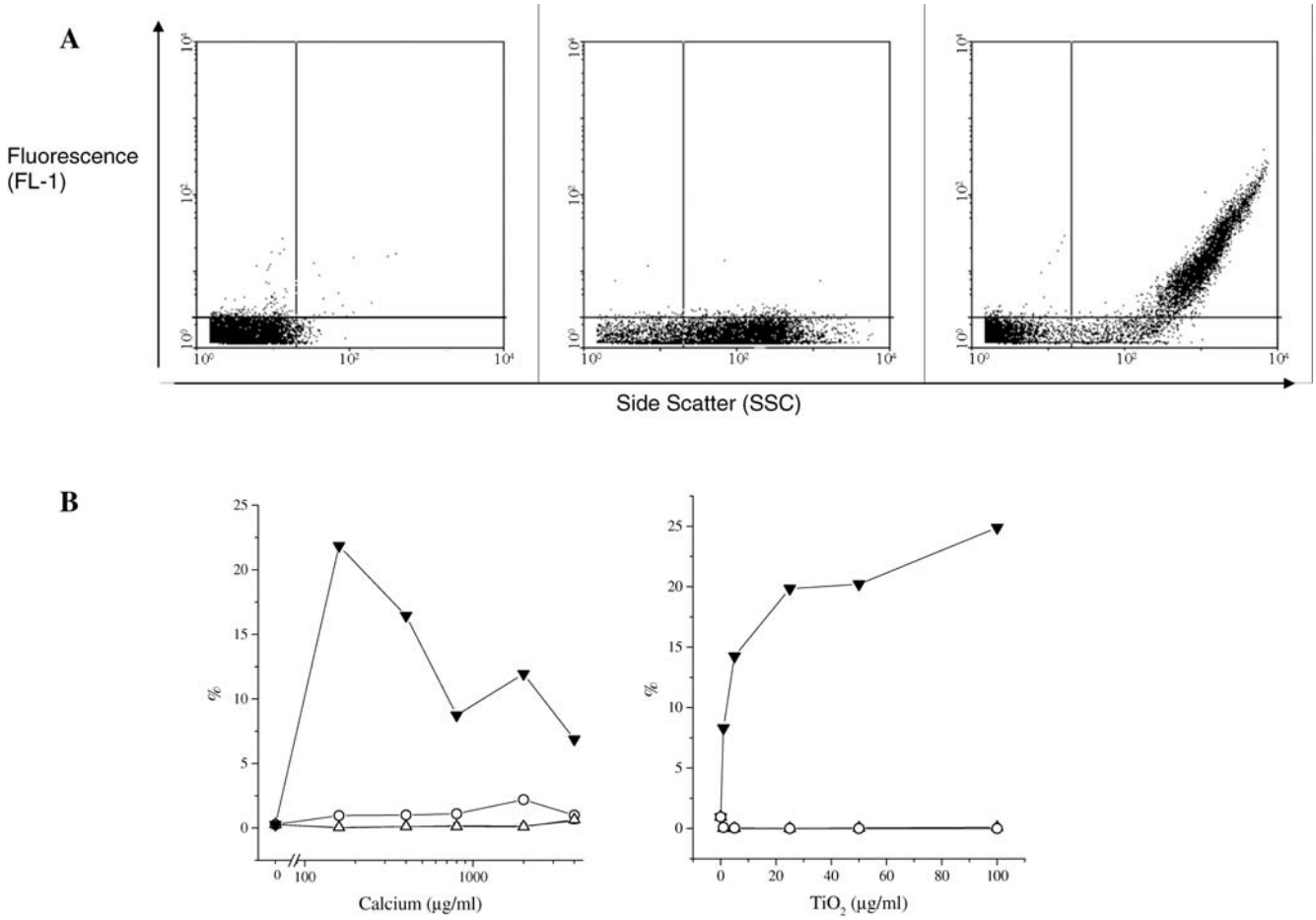

Figure 1. LPS adsorbs to fine particulate titanium dioxide with bridging calcium cations. (A) Interactions between 500 ng/ml FITC-LPS, 160 μg/ml Ca^{2+} (as $CaCl_2$), and 5 μg/ml TiO_2 in ultrapure water. Test solutions of FITC-LPS plus either (left panel) Ca^{2+} or (middle panel)TiO_2 were compared with (right panel) the conjugate of FITC-LPS plus Ca^{2+} plus TiO_2 (i.e., LPS-Ca^{2+}-TiO_2) for (upper right quadrant) the formation of fluorescent-associated particles/agglomerates. Data are expressed as $\log_{10}$ arbitrary units. (B) (Upper right quadrant) Fluorescent-associated particles/agglomerates assessed in solutions containing either (left)0–4000 μg/ml Ca^{2+} (as $CaCl_2$) or (right) 0–100 μg/ml TiO_2. For both graphs, the conjugate (FITC-LPS+Ca^{2+}+TiO_2) is represented by closed, down triangles and was formed (left-hand graph) with 500 ng/ml FITC-LPS plus 5 μg/ml TiO_2 when the calcium concentration was varied or (right-hand graph)with 500 ng/ml FITC-LPS plus 160 μg/ml Ca^{2+} (as $CaCl_2$) when the TiO_2 concentration was varied. For the left-hand graph, open circles are FITC-LPS (500 ng/ml) + Ca^{2+} and open triangles are TiO_2 (5 μg/ml) + Ca^{2+} or Ca^{2+} alone (overlapping symbols). For the right-hand graph, all open symbols overlap, representing FITC-LPS (500 ng/ml) + TiO_2 (5 μg/ml), Ca^{2+} + TiO_2 (5 μg/ml), or TiO_2 (5 μg/ml) alone. Data are representative of 4 experiments; the standard deviation between experiments was 4–7%.

LPS + TiO_2 exhibited no synergy for IL-1β secretion, or for any of the other cytokines measured (data not shown).

Cell death induced by particulate (i.e., whole) bacteria and specific concomitant secretion of IL-1β have been linked to caspase activation, notably activation of caspase 1 (also termed ICE) (27, 28). Using the caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F, we confirmed that conjugate-stimulated IL-1β secretion was inhibited in a dose-dependent fashion (Fig. 5).

Scavenger Receptor–Mediated Phagocytosis of the Particle-Ca-LPS Conjugate Is Required for Cell Death and Proinflammatory Signalling. Recent focus on the role of the scavenger receptor in particle uptake and cellular apoptosis (39) prompted us to investigate its role in the uptake of, and responsiveness to, the LPS-Ca^{2+}-TiO_2 conjugate. First we confirmed that, as for particulate calcium (24), blockade of conjugate phagocytosis with the general inhibitor cytochalasin D led to reduced IL-1β secretion (Fig. 6) and reduced PI incorporation into the subdiploid peak (Fig. 6). Next, using the competitive polyribonucleotide inhibitor poly I for the general blockade of scavenger receptors, we showed that phagocytosis, IL-1β secretion, and PI incorporation were all inhibited in a dose-dependent fashion (Fig. 7). The control polyribonucleotide, poly C, which does not block scavenger receptor–mediated uptake, had no effect (Fig. 7).

Discussion

Extrapolation of the *in vitro* findings presented here leads us to hypothesize that modern-day gastrointestinal exposure to inorganic dietary microparticles could lead to luminal conjugate formation with endogenous calcium ions

