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Activation of Macrophages by P2X7-Induced Microvesicles from Myeloid Cells Is Mediated by Phospholipids and Is PartiallyDependent on TLR4

L. Michael Thomas and Russell D. Salter

ATP-mediated activation of the purinergic receptor P2X7 elicits morphological changes and proinflammatory responses in macrophages. These changes include rapid shedding of microvesicles (MV) and the nonconventional secretion of cytokines, such as IL-1β and IL-18 following priming. In this study, we demonstrate the activation potential of P2X7–induced MV isolated from nonprimed murine macrophages. Cotreatment of nonprimed macrophages with ATP and calcium ionophore induced a rapid release of MV that were predominantly 0.5–1 μm in size. Exposure of primary murine bone marrow-derived macrophages to these MV resulted in costimulatory receptor upregulation and TNF-α secretion. Cell homogenates or supernatants cleared of MV did not activate macrophages. MV-mediated activation was p38 MAPK and NF-κB dependent, and partially dependent on TLR4 activity, but was high-mobility group box 1 independent. Biochemical fractionation of the MV demonstrated that the phospholipid fraction, not the protein fraction, mediated macrophage activation through a TLR4-dependent process. P2X7 activation is known to induce calcium-independent phospholipase A2, calcium-dependent phospholipase A2, and phospholipase D activities, but inhibition of these enzymes did not inhibit MV generation or shedding. However, blocking phospholipase D activity resulted in release of MV incapable of activating recipient macrophages. These data demonstrate a novel mechanism of macrophage activation resulting from exposure to MV from nonprimed macrophages, and identifies phospholipids in these MV as the biologically active component. We suggest that phospholipids delivered by MV may be mediators of sterile inflammation in a number of diseases. The Journal of Immunology, 2010, 185: 000–000.
To address this question, we induced MV shedding from non-prime mouse primary macrophages or cell lines through P2X7 activation and tested isolated MV for their ability to activate bone marrow-derived macrophages (BMDM). We found that MV were able to activate BMDM in a partially TLR4-dependent manner, and that the stimulatory component within the MV was found within phospholipid fractions. MV-derived phospholipids activated macrophase through TLR4. Furthermore, MV-induced activation is independent of observed loaded cargo, such as IL-1β, TNF-α, and high-mobility group box 1 (HMGB1).

Materials and Methods

**Cell culture and reagents**

J774A.1 (TIB-67; American Type Culture Collection, Manassas, VA), a murine macrophage cell line, and D25C-1, a murine splenic derived immature dendritic cell (DC) line (a gift of L. Kane, University of Pittsburgh, Pennsylvania), were maintained in DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA), 1% additional t-glutaminate (Lonza, Basel, Switzerland), and 1% penicillin and streptomycin (Lonza; hereafter called DMEM complete). FSDC, a murine fetal skin-derived immature DC line (a gift of P. Ricciardi-Castagnoli, Singapore Immunology Network, Singapore), and THP-1, a human monocyte cell line (American Type Culture Collection), were maintained in IMDM (Lonza) supplemented with 10% FBS, 1% additional t-glutaminate, and 1% penicillin and streptomycin (hereafter called IMDM complete). For experiments, THP-1 cells were treated with 20 μM PMA for 2 d to differentiate them to become more macrophage-like. RAW264.7 murine macrophage cell line transfected with NF-κB reporter plasmid pNF-κB-MetLuc Vector (BD Clontech, Mountain View, CA) encoding inducible Metridia luciferase protein expression and secretion was a gift of R. Binder (University of Pittsburgh) and was maintained in DMEM complete supplemented with 500 μg/ml G418. Murine BMDM were derived from C57BL/6 mouse bone marrow (23) as a gift of R. O'Doherty (University of Pittsburgh) and were differentiated with L-cell-supplemented media, as described previously (22). TLR4−/− mouse bone marrow (23) was a gift of R. O’Doherty (University of Pittsburgh). All other reagents include ATP (Thermo Fisher Scientific, Waltham, MA), A23187 (Sigma-Aldrich, St. Louis, MO), A 438079 (Toxic Bioscience, Ellishville, MO), A 740003 (Toxic Bioscience), brefeldin A (Sigma-Aldrich), SB203580 (EMD Chemicals, Gibbstown, NJ), BAPTA/AM (EMD Chemicals), sodium cholate (BD), EMD Chemicals), and 1,2-dimyristoyl-sn-glycerol-3-phosphorylcholine. MV were collected by centrifugation at 100,000 g for 10 min to remove cells and larger debris. MV were resuspended in 500 μl PBS and then homogenized with 10 passes through a 27-gauge needle. Samples were used immediately or stored at −20˚C.

