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Lipopolysaccharide induces multinuclear cell from RAW264.7 line with increased phagocytosis activity

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ABSTRACT

Lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria, induces strong pro-inflammatory responses, including the release of cytokines and nitric oxide from macrophage. In this study, we found that a murine macrophage-derived line, RAW264.7, became multinuclear through cell-cell fusion after incubation with highly purified LPS or synthetic lipid A in the presence of Ca^{2+}. The same cell line is known to differentiate into multinuclear osteoclast, which expresses a specific proton pumping ATPase together with osteoclast markers on stimulation by the extracellular domain of receptor activator of nuclear factor κB ligand (Toyomura, T., Murata, Y., Yamamoto, A., Oka, T., Sun-Wada, G.-H., Wada, Y. and Futai, M., 2003). The LPS-induced multinuclear cells did not express osteoclast-specific enzymes including tartrate-resistant acid phosphatase and cathepsin K. During multinuclear cell formation, the cells internalized more and larger polystyrene beads (diameter 6–15 μm) than mononuclear cells and osteoclasts. The internalized beads were located in lysosome-marker positive organelles, which were probably phagolysosomes. The LPS-induced multinuclear cell could be a good model system to study phagocytosis of large foreign bodies.

1. Introduction

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is recognized by the immune system as a sign of infection. LPS induces expression of a variety of pro-inflammatory cytokines and nitric oxide producing enzyme iNOS (inducible nitric oxide synthase) in macrophage [1]. These defense responses are important for the host to survive infection. Signal transduction by the LPS receptor TLR4 (Toll-like receptor-4) has been demonstrated in detail [1,2]. However, effects of LPS on morphology of macrophage are not studied intensively. To internalize large amount of un-necessaries including host cells damaged by strong inflammatory responses, macrophage may change their morphologies.

In response to multiple signals or cytokines, macrophages differentiate into different types of multinuclear cells. Osteoclasts are one of these cells, which are attached tightly to the bone surface, and secrete protons and lysosomal enzymes for bone resorption [3–7]. It is important for normal bone homeostasis to maintain equilibrium between resorption by osteoclasts and bonegenesis by osteoblasts. Reduced and increased resorbing activity of osteoclasts causes osteopetrosis and osteoporosis, respectively [8]. RANKL (receptor activator of nuclear factor κB ligand) and M-CSF (macrophage colony-stimulating factor) are required for osteoclastogenesis [9,10]. A membrane protein, RANKL, located on the surface of osteoblasts activates osteoclast progenitors through its receptor, RANK (receptor activator of nuclear factor κB) [9]. Upon incubation of murine macrophage line RAW264.7 cells with the extracellular domain of RANKL, osteoclast-like cells were observed within three days and their number reached a plateau within seven days [11,12].

Macrophages also become multinuclear giant cell (FBGC, foreign body giant cell) originally found internalizing orthopedic implant wear debris at the site of implantation [13,14]. They are believed to resorb large foreign bodies, which cannot be internalized or digested by mononuclear cells [14,15]. Bone marrow macrophages differentiate into similar multinuclear cells upon stimulation with IL4 (interleukin-4) and GM-CSF (granulocyte-monocyte colony-stimulation factor) *in vitro* [16].

In this study, we found that RAW264.7 cells differentiate into multinuclear cells on the addition of LPS. Sequential observations revealed that these cells were formed through cell–cell fusion in

Abbreviations: LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; TLR4, Toll-like receptor-4; RANKL, receptor activator of nuclear factor κB ligand; M-CSF, macrophage colony-stimulating factor; RANK, receptor activator of nuclear factor κB; FBGC, foreign body giant cells; IL4, interleukin-4; GM-CSF, granulocyte-monocyte colony-stimulation factor; MEMα, Minimum Essential Medium Alpha Medium; PBS, phosphate-buffered saline; TRAP, tartrate-resistant acid phosphatase; FITC, fluoresceinisothiocyanate; RT-PCR, reverse transcriptase-polymerase chain reaction; DC-STAMP, dendritic cell-specific transmembrane protein; OC-STAMP, osteoclast-specific transmembrane protein; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; lamp1, lysosomal associated membrane protein 1.

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the presence of Ca^{2+}. The average number of nuclei in this cell was about 90, similar to that of osteoclasts. The multinuclear cells induced by LPS could internalize polystyrene beads (diameter 6–15 μm) more efficiently than mononuclear cells or osteoclasts. Furthermore, the beads were located in phagolysosomes, suggesting that these multinuclear cells have increased phagocytosis activity.