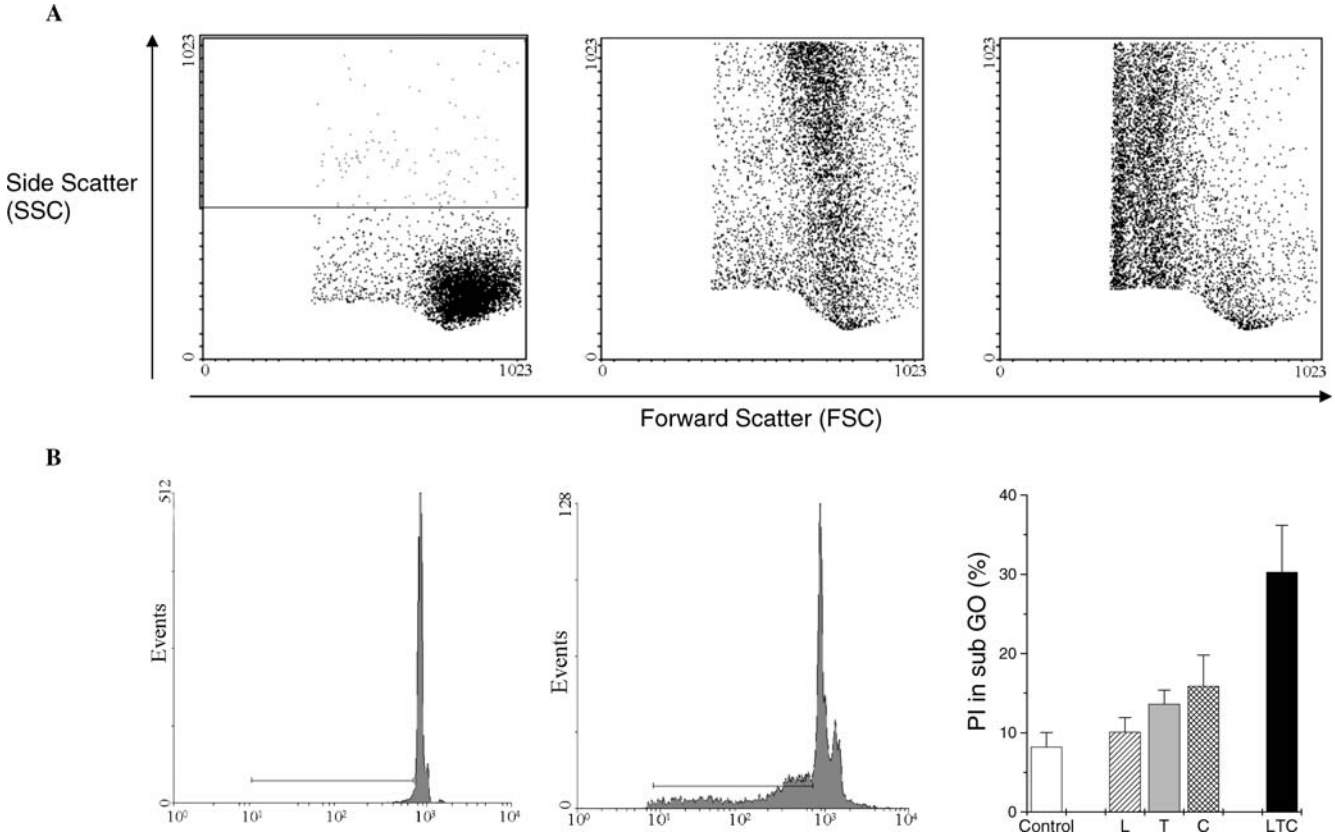

Figure 2. Fine particle–LPS conjugates induce phagocyte cell death. (A) (Middle panel) Phagocytosis of TiO_2 particles results in an increase in side scatter (*y* axis) but only little variation in cell-size forward scatter (*x* axis) when compared with (left panel) untreated monocytes. (Right panel) Stimulation with the LPS-Ca^{2+}-TiO_2 conjugate not only increases side scatter, indicating phagocytosis, but also reduces cell size, indicating shrinkage caused by cell death . (B) PI incorporation into the sub-G0/G1 peak. Comparison of monocyte cell death (left panel) in untreated PBMNC cultures (5%) and (middle panel) following LPS-Ca^{2+}-TiO_2 conjugate stimulation (40%) shows a characteristic pattern of cell death with reduced DNA content, depicted by an increase in events detected in the sub-G0/G1 peak following particle stimulation. (Right panel) Quantitation of cell death in peripheral blood monocytes following particle stimulation. Cells were cultured alone (control) or challenged with 1 ng/ml LPS (L), 5 μg/ml TiO_2 (T), 160 μg/ml $CaCl_2$ (C), or with the LPS-Ca^{2+}-TiO_2 conjugate (LTC). Data are expressed as the percentage of 10,000 cells within the "monocyte gate" that incorporated PI into the sub-G0/G1 peak. Mean ± SEM, $n = 10$.

and bacterial toxins from the gut flora. Unlike the resident gut flora, which are prevented from significantly translocating into gastrointestinal tissue, these particle-toxin conjugates may effect facile uptake by the Peyer's patches and other M cell–rich sites (3). What are the potential implications? The innate immune response relies upon the ability of cells of the immune system to recognize microbial products through pattern recognition receptors. Microbial recognition, for example through scavenger receptors and toll-like receptors (TLRs), controls pathogen uptake, killing, and the activation and coordination of the adaptive immune response. Metabolism of and responsiveness to LPS following cell surface recognition requires TLR4 and CD14. Classically, only short-term (<1 min) exposure to LPS is necessary for binding to CD14, with subsequent internalization leading to the activation of monocytes and stimulation of cytokine release (40). However, previous studies have also shown that monomeric LPS and aggregated LPS have distinct mechanisms of cellular uptake and activation. Large LPS aggregates are rapidly internalized into lysosomal compartments, whereas uptake of monomeric LPS is slower and involves transport to the Golgi apparatus (41, 42). In addition, it has recently been shown that uptake of soluble LPS is CD14-dependent and that TLR4 is involved in LPS recognition but not in cellular uptake (43). In contrast, internalized particulate PAMPs can engage Nuclear Oligomerization Domain (NOD) proteins or endosomal TLRs but not cell surface TLRs. Thus, in experiments using macrophages taken from CD14-deficient mice, challenges with *E. coli* bacteria but not *E. coli*–derived LPS were able to stimulate TNFα production and could be inhibited by pretreatment with phagocytic-blocking cytochalasins (44). Therefore, as has been well shown for antigens, the physical form of PAMPs will also, in part, determine their mechanisms of uptake, recognition, and cell-induced responses.