**Generating cell homogenate**

Ten million J774A.1 were washed twice in PBS, pelleted, and resuspended in homogenization buffer (100 mM KCl, 25 mM NaCl, 2 mM MgSO4, 12 mM sodium-citrate, 10 mM glucose, 25 mM HEPES, 5 mM ATP, 0.35% BSA (pH 7.0)) supplemented with protease inhibitor mixture (Sigma-Aldrich). Cells were lysed through four cycles of freeze-thawing and homogenized with 30 strokes within a tight-fitting dounce homogenizer. The homogenate was then ultracentrifugated at 100,000 × g for 1 h at 4˚C. Resulting pellets were resuspended in 500 μl PBS, and then homogenized with 10 passes through a 27-gauge needle. Samples were used immediately or stored at −20˚C.

**Measuring activation of treated BMDM**

BMDM (wild type [WT] or knockout derived where indicated) were harvested following differentiation and plated at 1 × 10³ cells/ml in IMDM complete (unless described differently) in petri dishes. Inhibitors where indicated were applied for at least 30 min prior to exposure to MV or other compounds, and were maintained throughout the experiment. Supernatants were tested for TNF-α ELISA (eBioscience, San Diego, CA), IL-12p70 ELISA (eBioscience), or IL-23 ELISA (eBioscience), according to manufacturer’s protocols.

**Flow cytometry studies to determine viability and expression of costimulatory receptors**

For flow cytometry studies to determine viability and expression of costimulatory receptors, cells were blocked with 1.5% normal goat serum diluted in 1% BSA in PBS for 15 min and then stained with allophycocyanin anti-CD80 (BD Biosciences, San Jose, CA), PE anti-CD83 (BD Biosciences), FITC anti-CD86 (BD Biosciences), allophycocyanin anti-CD86 (BD Biosciences), or PE anti-I-Α° (BD Biosciences) Abs for 40 min. Cells were stained with 1 μg/ml DAPI (Sigma-Aldrich) viability dye. Flow cytometry was performed using a BD Biosciences LSR II, and results were analyzed using FlowJo software. Mean fluorescent intensity (MFI) and population percentages of FITC, PE, and/or allophycocyanin were calculated for DAPI-negative cell populations (i.e., living cells), which were >85% of the total cell population for all results and treatments shown in this work.

**RAW264.7 NF-κB reporter assay**

For whole-cell culture supernatant studies, 2 × 10⁵ J774A.1 were plated in 1 ml DMEM complete with or without 1 μg/ml LPS for 4 h. Cells were washed twice in serum-free DMEM with no additions. Cells were then treated with 3 mM ATP and 10 μM A23187 in a final volume of 20 ml serum-free DMEM with no additions for 30 min at 37˚C. Supernatant was harvested and centrifugated at 309,1 × g for 10 min at 4˚C to remove cells and larger debris. MV were collected by centrifugation at 100,000 × g ultracentrifugation for 1 h at 4˚C. The pellet material from the 100,000 × g ultracentrifugation was resuspended in 500 μl PBS. MV were disrupted with 10 passes through a 27-gauge needle. Bradford assay (Thermo Fisher Scientific) was used to determine the protein concentration with each MV fraction, according to manufacturer’s specifications. MV were either used immediately or stored at −20˚C for later use.

For biochemical fractionation of MV, proteins and lipids were separated through the Bligh and Dyer method of protein/lipid extraction with 1:2 of chloroform and methanol (25). The protein fraction was harvested at the biphasic interphase and reconstituted in PBS. The lipid fraction was either dried by speed vacuum centrifugation or further separated through a lipid polarity extraction technique (26). Lipid extract was passed through a silica (Sigma-Aldrich) column (1 mg silicaic acid/1 ml vol lipid extract). The pass through was collected as a sample. Bed layer volumes of chloroform, methanol, and then ether were passed sequentially in sequential fashion to elute off potential neutral lipids, glycolipids/sphingolipids, and phospholipids, respectively. Samples were harvested from each elute, dried with speed vacuum centrifugation, and stored at −20˚C.