2. Materials and methods

2.1. Cell culture

RAW264.7 cells obtained from the European Collection of Cell Culture were grown in Dulbecco's Modified Eagle Medium as previously described [11]. For multinuclear cell induction with LPS, cells were harvested by pipetting, and seeded at 8×10^5 cells per well (1.77 cm^2) in Minimum Essential Medium Alpha Medium (MEMα) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Fetal bovine serum was from Sigma (St. Louis, MO) and the other reagents were purchased from Invitrogen (Gaithersburg, MD).

After incubation with 100 ng/ml LPS from *Escherichia coli*, O55:B5 (TLR grade, Alexis Biochemicals, San Diego, CA), in the presence of 6.8 mM $CaCl_2$ for 16 h, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS, and then stained with Hoechst 33343 (Invitrogen) and FITC-conjugated phalloidin (Fluka Biochemika, Buchs, Switzerland). LPS from other bacteria or its derivatives (Sigma) and synthetic lipid A (Peptide Institute, Inc. Osaka, Japan) were tested as described above.

For sequential observation of cell–cell fusion, images of live cells were acquired every minute using a microscope IX81 with a time-lapse system (Olympus, Tokyo, Japan). Differentiation into osteoclasts and TRAP (tartrate-resistant acid phosphatase) staining were performed as described previously [17]. Calcium phosphate resorption assay was performed using BD BioCoat™ Osteologic™ Bone Culture System (BD Biosciences, Bedford, MA).

2.2. Western blot analysis

Western blotting was performed as described previously [11]. Immunodetection was carried out using horseradish peroxidase-conjugated antibodies and an ECL chemiluminescence detection system (GE Healthcare, Buckinghamshire HP7 9NA, UK). Antibodies against the mouse a3 isoform of V-ATPase were prepared using a synthetic peptide corresponding between His660 and Glu676 of the a3 isoform. Antibodies against the d2 isoform and cathepsin K were purchased from Abnova (Taipei, Taiwan) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

2.3. RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using a SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen). PCR was performed using specific primers: for DC-STAMP (dendritic cell-specific transmembrane protein), forward primer, 5′-ACA ATT TGT TCG GTT TGA TG-3′, reverse primer, 5′-GTT TCC CGT CAG CCT CTC TC-3′; for OC-STAMP (osteoclast-specific transmembrane protein), forward, 5′-ACA CCA TCG CAG CCT CCT TC-3′, reverse, 5′-ACT AAA CGT CAG AAG TCT AG-3′; for the V-ATPase d2 isoform, forward, 5′-GGG AGC TAA CAT ACA CCA TG-3′, reverse, 5′-ACA TTA TTT CTG TGC CCT TC-3′; and for GAPDH, forward, 5′-TCT GGA AAG CTG TGG CGT GAT G-3′, reverse, 5′-GGT GCA CGA ACT TTA TTG ATG G-3′.

2.4. Internalization of large beads

Polystyrene beads (Polysciences, Warrington, PA) with a diameter of 6, 10, or 15 μm were fed to cells ($2.5 \times 10^5/cm^2$) six hours after the addition of LPS. They were further incubated for 10 h, fixed with 4% paraformaldehyde in PBS, and stained with Hoechst 33343 and FITC-conjugated phalloidin. Internalized beads were observed under a LSM510 microscope (Carl Zeiss, Oberkochen, Germany). In the case of osteoclasts, beads ($2.5 \times 10^5/cm^2$) were also fed six hours after extracellular domain of RANKL addition. After 3 days, the internalized beads were observed.

3. Results

3.1. Formation of multinuclear cells from RAW264.7 cells on LPS addition

Macrophages become multinuclear during differentiation into osteoclasts, which resorb the bone matrix for bone homeostasis. Similar giant cells are formed when they internalize foreign materials, including orthopedic implant wear debris [13,14]. Macrophages also respond to the bacterial infection, thus, it became of interest to determine whether bacterial components can induce multinuclear cells. To address this question, we incubated macrophage-derived RAW264.7 cells with highly purified *E. coli* LPS, an outer membrane component of Gram-negative bacteria. The cells became multinuclear, whereas the control ones without LPS remained mononuclear (Fig. 1A).