We have proposed that the inadvertent adsorption of bacterial fragments to environmental fine particles may alter the physicochemical form and hence the cellular activity of both components. This could occur, for example, in the environment or in lung lining fluid, but, as noted above, the most obvious target site is the gastrointestinal tract as 10^{12}–

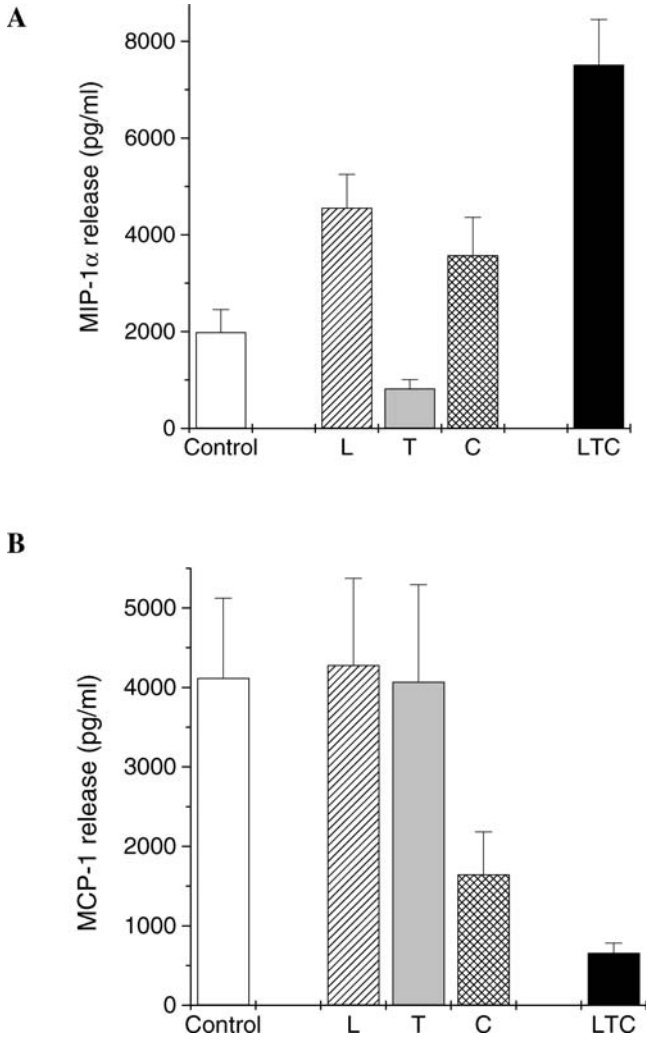

Figure 3. Fine particle–LPS conjugates skew chemokine secretion.The release of the chemokines (A) MIP-1α and (B) MCP-1 from PBMNC cultures either without stimulation (controls) or stimulated with 1 ng/ml LPS (L), 5 μg/ml TiO_2 (T), 160 μg/ml Ca^{2+} as $CaCl_2$ (C), or the LPS-Ca^{2+}-TiO_2 conjugate (LTC). Chemokine levels (pg/ml) were measured in culture supernatants following 24 hrs culture at 37°C in 5% CO_2 /95% air. Mean ± SEM, $n = 10$.

10^{14} fine particles are ingested per person per day (23) and bacterial components are ubiquitous within the lumen. For example, 200-nm–sized particles of the food additive titanium dioxide are widely ingested in the Western world and are likely to interact with luminal biomolecules (23). Indeed, the *in vivo* binding of biomolecules to wear particles of oxidized titanium surfaces is thought to be responsible for the failure of titanium-based surgical implants (14, 15). Bridging calcium cations are required for wear particle–biomolecule interaction, and these conjugates are apparent in tissue macrophages lining the prosthesis, where they may stimulate the production of a proinflammatory cytokine cascade, leading to inflammation and, ultimately, the loosening and failure of the implant (15, 45). Similarly, LPS from *Pophyromonas gingivalis* or *E. coli* shows high affinity for titanium compounds used in dental implants (46) and adherence is positively associated with the amount of calcium bound to the titanium surface (47). In this work, we have also shown that the adsorption of bacterial LPS to particles of dietary TiO_2 (mean diameter 200 nm) requires bridging cations, most effectively calcium and especially at typical gut luminal levels of around 4–5 m*M* Ca^{2+}.

