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Lipopolysaccharide-induced multinuclear cells: Increased internalization of polystyrene beads and possible signals for cell fusion

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ABSTRACT

A murine macrophage-derived line, RAW264.7, becomes multinuclear on stimulation with lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria. These multinuclear cells internalized more polystyrene beads than mononuclear cells or osteoclasts (Nakanishi-Matsui, M., Yano, S., Matsumoto, N., and Futai, M., 2012). In this study, we analyzed the time courses of cell fusion in the presence of large beads. They were internalized into cells actively fusing to become multinuclear. However, the multinuclear cells once formed showed only low phagocytosis activity. These results suggest that formation of the multinuclear cells and bead internalization took place simultaneously. The formation of multinuclear cells was blocked by inhibitors for phosphoinositide 3-kinase, phospholipase C, calcineurin, and c-Jun N-terminal kinase. In addition, interleukin 6 and 10 also exhibited inhibitory effects. These signaling molecules and cytokines may play a crucial role in the LPS-induced multinuclear cell formation.

1. Introduction

In response to cytokines and growth factors, macrophages differentiate into multinuclear cells, including osteoclasts, involved in bone homeostasis, and FBGC (foreign body giant cells), which internalize orthopedic implant wear debris [1–4]. Osteoclasts are formed from bone marrow macrophages after stimulation with RANKL (receptor activator of nuclear factor κB ligand), and FBGCs are formed with IL4 (interleukin 4) [1,5]. Murine macrophage-derived line RAW264.7 also differentiates into osteoclast-like cells upon stimulation with RANKL [6,7].

We found previously that the same cell line forms multinuclear cells through cell–cell fusion on addition of highly purified LPS (lipopolysaccharide) or synthetic lipid A [8]. The LPS-induced multinuclear cells (average 90 nuclei/cell), observed within 16 h after stimulation, did not express osteoclast-specific enzymes and showed no bone resorption [8]. However, these cells can internalize more polystyrene beads than mononuclear cells and osteoclasts [8].

Upon infection by Gram-negative bacteria, macrophages should recognize LPS in the bacterial outer membrane, which leads to inflammatory responses including phagocytosis. Macrophages may also form multinuclear cells when stimulated *in vivo* by the infection. In this regard, LPS-induced multinuclear cells could be a good model system for studying the role of macrophages in infection.

In this study, we analyzed the time courses of multinuclear cell formation in the presence of polystyrene beads, and found that large sized beads were internalized by cells actively fusing, suggesting that formation of the multinuclear cells and bead internalization took place simultaneously. We have also tested a series of inhibitors, and discuss possible signals involved in multinuclear cell formation with LPS.

2. Materials and methods

2.1. Materials

All chemicals used were of the highest grade commercially available: NSC23766, Tocris Bioscience (Park Ellisville, MO); Y-27632, Sigma–Aldrich (St. Louis, MO); PD98059, Cayman Chemical Company (Ann Arbor, MI); and cyclosporine A, Wako (Osaka, Japan). Cycloheximide, SP600125, SB203580, wortmannin, and 21U-73122 were from Calbiochem (La Jolla, CA). Recombinant

Abbreviations: LPS, lipopolysaccharide; TLR4, toll-like receptor-4; RANKL, receptor activator of nuclear factor κB ligand; RANK, receptor activator of nuclear factor κB; FBGC, foreign body giant cell; MEMκ, minimum essential medium alpha medium; PBS, phosphate-buffered saline; TRAP, tartrate-resistant acid phosphatase; FITC, fluorescein isothiocyanate; IL6, interleukin 6; IL10, interleukin 10; TNFα, tumor necrosis factor α; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2; PI3K, phosphoinositide 3-kinase; TRAF6, tumor necrosis factor receptor associated factor 6; PLCγ, phospholipase C-γ; NFκB, nuclear factor κB; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1.