We followed the formation of multinuclear cells quantitatively by observing ~4000 nuclei, and determined a "multinuclear index" (% of nuclei found in multinuclear cells against total nuclei in the same culture). Those with two nuclei, less than 0.5% of total cells, were not regarded as multinuclear. The time course of the index was essentially similar to that of the number of multinuclear cells; the index started to increase about four hours after LPS addition and became maximum within 16 h (Fig. 1B). However, fusion between two cells was completed within 10 min (Fig. 1C). It should be noted that LPS induced multinuclear cells much faster than the time necessary for the same cells to differentiate into osteoclasts depending on RANKL. As shown previously, osteoclasts were observed 3–7 days after the ligand addition [11].

The numbers of nuclei in individual cells were determined under a microscope, and summarized as histograms that showed two populations: cells with about 90 nuclei (Fig. 1D, closed bars), and others with 3–20 nuclei (Fig. 1D, gray bar and inset). The numbers of nuclei ranged from 3–300 per cell, the average being 88. The average number of nuclei in osteoclasts formed with RANKL was 74, thus being almost the same as that for LPS-induced ones.

3.2. Requirement of Ca^{2+} and the lipid A moiety for multinuclear cell formation

Multinuclear cell formation was dependent on the LPS concentration: the multinuclear index became maximum with 10–100 ng/ml LPS (Fig. 2A, open bars). Thus, cells were incubated with 100 ng/ml LPS for 16 h in the following studies. The cells also became multinuclear when they were incubated with LPS for 4 h, washed with the medium without LPS, and then incubated for another 12 h in the absence of LPS (Fig. 2A, gray bar). This result suggests that LPS is required for conditioning for differentiation, which takes about four hours.

Multinuclear cell formation required a high Ca^{2+} concentration in the culture medium: the indices with 1.8 and 6.8 mM $CaCl_2$ were 36 ± 4 and 70 ± 8%, respectively (Fig. 2B). The index became ~0% when cells were incubated with 1.8 mM $CaCl_2$ and 2 mM EGTA,