The central role of phagocytosis of the LPS-Ca^{2+}-TiO_2 conjugate in cell stimulation was illustrated by blocking experiments, first of phagocytosis *per se*, using an inhibitor of actin polymerization, namely cytochalasin D (48), and second, by blocking negatively-charged particle uptake mediated by scavenger receptors with the competitive inhibitor poly I (49, 50) resulting in dramatic decreases in cell death and the production of proinflammatory signalling. Without inhibition of phagocytosis, however, the LPS-Ca^{2+}-TiO_2 conjugate effectively induced the release of the proinflammatory cytokines IL-1β, IL-6, and TNFα in the absence of a corresponding elevation in the regulatory cytokines IL-1ra and TGF-β1. Skewing of chemokine secretion was also observed. However, secretion of IL-1β was especially striking, and, unlike the other proinflammatory cytokines, IL-1β was secreted as a clear result of synergy between particle, calcium, and LPS rather than purely as an additive effect of these components. Previously, the ratios between IL-1–related proinflammatory and regulatory signals, such as the IL-1ra:IL-1β ratio, have been used to indicate the balance of inflammatory cytokine production in a system, and are often used to predict the extent and severity of inflammation (38, 51). Although we have shown that these ratios oversimplify complex competing cytokine interactions at a cellular level (38), the magnitude of the reduction in the IL-1ra:IL-1β ratio in this work, from 50 to 1, following exposure to the LPS-Ca^{2+}-TiO_2 conjugate, strongly suggests induction of a proinflammatory phenotype. Even more interestingly, the conjugate exhibited proinflammatory properties that mimic those of invasive, whole bacteria.

Cell death and specific caspase-1–induced IL-1β secretion are hallmark responses of phagocytes to invasive bacteria, especially pathogenic enteric organisms such as *Shigella flexneri* and *S. typhimurium* (27, 28). Similar results have been found for *C. jejuni*, although, in this case, IL-1β secretion and apopotosis are concomitant but independent events, with only the former requiring caspase-1 activation (29). A subfamily of "inflammatory" caspases such as caspases 1 and 5 are associated with immune responses to microbial products and are activated upon the formation of an intracellular complex termed the inflammasome (52). Activation of the caspases is initiated by oligomerization of adaptor molecules and the formation of the inflammasome is central to the production and activation of IL-1β. Here, the phagocytosis of LPS-Ca^{2+}-TiO_2 and subsequent IL-1β release occurred concomitantly with an increase in the incorporation of PI in the sub-G0/G1 peak, cell shrinkage, and the downregulation of CD14. These features are consistent with cell death by apoptosis,