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murine IL6, IL10 and TNFα were obtained from Pepro Tech Inc. (Rocky Hill, NJ). Fetal bovine serum was from Sigma (St Louis, MO), and other reagents for cell culture were purchased from Life Technologies (NY, USA). Polystyrene beads of 6 and 15 μm in diameter were from Polyscience (Warrington, PA).

2.2. Cell culture, induction of multinuclear cells and determination of cytokines

RAW264.7 cells obtained from the European Collection of Cell Culture (ECACC Cat. 91062702) were grown in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. Multinuclear cells were induced with LPS as previously described [8]. Briefly, cells were seeded at 8×10^5 cells per well (1.77 cm^2), and then incubated with 100 ng/ml highly purified LPS from *E. coli*, O55:B5 (TLR grade., Alexis Biochemicals, San Diego, CA), in the presence of 6.8 mM $CaCl_2$ for 16 h. They were then washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS, and stained with Hoechst 33343 (Life Technologies) for nuclei and FITC-conjugated phalloidin (Fluka Biochemika, Buchs, Switzerland) for actin. Stained cells were observed under a LSM510 microscope (Carl Zeiss, Oberkochen, Germany). For determination of cytokine concentrations, culture medium was collected, and IL6, IL10, or TNFα was detected using an ELISA kit from Thermo Scientific (Rockford, IL, USA).

2.3. Internalization of polystyrene beads

Internalization of beads was estimated as described previously [8]. Polystyrene beads of 6 or 15 μm in diameter were fed to cells ($2.5 \times 10^5/cm^2$) 6 h after the addition of LPS. They were further incubated for 10 h, fixed, and stained as described above. The 10 h incubation was required to obtain maximum internalization of 15-μm beads.

3. Results

3.1. Internalization of polystyrene beads by LPS-induced multinuclear cells

We observed previously that LPS-induced multinuclear cells could internalize more beads (6- or 10-μm diameter) than osteoclasts and mononuclear cells: LPS-induced multinuclear cells