**Western blotting**

For whole-cell culture supernatant studies, 2 × 10⁵ J774A.1 were plated in 1 ml DMEM complete with or without 1 μg/ml LPS for 4 h. Cells were washed twice in serum-free DMEM with no additions. Cells were then treated with or without 1 μg/ml LPS or 25 μg protein equivalents/ml J774A.1-derived MV for 2, 10, 30, or 60 min. Following the time course, cells were washed once with PBS and then given 100 μl 1% Triton X-100 lysis buffer in the presence of...
protease inhibition mixture (Sigma-Aldrich) and phosphatase inhibition (Sigma-Aldrich) for 15 min on ice. Lysates were collected and centrifuged at 10,000 × g for 10 min at 4 °C before addition of sample buffer and loading onto 11% SDS-PAGE gels.

Western blot was performed using the SNAP-ID, according to manufacturer’s procedures (Millipore, Billerica, MA). Abs for Western blotting included 0.5 μg/ml mouse anti-HMGB1 Ab (Abcam, Cambridge, MA), 6 μg/ml mouse anti–IL-1β Ab (3ZD; National Cancer Institute Biological Resources Branch, Frederick, MD), 0.1 μg/ml rabbit anti–phospho-p38 (Thr180/Tyr182 epitope; Millipore), 1/2000 diluted rabbit anti-p38 (Poly2624; BioLegend, San Diego, CA), 1/1666.7 diluted HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA), and 1/1666.7 diluted HRP-conjugated donkey anti-rabbit IgG (BioLegend, San Diego, CA). Signals were developed using Western blotting luminal reagent (Santa Cruz Biotechnology). Imaging was performed with KODAK Image Station 4000MM and its accompanying KODAK M1 SE Software Informer (Carestream Molecular Imaging, New Haven, CT). In some instances, the membrane was stripped for Western blot reprobing with Restore Western blot stripping buffer (Thermo Scientific), according to manufacturer’s protocols.

Bioluminescence and Cy5 labeling of MV and uptake by BMDM

MV were labeled with biotin or Cy5 using EZ-Link Sulfo-NHS-SBiotin reagent (Thermo Scientific) or monoreactive Cy5 dye (GE Healthcare, Piscataway, NJ), respectively, for 1 h in the 500 μl PBS reconstitution using the protocols supplied by the manufacturers. An additional wash with PBS followed by ultracentrifugation at 100,000 × g for 1 h at 4 °C was used to remove nonconjugated biotin or Cy5 reagent. Sizing of MV was performed with BD Biosciences FACSAria with various sized YG beads (Polysciences, Warrington, PA).

For assessing MV association with recipient BMDM, 1 × 10⁶ BMDM were plated on 12-mm poly(t-lysine)-coated coverslips in petri dishes. Cells were allowed to adhere for at least 4 h. Indicated amounts of Cy5-labeled or biotinylated MV were given to the BMDM for varying times. Cells not associated to the coverslips were harvested and processed for FACS analysis of CD86 and biotin/Cy5 label. For some experiments, coverslip-associated cells were further stained with 5-chloromethylfluorescein diacetate as a cytoplasmic counterstain (Invitrogen) and LysoTracker Red to label lysosomal compartments (Invitrogen), according to manufacturer’s protocols. Coverslips were then washed, 2% paraformaldehyde fixed, permeabilized, and blocked with 0.5% saponin, 1.5% normal goat serum, and 1% BSA in PBS for 30 min. Alexa647-conjugated streptavidin (Invitrogen) and FITC anti-CD86 (BD Biosciences) were used at 1/100 dilution for 1 h to visualize biotin and CD86 expression, respectively. After successive washes, the coverslips were stained for nuclei with 1 μg/ml DAPI and then mounted with gelvatol. Confocal microscopy images were taken with an Olympos FluoView 1000 (Inverted) and accompanying software (Olympus America, Center Valley, PA). Laser excitations and emissions were performed sequentially for DAPI, Cy5/Alexa647, LysoTracker Red, and FITC/5-chloromethylfluorescein diacetate, and background noise was minimized. Differential interference contrast microscopy images for each field of view were also taken. Final images were then directly exported to Adobe Photoshop CS2 (Adobe, San Jose, CA).

Statistical analyses

Unpaired Student t test or one-way ANOVA analyses were performed using Graph Pad Prism (GraphPad, La Jolla, CA). Values of p were calculated where indicated, and for all statistical studies p < 0.05 was considered as significant.