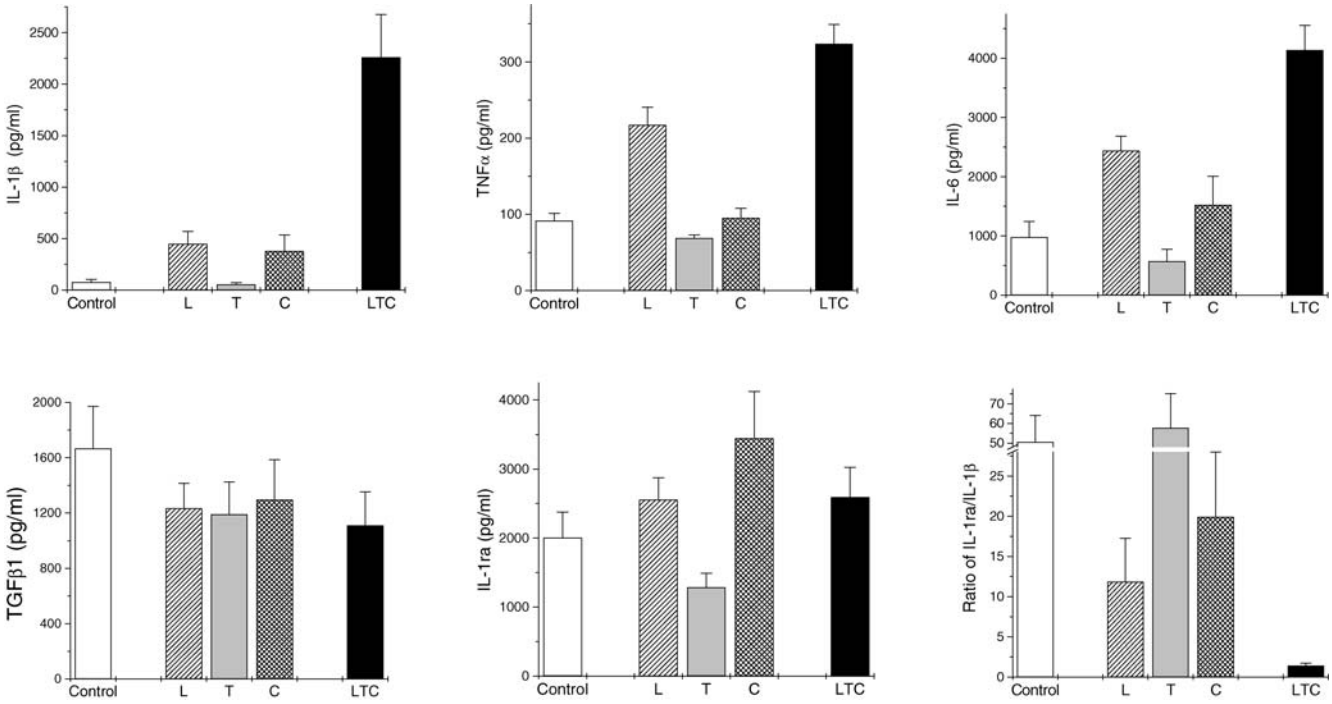

Figure 4. Fine particle–LPS conjugates induce marked proinflammatory cell signalling, especially IL-1β secretion. The release of cytokines from isolated PBMNCs (1×10^6 cells) without treatment (controls) or following stimulation with either 1 ng/ml LPS (L), 5 μg/ml TiO_2 (T), 160 μg/ml Ca^{2+} as $CaCl_2$ (C), or the LPS-Ca^{2+}-TiO_2 conjugate (LTC). The release of proinflammatory IL-1β, TNFα, and IL-6 or anti-inflammatory TGF-β1 and IL-1ra were assessed from culture supernatants following 24 hrs incubation at 37°C in 5% CO_2/95% air. The balance between pro- and anti-inflammatory signals is depicted as the IL-1ra:IL-1β ratio (lower right panel). Mean ± SEM (pg/ml), $n = 10$.

although increasingly it is recognized that apoptosis and necrosis form polar and complex aspects of a broad spectrum of cell death and that an understanding of the cellular environment is more important than nomenclature. Hence, we have tended in this work to use the broader term "cell death." Several previous studies have linked cell death in monocytes with an increase in the release of IL-1; only apoptotic cell death appears to produce mature IL-1β production, whereas necrotic death releases pro IL-1β and IL-1α (53). Taken together our data indicate that the dietary-derived LPS-Ca^{2+}-TiO_2 conjugate induces apoptotic-like cell death and mature IL-1β release, and as such mimics the innate inflammatory effect of certain invasive, enteric organisms. In support of this conclusion, we recognize the importance of unambiguously identifying