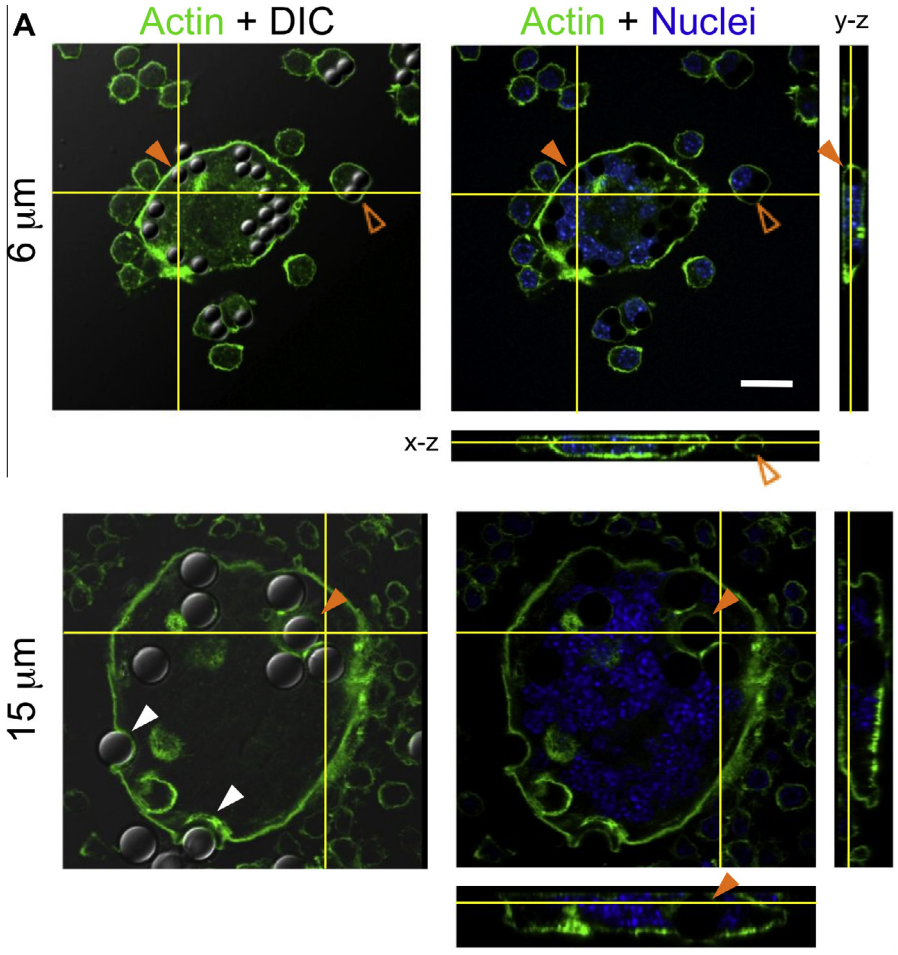

Fig. 1. Internalization of polystyrene beads by LPS-induced multinuclear cells. (A) Polystyrene beads internalized into multinuclear cells. Cells were grown with 100 ng/ml highly purified LPS, and fed with polystyrene beads ($2.5 \times 10^5/cm^2$) of two sizes (diameter 6 or 15 μm) after 6 h incubation. They were incubated for a further 10 h, fixed, and then stained for actin filaments (green) and nuclei (blue). Cells were observed under a confocal microscope. Actin staining is shown with differential interference contrast (DIC) (left) or with nuclei stained (right). Closed and open orange arrowheads indicate beads within multinuclear and mononuclear cells, respectively. White arrowheads indicate beads seem to be undergoing the internalization process. The positions of lateral images (x–z and y–z sections) are shown by yellow lines in horizontal views (x–y sections). Scale bar, 20 μm. (B) Mononuclear and small multinuclear cells attached to large beads. 15 μm-beads were introduced as in A, and cells were fixed 4 h later, and then stained for actin (green) and nuclei (blue). Six typical beads attached to mononuclear and small multinuclear cells are shown. In the merged field (*Merged*, right), beads (white circles) and cells with more than two nuclei (white arrowheads) are shown. Dotted lines indicate the shapes of cells based on DIC and the actin cytoskeleton. Scale bar, 20 μm.

internalized about 6-fold more 6-μm beads than mononuclear cells [8]. Furthermore, they could incorporate larger beads (15 μm) that other cells could not. Thus, it became of interest to determine whether or not beads were internalized concomitantly with the fusion process, or by multinuclear cells formed on LPS stimulation.

To address this, we first examined bead internalization using essentially the same protocol as previously reported [8]: polystyrene beads (6- or 15-μm diameter) were introduced 6 h after LPS stimulation, and their internalization was observed after a further 10 h incubation. We paid special attention to cell–cell interactions and the process of bead internalization, and representative fields