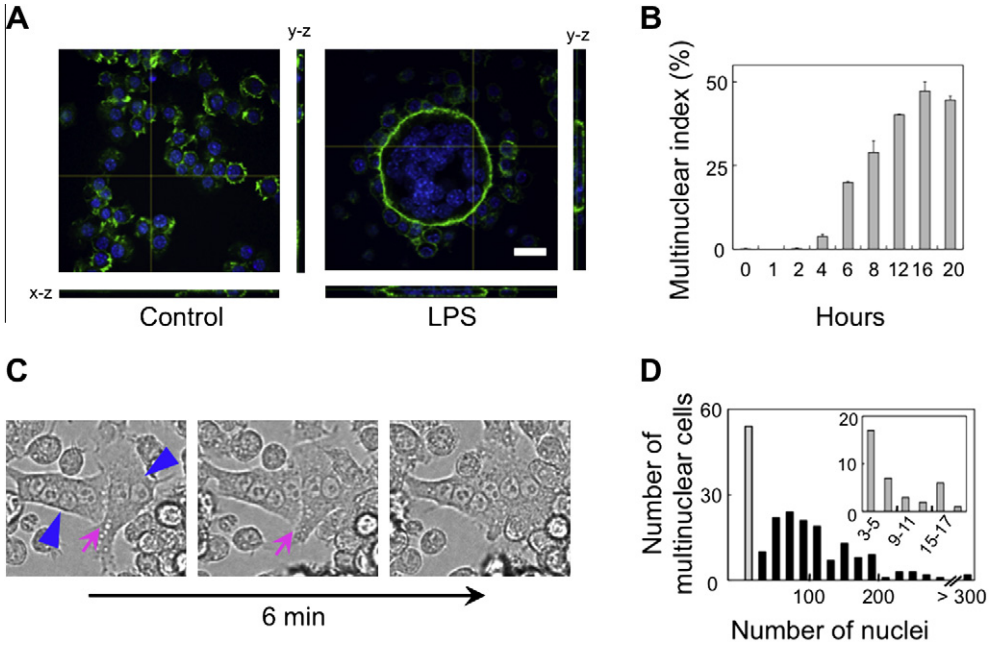

Fig. 1. Formation of multinuclear cells from RAW264.7 on the addition of LPS. (A) Multinuclear cells formed on LPS addition. Cells were incubated with 100 ng/ml highly-purified LPS from *E. coli* O55:B5 for 16 h, fixed, and then stained with Hoechst 33343 (blue) and FITC-conjugated phalloidin (green). Cells incubated with (right) or without (left) LPS are shown. Yellow lines in the horizontal views indicate the positions of lateral images (x-z and y-z sections). Scale bar, 20 μm. (B) Time course of multinuclear cell formation. Cells were incubated as in A for various times, and then the multinuclear index (% of the number of nuclei in multinuclear cells against the total number of nuclei) was determined and shown with standard deviation. (C) Sequential observation of cell fusion. Two multinuclear cells (blue arrowheads) became fused in 6 min (from 7 h 8 min to 7 h 14 min after LPS addition). Plasmamembrane (pink arrows) disappeared in 6 min. (D) Number of nuclei in multinuclear cells. Cells were incubated as in A, and then more than 1.4×10^3 nuclei were observed under a microscope. The numbers of nuclei in individual multinuclear cells were determined and summarized as histograms (closed bars). A gray bar and the inset show cells with 3–20 nuclei.

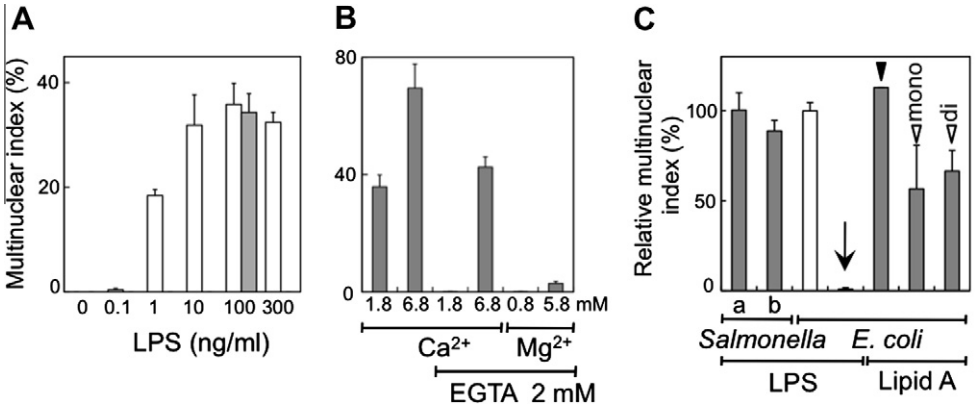

Fig. 2. Effects of Ca^{2+} and LPS derivatives on multinuclear cell formation. (A) Effect of the LPS concentration on multinuclear cell formation. Cells were incubated for 16 h in the presence of 1.8 mM $CaCl_2$ with various concentrations of LPS (open bars). A gray bar indicates cells incubated with 100 ng/ml LPS for four hours, washed, and incubated with medium without LPS for another 12 h. Cells were then fixed and stained with Hoechst 33343, and the multinuclear index was calculated. (B) Ca^{2+} dependency of multinuclear cell formation. Cells were incubated with $CaCl_2$ (1.8 or 6.8 mM) or $MgCl_2$ (0.8 or 5.8 mM) in the presence of 100 ng/ml LPS. 2 mM EGTA was also added as indicated. (C) Multinuclear cell formation with LPS derivatives. After incubation with 100 ng/ml LPS derivatives for 16 h, the relative multinuclear index was calculated, taking the multinuclear index with LPS from *E. coli* O55:B5 as 100% (open bar). The two gray bars for *Salmonella* represent *Salmonella typhosa* (a) and *Salmonella enterica* (b), respectively. The arrow, and closed and open arrowheads (mono or di) indicate LPS without fatty acids, chemically synthesized lipid A, and mono- or di-phosphoryl lipid A, respectively.

whereas it was ~40% with 6.8 mM $CaCl_2$ and 2 mM EGTA (Fig. 2B). These results indicate that free Ca^{2+} is essential for multinuclear cell formation. The following studies were carried out in the presence of 6.8 mM $CaCl_2$, unless otherwise indicated. Ca^{2+} was required in the late stage of multinuclear cell formation, since the presence of EGTA at 4–16 h after LPS addition prevented the multinuclear cell formation, whereas its presence was not inhibitory at an early stage (0–4 h) (data not shown). It is noteworthy that $MgCl_2$ did not reverse the inhibition by EGTA (Fig. 2B).