secreted IL-1β as the mature 17-kDa form. Indeed, the ELISA used in these experiments does not definitively identify the mature form of IL-1β, although the pro form of IL-1β is considerably underestimated (<10%; personal communication). Moreover, unlike with cell lysates, the large majority of secreted IL-1β should be present in the processed mature form. To confirm this, we inhibited caspase-1 activity and thus the processing of pro–IL-1β, which led to a dramatic reduction in ELISA-detectable IL-1β (Fig. 5). Thus, we are confident that our findings indicate that cell death is accompanied by secretion of the processed form of IL-1β when phagocytes are challenged with the LPS-Ca^{2+}-TiO_2 conjugate.

The exact mechanisms remain unclear but recent data confirm that the adaptor molecules ASC and Ipaf, but not, as once thought, RIP2, regulate pro–caspase-1 cleavage, leading to activation of the inflammasome (28). Importantly, healthy cells challenged with wild-type *S. typhimurium*

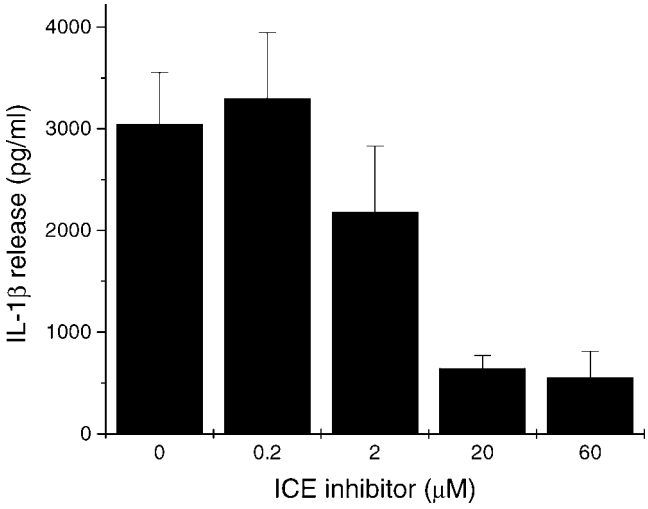

Figure 5. The induction of IL-1β by fine particle–LPS conjugates requires caspase-1 (ICE) activation. Inhibition of the secretion of ICE-cleaved IL-1β, induced by exposure to the LPS-Ca^{2+}-TiO_2 conjugate. PBMNC cultures were pretreated with increasing doses of the caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F prepared in DMSO for 30 mins before 24 hrs incubation with the LPS-Ca^{2+}-TiO_2 conjugate at 37°C in 5% CO_2/95% air. IL-1β release (pg/ml) into culture supernatants was assessed by specific ELISA. Mean ± SEM, $n = 8$.