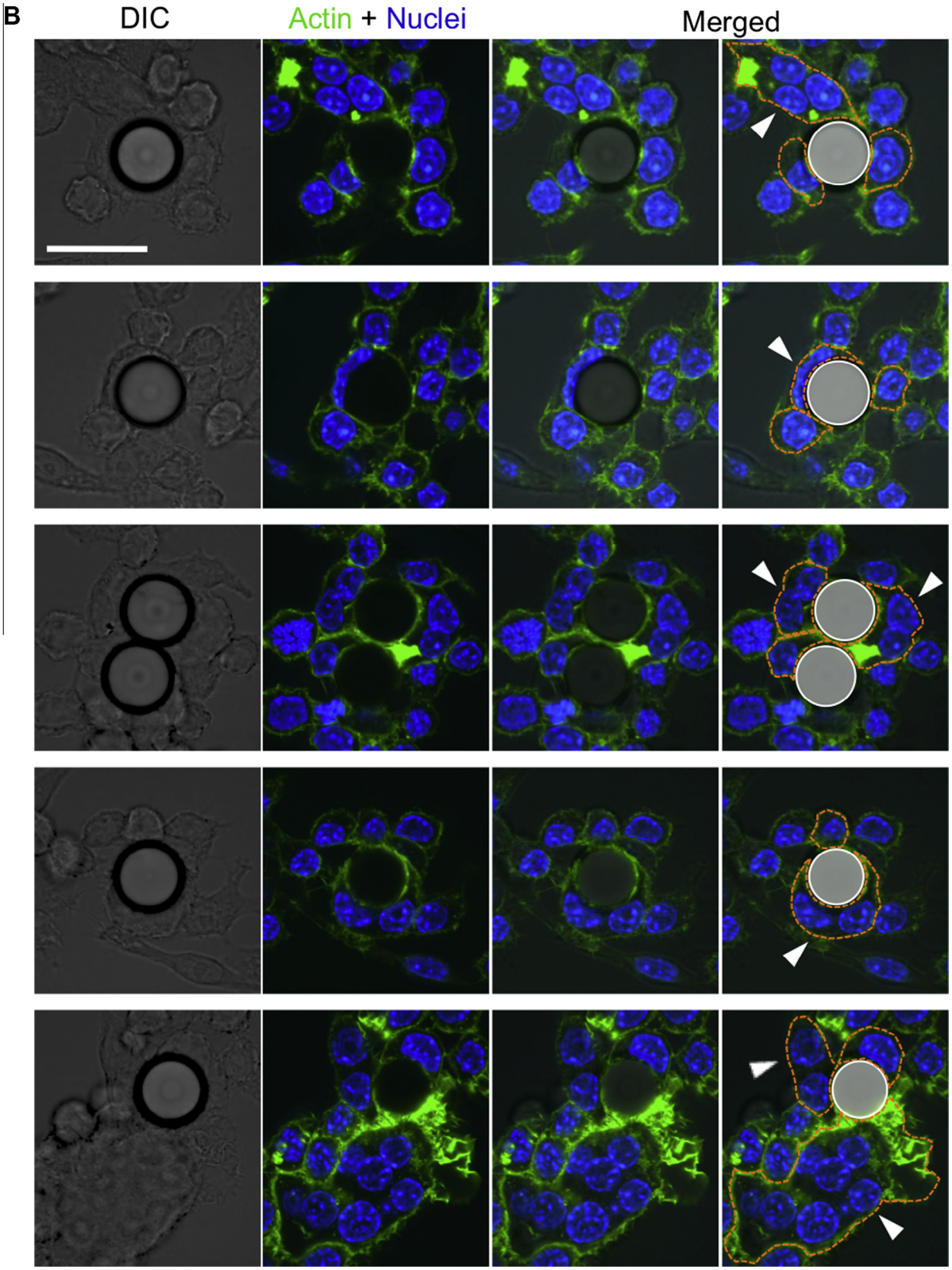

Fig. 1 (*continued*)

are shown (Fig. 1A). The 15-μm beads were completely observed inside the actin cytoskeleton of multinuclear cells, as shown on three-dimensional analysis (Fig. 1A, closed orange arrowheads), confirming that these cells can internalize the large beads [8]. As expected, mononuclear cells could internalize 6-μm, but not 15-μm beads (Fig. 1A, open orange arrowheads). The number of beads internalized decreased with an increase in their size (Fig. 1A, compare 6 and 15 μm beads), confirming the previous results [8]. We occasionally observed beads surrounded partially by cells probably undergoing internalization process (e.g., Fig. 1A, 15 μm, white arrowheads).

Next, we fixed cells 4 h after the addition of beads to follow their internalization and cell fusion at an early stage. As expected, 15-μm beads were often attached to mononuclear cells and multinuclear cells with a few nuclei (Fig. 1B, five examples). About 6% of the cells were found to be attached to the beads when about 600 cells were observed. These results suggested that significant numbers of beads might be internalized during the fusion process. To confirm this, we tested different protocols for observation.