Results

ATP-induced MV drive de novo TNF-α secretion and upregulate costimulatory receptor surface expression in macrophages

J774A.1, a murine macrophage cell line that expresses P2X7 (27), was used to produce MV in response to 3 mM ATP plus 10 μM A23187. Whereas ATP alone was sufficient for generating detectable levels of MV, the addition of the calcium ionophore A23187 produced greater quantities of MV as judged by Bradford assay of collected material (data not shown). A23187 alone did not induce MV release. These results suggested that MV release is P2X7-dependent, and enhanced by A23187. To test this, J774A.1 cells were treated with P2X7 inhibitor A 740003 or A 438079 prior to generating MV. To confirm that the inhibitors blocked P2X7 activity, we measured Yo-Pro-1 uptake, a common measure of P2X7-induced pore formation (28). Whereas treatment with 3 mM ATP alone was sufficient to induce Yo-Pro-1 uptake, cotreatment with 10 μM A23187 and 3 mM ATP greatly increased the signal (Fig. 1A). Furthermore, Yo-Pro-1 uptake by cells exposed either to 3 mM ATP or to 3 mM ATP plus 10 μM A23187 was blocked by pretreatment with A 740003 inhibitor. Both P2X7 inhibitors either completely abolished or significantly decreased MV shedding, as determined through protein concentration determination by Bradford assay (Fig. 1B).

To study the effect of MV on macrophage function, purified MV were incubated with BMDM for 18 h and supernatants were analyzed by ELISA for TNF-α. MV induced TNF-α secretion in a dose-dependent fashion, with the highest dose of MV (75 μg protein equivalents) stimulating more TNF-α secretion than LPS (Fig. 1C, 1D). Importantly, an equivalent amount of cell homogenate from J774A.1 cells did not induce TNF-α secretion at significant levels. MV-depleted ultracentrifugation supernatant from MV generation also did not induce TNF-α at significant levels.

CD86 surface expression was also upregulated in a dose-dependent fashion following exposure to MV (Fig. 1E). Importantly, cell homogenates and MV-depleted supernatants did not increase CD86 levels compared with nontreated control BMDM (Fig. 1E). To exclude the possibility that CD86 upregulation reflected a passive uptake of the protein from MV, BMDM were pretreated with brefeldin A. This significantly diminished CD86 upregulation induced by MV, suggesting that observed increase of CD86 was due to transport of endogenously synthesized CD86 to the cell surface (Fig. 1F). Similar results of CD86 upregulation were also observed for BMDM treated with MV generated from BMDM, human monocyte cell line THP-1, the murine splenic DC line D2SC-1, and the murine fetal skin-derived DC line FSDC (Fig. 1G).

Exposure of cells to MV also upregulated the expression of other markers of activation, specifically CD80, CD83, and I-Aα (Fig. 2A). We next characterized the potential for MV to elicit the production of cytokines known to have roles in polarizing Th1 and Th17 responses. In contrast to TNF-α, however, no increase in IL-12p70 or IL-23 was observed following treatment (Fig. 2B). These data demonstrate that P2X7-induced MV contain a stimulatory activity that can activate macrophages and are not present in equivalent amounts of homogenates of the cells from which the MV derive. This also suggests that MV primarily stimulate innate immune responses in myeloid cells as opposed to directly influencing adaptive responses.

MV bind rapidly to BMDM and are largely retained at the plasma membrane before inducing TNF-α secretion and CD86 upregulation

To examine their interaction with BMDM, MV were first biotinylated. After washing and repelleting, biotinylated MV were incubated with BMDM for 18 h and MV-depleted ultracentrifugation supernatant from MV generation also did not induce TNF-α at significant levels.
MV-induced activation is partially TLR4 dependent and is independent of HMGB1

Damaged cells can release a number of compounds (such as damage-associated molecular patterns) that can activate immune cells through TLR engagement. In particular, HMGB1 (29), hyaluronic acid (30), and S100A8/S100A9 complex (31), among others, activate TLR4 for cellular activation. To examine whether MV activate through a TLR4-dependent pathway, BMDM were generated from WT and TLR4−/− mice. TNF-α induction by MV was reduced in TLR4−/− BMDM, which produced 35–81% less TNF-α than WT BMDM; however, the decrease was not statistically significant (Fig. 4A). TLR4−/− BMDM were significantly impaired in CD86 upregulation when treated with MV compared with WT BMDM (Fig. 4B). In contrast, CD86 upregulation induced by the TLR4 agonist MPLA was completely dependent on TLR4, whereas poly (I:C) stimulation was shown to be TLR4 independent, as expected.