Multinuclear cells were observed with LPS purified from *Salmonella typhosa* and *Salmonella enterica* (Fig. 2C, *Salmonella*, a and b, respectively), as shown by the relative multinuclear index, that of *E. coli* O55:B5 being taken as 100% (Fig. 2C, open bar). Basically the same results were obtained with LPS from *E. coli* O26:B6, O111:B4, and O127:B8 (data not shown), indicating that O-antigen (sugar moiety of LPS) is not affecting the activity to induce fusion. The *E. coli* O55:B5 LPS lacking fatty acid moieties exhibited no activity (Fig. 2C, arrow). Chemically synthesized *E. coli*-type lipid

A was effective, similar to LPS (Fig. 2C, closed arrowhead), indicating that the lipid A moiety of LPS induced multinuclear cells. Mono- and diphosphoryl lipid A prepared from *E. coli* were slightly less effective (Fig. 2C, mono and di, respectively, open arrowheads).

3.3. Comparison of LPS-induced multinuclear cells with osteoclasts

Since this cell line differentiates into osteoclasts with stimulation of RANKL [11], we compared LPS-induced multinuclear cells with osteoclasts. LPS-induced multinuclear cells could not form resorption areas on calcium phosphate-coated discs (Fig. 3A, left), whereas osteoclasts clearly formed such areas (Fig. 3A, right, arrowheads). Osteoclasts showed positive staining for TRAP (Fig. 3B, right), and increased expression of V-ATPase isoforms (a3 and d2) and cathepsin K (Fig. 3C, right). As expected, LPS-induced multinuclear cells did not synthesize these enzymes involved in bone resorption (Fig. 3B, left, and C, left). Taken together, our results clearly indicate that LPS-induced cells do not exhibit bone resorption activity.

We then examined whether or not fusion factors for osteoclasts also play roles during LPS-induced multinuclear cell formation. The mRNA of the fusion factors, DC-STAMP and OC-STAMP, was induced 3 h after LPS addition (Fig. 3D, left). The two membrane proteins are strongly induced during osteoclast differentiation [18–20] (Fig. 3D, right). Essentially the same results were obtained on DNA microarray analysis (data not shown). These results indicate that DC-STAMP and OC-STAMP are probably involved in cell fusion induced by LPS as well as that by RANKL. Macrophages derived from knock-out mice as to the d2 gene for the V-ATPase subunit are defective in cell fusion, resulting in formation of small osteoclasts [21]. However, d2 mRNA was not induced by LPS, consistent with no corresponding protein expression (Fig. 3C, left, and D, left).

3.4. Phagocytosis by multinuclear cells

The LPS-induced multinuclear cells may have enhanced ability as to phagocytosis since they have a large volume and wide surface area. To address this question, we added polystyrene beads (diameter 6–15 μm) to RAW264.7 cells six hours after LPS addition. After a further 10 h incubation, internalized beads were observed under a microscope and the numbers of beads were determined based on those of nuclei in individual cells: we randomly analyzed cells until more than 2000 nuclei had been observed in total, and summarized phagocytosis as the number of internalized beads per 1000 nuclei. LPS-induced multinuclear cells (Fig. 4A, dark blue bars) internalized about 350 beads (6 μm diameter)/1000 nuclei. On the other hand, mononuclear cells with and without LPS stimulation internalized ~90 and ~60 beads/1000 nuclei, respectively (Fig. 4A, light blue and open bars, respectively), indicating that their phagocytosis activity is about 6-fold less than that of multinuclear cells. This finding also indicates that mononuclear cells without any stimulation took up almost the same number of 6 μm beads as the LPS-stimulated mononuclear cells. Twenty μm beads could not be internalized even by LPS-induced multinuclear cells (data not shown). It should be noted that all beads tested did not have an effect on multinuclear cell formation or multinuclear index.

Osteoclasts internalized ~17 beads (6 μm diameter)/1000 nuclei (Fig. 4A, red bar), indicating that their phagocytosis activity is ~20 times less than that of LPS-induced multinuclear cells. The average numbers of beads internalized into one LPS-induced multinuclear cell and osteoclast were estimated to be ~30 and ~1, respectively. Furthermore, osteoclasts did not take up 10 or 15 μm beads. In contrast, LPS-induced cells internalized 61 and 54 beads/1000 nuclei, respectively (Fig. 4A, dark blue bars).