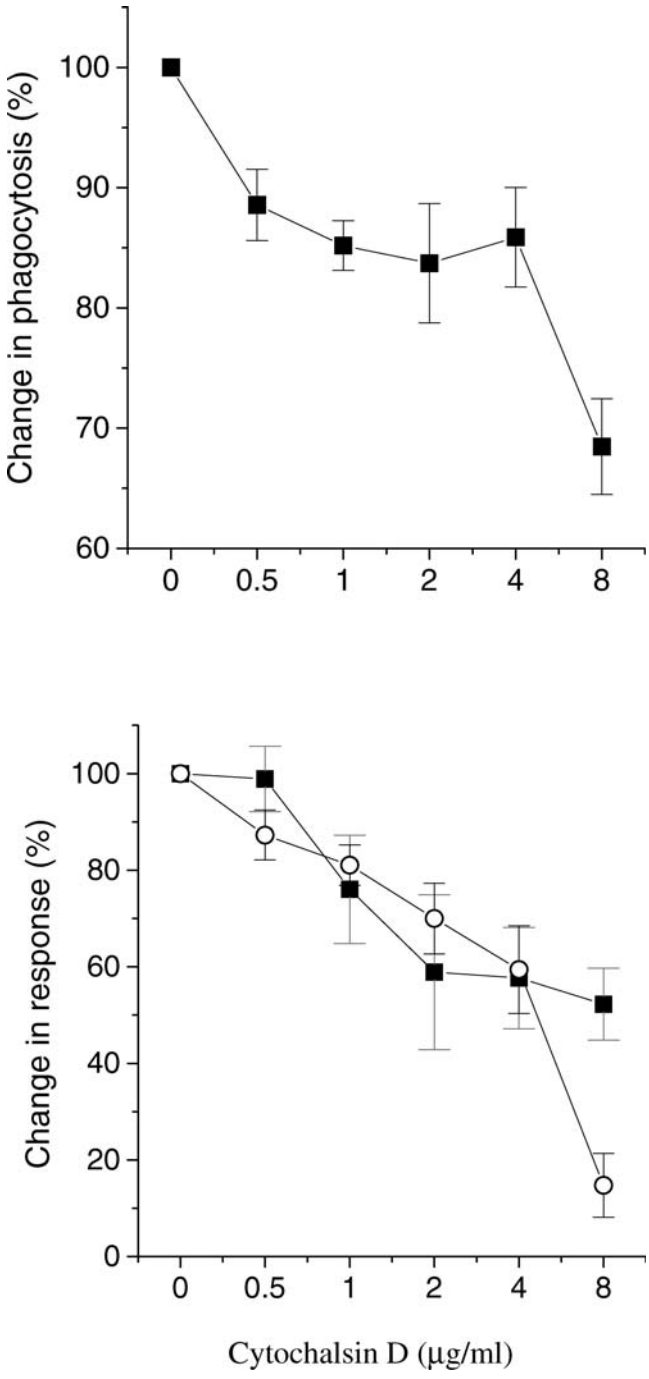

Figure 6. Cellular responsiveness to fine particle–LPS conjugates requires phagocytosis. The effect of phagocytosis inhibition, with cytochalasin D pretreatment, on (top graph) the uptake of the LPS-Ca^{2+}-TiO_2 conjugate and (bottom graph) subsequent cell stimulation. PBMNC (1×10^6 cells) cultures were pretreated with increasing doses of cytochalasin D for 30 mins at 37°C in 5% CO_2/95% air prior to stimulation with the LPS-Ca^{2+}-TiO_2 conjugate for 24 h. Culture supernatants were (bottom graph; closed squares) analyzed by specific ELISA for IL-1β (pg/ml) and the monocyte population assessed for (top graph) phagocytosis and (bottom graph; open circles) PI incorporation by flow cytometry analysis of 10,000 events within the "phagocyte gate." Data are expressed as the percentage change in response compared with no cytochalasin D pretreatment. Mean ± SEM, $n = 8$.