3.2. Uptake of beads during multinuclear cell formation

As shown in the time course of the fusion index (Fig. 2A), multinuclear cells started to increase about 4 h after LPS addition and became maximum within 16 h. Thus, beads were introduced to cultures at 6, 10 and 16 h after LPS addition, and internalized beads were counted after a further 10 h incubation (Fig. 2B). These protocols enabled us to study bead internalization by three different cell populations: cells actively fusing, beads added at 6 h; a mixed population of ones fused (~80%) and still in the process of fusing, beads added at 10 h; and ones showing no further fusion, beads added at 16 h.

The numbers of internalized 15-μm beads fed at 6, 10, and 16 h after LPS addition were 150 ± 31, 19 ± 13, and 2 ± 1 per 1000 nuclei, respectively (Fig. 2C, closed bars). Similarly, the highest number of 6-μm beads was internalized when they were introduced at 6 h (Fig. 2C, open bars). We often observed 15-μm beads surrounded by mononuclear cells and small multinuclear cells with a few nuclei when observed at 4 h after bead addition (Fig. 1B). These results suggest that mononuclear and/or small multinuclear cells attached to a large bead, and fused with each other, resulting in multinuclear cells with beads inside them.

Essentially no intracellular beads were observed in the population fed beads at 16 h, indicating that large multinuclear cells have no phagocytosis activity, although these cells looked healthy when observed under a microscope: they hardly detouched from the culture dishes. The reduced number of beads in those fed at 10 h supports this finding. These cells showed a relative fusion index of ~80% (Fig. 2A), and internalized 15-μm beads (~10% of those fed at 6 h) (Fig. 2C). These results together with the microscopic observation (Fig. 1) suggest that beads were internalized during the formation of multinuclear cells, and that fusion and bead internalization took place simultaneously.

3.3. Effects of signal transduction inhibitors on multinuclear cell formation

As shown in Fig. 2A, multinuclear cells started to appear about 4 h after LPS addition and became maximum within 16 h, indicating that a series of biochemical events within 4 h after stimulation lead to cell fusion. Consistently, multinuclear cells were not observed on incubation with cycloheximide (Fig. 3), indicating that *de novo* protein synthesis is required. The multinuclear index significantly decreased with the addition of NSC23766 and Y-27632 (Fig. 3), inhibitors of Rac and Rho kinase, respectively [6]. Consistent with the roles of these small GTPases [9], remodeling of the