In addition, the immunofluorescence study revealed that the internalized beads were surrounded by the lysosomal membrane, as indicated by lamp1 (lysosomal associated membrane protein 1) antibodies (Fig. 4B, blue arrowheads). This finding indicates that internalized beads are in phagolysosomes, possibly at the final stage of phagocytosis.

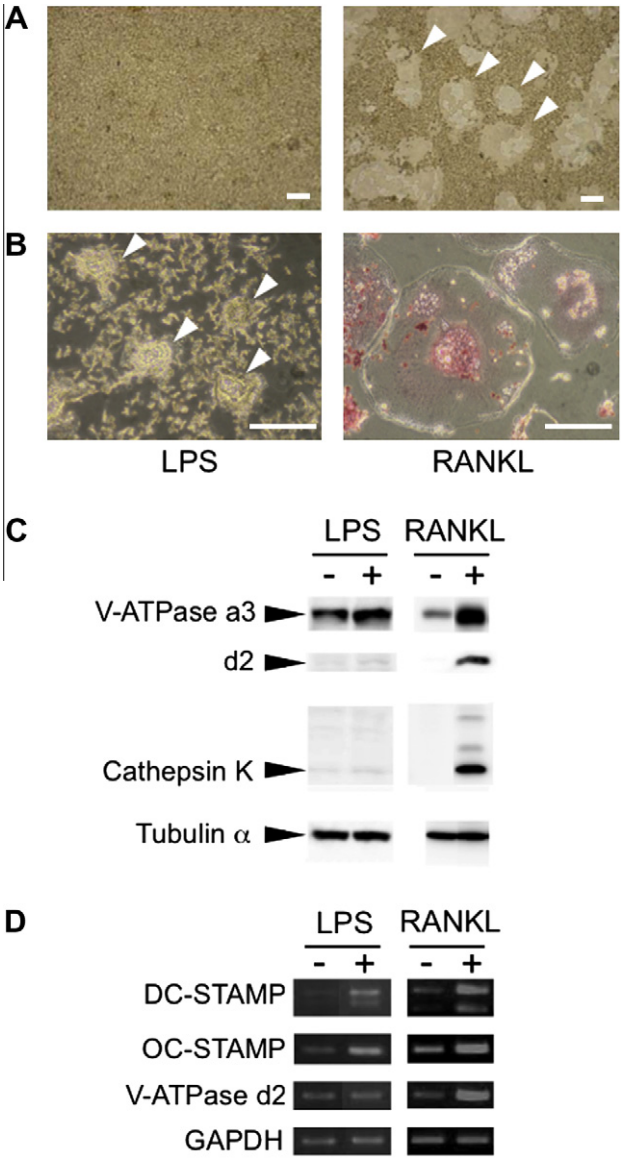

Fig. 3. Comparison of LPS-induced multinuclear cells with osteoclasts. (A) Resorption of calcium phosphate. Cells were seeded on calcium phosphate-coated discs, and then incubated with LPS for 16 h or with 100 ng/ml extracellular domain of RANKL for 3–4 days to allow the formation of multinuclear cells. The cells were then further incubated for 24 h to allow resorption of calcium phosphate, and removed with 6% NaOCl. The resorption areas were observed under a microscope: arrowheads, examples of the areas. Scale bar, 100 μm. (B) Multinuclear cells stained with TRAP (tartrate-resistant acid phosphatase). An LPS-induced multinuclear cell (left, arrowheads) and an osteoclast (right) are shown. Scale bar, 100 μm. (C) Expression of proteins after incubation with LPS or RANKL. After stimulation with LPS (16 h) or RANKL (4 days), cells were lysed with the sample buffer and then subjected to Western blotting. They were then decorated with the corresponding antibodies, followed by immunodetection. (D) RNA expression of factors involved in cell fusion. Total RNA was prepared from cells treated with LPS (3 h for OC-STAMP or 16 h for DC-STAMP and d2) or RANKL (4 days), and then RT-PCR was performed. GAPDH (glycerolaldehyde phosphate dehydrogenase) is a positive control.