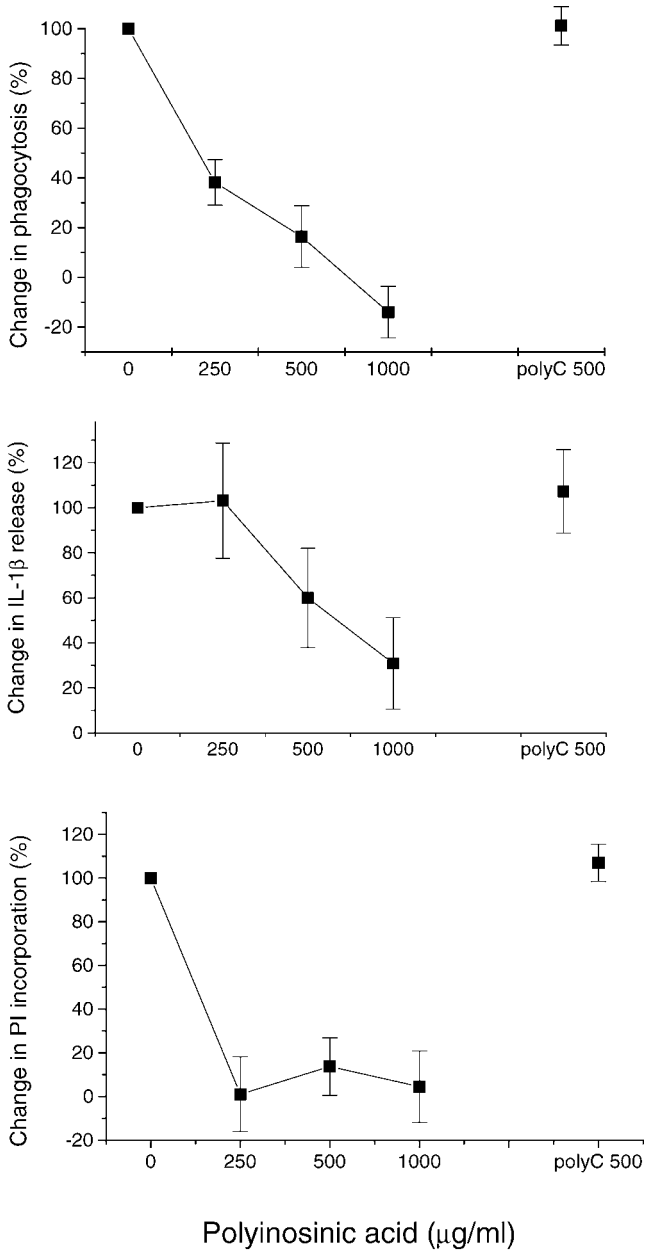

Figure 7. Phagocytosis and cellular activity of fine particle–LPS conjugates is scavenger receptor dependent. PBMNCs (1×10^6 cells) were pretreated with increasing doses of the scavenger receptor blocker, poly I, or 500 μg/ml of the control polynucleotide, poly C, before incubation with the LPS-Ca^{2+}-TiO_2 conjugate at 37°C in 5% CO_2 and 95% air. The monocyte population was analyzed by flow cytometry for phagocytosis and PI incorporation. In addition, IL-1β release was detected by ELISA in culture supernatants. Data are expressed as the percentage change in response compared with LPS-Ca^{2+}-TiO_2 conjugate treatment alone. Mean ± SEM, $n = 8$.

undergo caspase-1–induced cell death and secrete IL-1β, whereas cells exposed to LPS or other soluble TLR agonists may secrete IL-1β but do not exhibit cell death even in the presence of most secondary stimuli (28). ASC and especially Ipaf appear to play specific roles in caspase-1 activation and cell death triggered by intracellular pathogens, but only ASC has a role in activation of caspase-1

through the TLRs (28). Although this is not yet clear, ASC may be involved in early pathogen-induced cell death (typically apoptosis) and Ipaf in late cell death (aponecrosis or secondary necrosis). Whatever the exact mechanism, intracellular signalling through particulate PAMPs appears to result in distinct recruitment of adaptor molecules that activate the inflammasome and lead to cell death. In the findings presented here it is unclear whether conjugate-induced cell death and IL-1β release are co-ordinated, as for *S. typhimurium* and *S. flexneri,* or simply concomitant events as for *C. jejuni*, although clearly caspase-1 cleavage is required in at least part of the process (Fig. 5), and further work is required in this area.

In conclusion, fine particle–LPS conjugation, such as could occur in the gastrointestinal lumen, is facilitated by bridging calcium ions and leads to the formation of a complex that undergoes ready phagocytosis in a scavenger receptor–dependent fashion with marked proinflammatory properties. Whether this could initiate inflammation in susceptible individuals will be examined in future work, but, in addition to initiation of ROS or skewing of immune responses, it provides an additional potential mechanism for the toxicity of environmental fine particles.

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