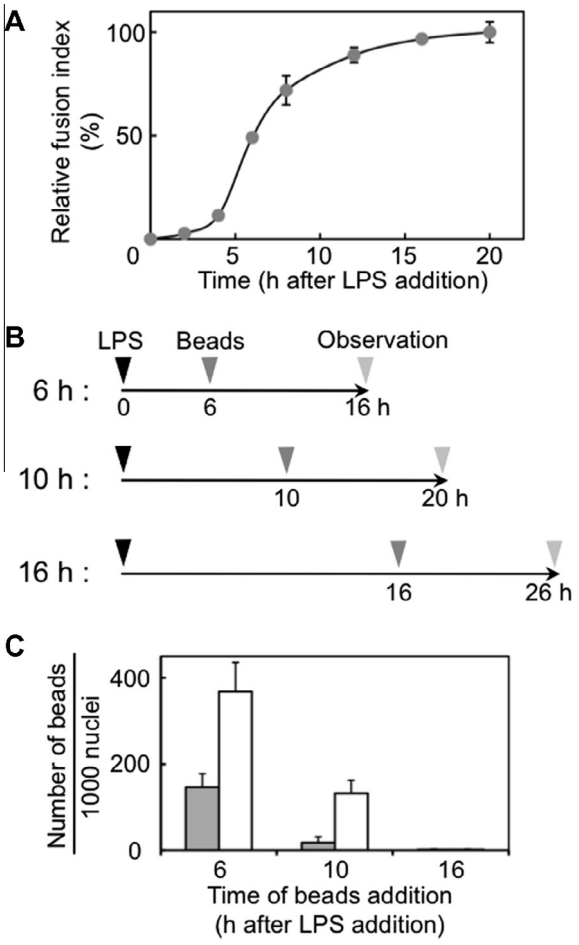

Fig. 2. Beads internalization during multinuclear cell formation. (A) Time course of multinuclear cell formation. Cells were incubated in the presence of LPS for various times, and then the relative fusion index (number of nuclei in multinuclear cells against the total number of nuclei, taking the fusion index at 20 h as 100%) was determined and is shown with standard deviation [8]. (B) Experimental protocols for observing bead internalization. Cells were fed with beads at 6, 10, or 16 h after LPS addition, and then incubated a further 10 h for observation. (C) The number of beads internalized into multinuclear cells. Cells were fixed and stained after incubation, and the beads and nuclei in multinuclear cells (with more than two nuclei) were observed under a microscope, and the number of internalized beads per 1000 nuclei was determined. Open and closed bars represent 6- and 15-μm diameter beads, respectively.

cytoskeleton is probably necessary for cell–cell fusion. Using time-lapse live-cell imaging, we observed that LPS-stimulated cells obviously changed in shape more frequently and moved to another site more actively than ones without stimulation (data not shown).

MAPKs (mitogen-activated protein kinases) play important roles in pro-inflammatory responses and osteoclast differentiation [10–12]. Of three kinase inhibitors, SP600125 [inhibitor for JNK (c-Jun N-terminal kinase)] decreased the LPS-dependent multinuclear index significantly, whereas SB203580 [for p38 MAPK] and PD98059 [for ERK1/2 (extracellular signal regulated kinase 1/2)] had no effect (Fig. 3). An inhibitor for PI3K (phosphoinositide 3-kinase), wortmannin (100 nM), significantly reduced the index (Fig. 3). These results suggest that JNK and PI3K mediate signaling for multinuclear cell formation. These kinases are known to be activated through TRAF6 (tumor necrosis factor receptor associated factor 6) employed by TLR4 (toll-like receptor 4), a receptor for LPS, and to be involved in activation of transcription factors AP1 and NFκB, respectively [10,11]. These transcription factors probably activate genes leading to the synthesis of proteins important for cell fusion or multinuclear cell formation.

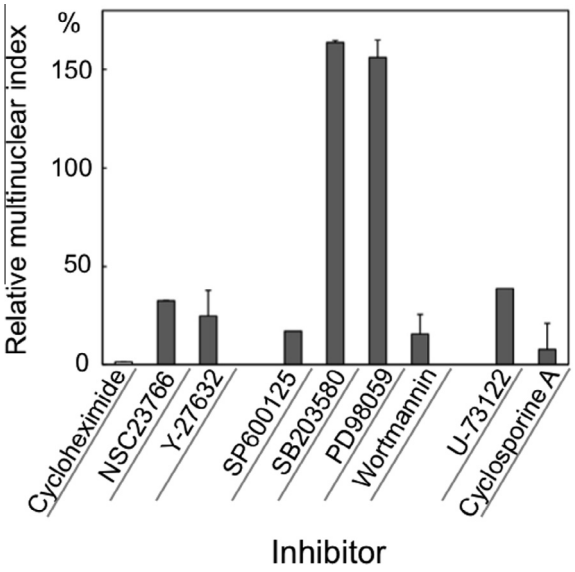

Fig. 3. Effects of various compounds on multinuclear cell formation. After 30 min incubation with 0.1 μg/ml cycloheximide, 100 μM NCS23766, 30 μM Y-27632, 10 μM SP600125, 3 μM SB203580, 10 μM PD98059, 100 nM wortmanin, 1 μM U-73122, or 5 μg/ml cyclosporin A, LPS and 6.8 mM $CaCl_2$ were added to cells as described in the legend to Fig. 1. Nuclei in mononuclear cells and multinuclear cells were counted after 16 h incubation, and the relative fusion index were determined, taking those for incubation without an inhibitor as 100 (%).