4. Discussion

In this study, we have demonstrated that murine macrophage line RAW264.7 forms multinuclear cells on the addition of *E. coli*

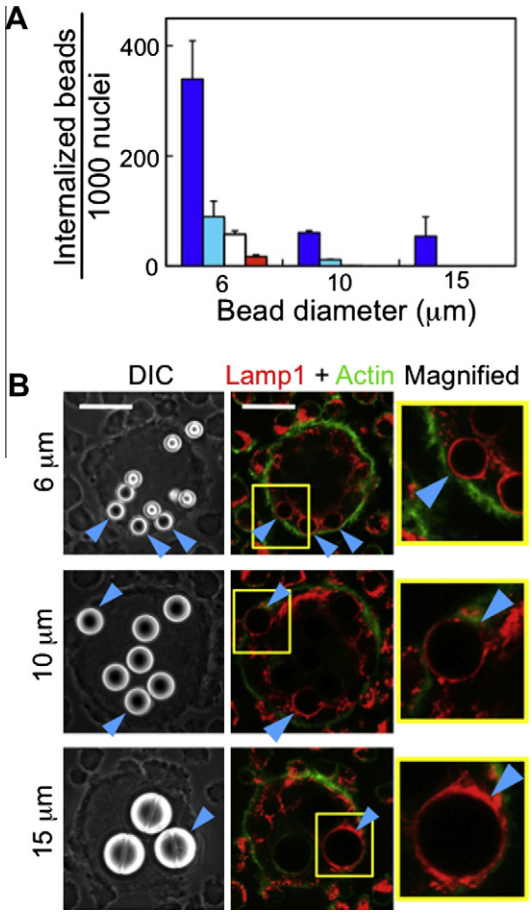

Fig. 4. Phagocytosis activity of LPS-induced multinuclear cells. (A) The number of polystyrene beads internalized. Polystyrene beads (2.5×10^5/cm^2) of various sizes (diameter 6, 10, or 15 μm) were added six hours after LPS addition. Following further incubation for 10 h, actin filaments and nuclei were stained with FITC-conjugated phalloidin and Hoechst 33343, respectively. Beads and nuclei in multinuclear cells were observed under a microscope, and the number of internalized beads per 1000 nuclei was calculated. Blue, light blue, open and red bars represent LPS-induced multinuclear cells, LPS-stimulated mononuclear cells, cells without any stimuli, and osteoclasts, respectively. LPS-stimulated mononuclear cells are those stay mononuclear even after incubation with LPS for 16 h. (B) Cells were treated as in A, fixed, and lamp1 and actin were stained with a specific antibody (red) and FITC-conjugated phalloidin (green), respectively. Then, cells were observed under a confocal microscope. Images of DIC (differential interference contrast) and immunofluorescence are shown. The areas indicated by yellow squares were magnified. Blue arrowheads indicate beads surrounded by the lamp1 signal. Scale bar, 20 μm.

or *Salmonella* LPS, or synthetic lipid A. Although osteoclasts and FBGCs (foreign body giant cells) are multinuclear cells, they are different from LPS-induced multinuclear cells: (1) The formation of osteoclasts or FBGC were observed 3–7 days after stimulation. On the other hand, multinuclear cells are formed within 16 h after LPS addition. This rapid formation should be reasonable because the response to LPS should correspond to that to bacterial infection. (2) LPS-multinuclear cell formation does not require the assistance of other cells, whereas osteoclasts and FBGC require RANKL and IL4 secreted from osteoblasts and helper T cells, respectively. Overall, we conclude that LPS induces novel type multinuclear cells from macrophages.

It is unlikely that multinuclear cells are induced by cytokines and/or growth factors secreted from macrophages upon LPS stimulation. Macrophages differentiate into osteoclasts and multinuclear giant cells *in vitro* upon stimulation by RANKL and IL4, respectively [9,10,13,16]. DNA microarray analysis revealed that LPS-stimulated cells express neither RANKL nor IL4 (data not shown), indicating that LPS induces multinuclear cells through a process not mediated by these ligands. Moreover, multinuclear cells were not formed on the addition of recombinant cytokines or growth factors expressed on the addition of LPS, including TNFα, IL6, IL1, IL10, M-CSF, GM-CSF, and IFNβ (data not shown).

LPS-induced multinuclear cells are functionally different from osteoclasts, since they do not form a resorption area on a calcium phosphate disc, and do not express osteoclast-specific enzymes including cathepsin K and TRAP. Because these multinuclear cells take up more and larger polystyrene beads than mononuclear cells and osteoclasts, they are probably involved in the elimination of foreign bodies. Upon infection by Gram negative bacteria, LPS, a component of their outer membrane, triggers strong inflammation processes that damage host cells and tissues as well as infecting bacteria. LPS-induced multinuclear macrophages rapidly respond and eliminate these large foreign materials released through the inflammation process.

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