The formation of multinuclear cells was also inhibited by U-73122 and cyclosporine A, inhibitors of PLCγ (phospholipase C-γ) and calcineurin, respectively (Fig. 3). Both proteins are known to mediate Ca^{2+} signaling. Since they are not employed by TLR4, they are probably activated indirectly. We titrated all inhibitors used and the data are shown for concentrations of inhibitors that have no effect on cell viability.

3.4. Effects of IL6, IL10, and TNFα on LPS-induced multinuclear cell formation

The formation of osteoclasts from RAW264.7 cells is regulated by various cytokines and growth factors: it is inhibited by IL6 and IL10, the concentrations for ~80% inhibition being 100 and 10 ng/ml, respectively [13,14]. TNFα induces osteoclast differentiation through a mechanism independent of the RANKL–RANK interaction (20 ng/ml TNFα for similar induction to 100 ng/ml RANKL) [15].

LPS-induced multinuclear cell formation was inhibited by IL6 (100 ng/ml) (Fig. 4A, open triangles). On the other hand, IL10 was highly inhibitory, 1 ng/ml giving ~75% inhibition, which is an about 10 times lower concentration than that required for inhibition of osteoclast formation (Fig. 4A, open circles). This inhibition was completely abolished by the addition of mouse IL10-specific monoclonal antibodies (data not shown), confirming that the inhibition was due to IL10. These results suggest that IL6 and IL10 negatively regulate LPS-signaling for multinuclear cell formation. In contrast to its effect on osteoclast formation, TNFα showed no effect even at 100 ng/ml (Fig. 4A, closed squares).

Since these cytokines may be secreted from macrophages upon LPS stimulation, we determined their concentrations in the culture medium after LPS addition. The concentrations of IL6, IL10, and TNFα increased with incubation, the values being 2.3 ± 0.5, 0.3 ± 0.1, and 27 ± 4 ng/ml, respectively, after 16 h stimulation (Fig. 4B, triangles, circles, and squares, respectively). These secreted cytokines should have no effect on multinuclear cell formation (compare Fig. 4A and B): the maximum concentrations obtained after 16 h stimulation were at most one-third of those required for 80% inhibition, although multinuclear cell formation had been completed by that time.

4. Discussion

In this study we found that substantial numbers of polystyrene beads were internalized when cells were fusing actively. Kinetic studies involving different protocols suggested that cell fusion and bead internalization took place simultaneously: mononuclear and small multinuclear cells became attached to large beads and then fused with each other, resulting in multinuclear cells with an internalized beads. Sequential observation of LPS-treated cells, such as of their shape and migration, revealed that rearrangement of the cytoskeleton increases during cell fusion, which may allow bead internalization and fusion to take place simultaneously. This mechanism should be advantageous for macrophages to internalize large objects such as microorganisms, cells damaged through inflammation processes, and orthopedic implant wear debris at the site of implantation [3,4].

LPS is recognized by a membrane receptor, TLR4, which stimulates transcription factor NFκB (nuclear factor κB) through adaptor proteins and kinases, such as TRAF6 and PI3 K [11]. TLR4 also stimulates transcription factor AP1 through TRAF6 and MAPKs

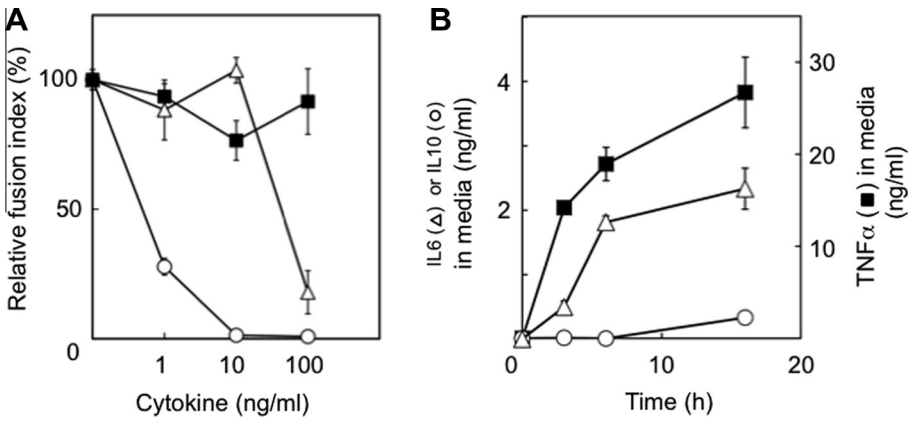

Fig. 4. Effects of IL6, IL10, and TNFα on multinuclear cell formation. (A) Effects of cytokines on multinuclear cell formation. Cells were incubated for 16 h with LPS in the presence of various cytokines, and then fixed for observation. The relative fusion indices are shown, taking those without the cytokines as 100%. Triangles, circles, and squares indicate IL6, IL10, and TNFα, respectively. (B) Release of cytokines to the culture medium. Cells were incubated with LPS for various times, and culture medium was recovered by centrifugation at 1000 × *g* for 10 min. Cytokines in the medium were assayed immunochemically (triangles, circles, and squares indicate IL6, IL10, and TNFα, respectively). Note that the scale for TNFα is different from that for IL6 and IL10.

[10,11]. Activated NFκB and AP1 induce genes involved in inflammatory responses [10,11]. It was of interest to determine whether or not the multinuclear cells were induced through a similar signal transduction pathway. The results obtained for inhibitors suggest that JNK, PI3K, PLC-γ, and calcineulin are involved in signal transduction for multinuclear cell formation. Only JNK, one of three MAPKs, is required for signaling to induce multinuclear cells, whereas another MAPK, p38, is also involved in inflammatory responses [10].

PLC-γ and calcineurin are essential for Ca^{2+} signaling in macrophages [1,12]. Ca^{2+} oscillation is probably required to induce multinuclear cells. Since PLC-γ and calcineurin are not located downstream of TLR4 [10], their activation may be triggered by molecules induced upon LPS stimulation. MAPKs, PLC-γ, and calcineulin are involved in induction of NFATc1 (nuclear factor of activated T-cells, cytoplasmic 1), an essential transcription factor for the formation of osteoclasts from pogenitor cells [1,12]. However, NFATc1 was not induced in LPS-stimulated RAW264.7 cells (data not shown), consistent with that they do not express osteoclast markers. Thus, the signal transduction pathway for inducing multinuclear cells may be slightly different from that for osteoclast formation or inflammatory responses.

IL6 is a pro-inflammatory cytokine that triggers strong inflammatory responses, whereas IL10 is anti-inflammatory and is induced at a late stage to restrain inflammation [16,17]. We found that IL10 inhibits the induction of multinuclear cell formation at low concentrations (∼1 ng/ml), whereas IL6 does not at the concentration released from LPS-stimulated macrophages. These results suggest a regulation mechanism by negative feedback for multinuclear cells: their formation is not inhibited during inflammation, but strongly inhibited by anti-inflammatory IL10 at a late stage of inflammation when the elimination of large objects has finished. This inhibition should have an important physiological role for macrophage regulation, since an excess activation of the phagocytosis causes severe diseases, such as hemophagocytic lymphohistiocytosis [18]. Because IL10 decreases the phosphorylation of JNK [14], it probably decreases LPS-induced multinuclear cell formation at least partly through an inhibitory effect on JNK. Similar to osteoclast formation, LPS-induced multinuclear cell formation is probably controlled by these cytokines.

Overall, our findings suggest a novel mechanism underlying the internalization of large beads, and signaling molecules and cytokines involved in multinuclear cell formation. In this study, we have studied phagocytosis activities of LPS-induced cells using polystyrene beads. It should be noted that the LPS-induced multinuclear cells are good model systems for studying phagocytosis of various materials, including microorganisms, cells damaged through inflammation processes, and orthopedic implant wear debris at the site of implantation.

Acknowledgments

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