Given the partial TLR4 response from macrophage-derived MV, HMGB1 could mediate MV-induced activation. HMGB1 can activate monocytes and macrophages (32) through TLR2, TLR4, and RAGE (29). Also, HMGB1 can be released from activated monocytes and macrophages (33) or necrotic cells (34) and is expressed within secretory lysosomes that are released from monocytes following stimulation with ATP (35). Furthermore, unlike hyaluronic acid and S100A8/S100A9 complexes, which require prior priming through agents like IFN-γ or LPS (36–38), HMGB1 can be pas-
suggesting that HMGB1 does not mediate MV activation (Fig. 5).

Characterizing MV-induced signaling pathways

NF-κB and p38 MAPK activation pathways are commonly initiated through TLR engagement (40). Indeed, in BMDM, p38 phosphorylation was induced within minutes of exposure to MV, and then declined over time, similar to the response to LPS (Fig. 6A). To address whether p38 MAPK blockade was sufficient to diminish MV-mediated CD86 upregulation, BMDM were preincubated with a titration of the phosphorylated p38 inhibitor SB203580 before exposure to MV. In the presence of the drug, CD86 upregulation was strongly inhibited, supporting a role for p38 MAPK in MV-mediated activation (Fig. 6B). In contrast, LPS-induced CD86 expression increased in the presence of 1 and 10 μM inhibitor, with significant inhibition only observed at 50 μM, suggesting differences in the two signaling pathways (data not shown).

We also tested whether MV were able to activate NF-κB, using a RAW264.7 reporter cell line (Fig. 6C). The kinetics of the response induced by MV were similar to LPS, with activation evident after 4 and 18 h, but not after 1 h. The magnitude of the

FIGURE 2. MV upregulate multiple costimulatory markers, but do not induce IL-12p70 or IL-23 secretion. A, BMDM were treated with 1 μg/ml LPS (solid line) or 25 μg protein equivalents of MV (dotted line), or were left nontreated (gray filled) for 18 h. Cells were analyzed for CD80, CD83, and CD86 or I-Ak surface expression. Isotype control is shown filled in black. Data are representative of multiple experiments. B, BMDM were treated with 1 μg/ml LPS or 25 μg protein equivalents of MV, or were left nontreated for 18 h. The histogram indicates IL-12p70 or IL-23 means ± SEM of n = 3. The statistical comparison is made to nontreated BMDM. The dotted line indicates the bottom limit of detection for each respective ELISA. **p < 0.001. n.s., not significant; NT, nontreated.

FIGURE 3. Differential kinetics of TNF-α and CD86 expression relative to surface binding of MV to BMDM. A, 5 × 10⁵ BMDM were left untreated or treated with 75 μg protein equivalents of MV for 4, 10, or 18 h. The histogram indicates means ± SEM of TNF-α released into the supernatant of n = 3, B, BMDM were left untreated or exposed to 25 μg biotinylated MV for 4, 10, or 18 h. Surface CD86 and biotin MFI changes over time are shown. The histogram indicates MFI means ± SEM of n = 3, C, BMDM were incubated with 25 μg biotinylated MV for 0.5 or 18 h. Cells were analyzed for nucleus (blue), CD86 (green), and biotin (red), and expression by confocal microscopy. Differential interference contrast image is also shown. Overlay of three fluorescent signals and differential interference contrast is shown in the far right image on the panel. Images are representative of 10 random fields of view from two separate experiments. Scale bar, 10 μm. *p < 0.05. DIC, differential interference contrast; NT, not treated.

FIGURE 4. Partial TLR4 dependence of MV-induced BMDM activation. A, 5 × 10⁵ WT or TLR4−/− BMDM were treated with 10 μg/ml poly (I:C), 5 μg/ml MPLA, or 75 μg protein equivalents of MV, or were left untreated for 18 h. The histogram indicates TNF-α means ± SEM of n = 3. B, WT or TLR4−/− BMDM were treated with 10 μg/ml poly (I:C), 5 μg/ml MPLA, or 25 μg protein equivalents of MV, or were left untreated for 18 h. Surface CD86 MFI means ± SEM of n = 3 are shown. ***p < 0.001. n.s., not significant; NT, nontreated.
response was less with MV than with LPS, however, and declined by 18 h posttreatment.

Protein kinase A (PKA) and PKC can influence p38 and NF-κB in immune cells (40). PKC is largely modulated with intracellular calcium or diacylglycerol (41), whereas PKA is cAMP controlled (42). To indirectly inhibit PKC, BMDM were treated with 30 μM BAPTA-AM to chelate intracellular calcium. Treatment did not impair CD86 upregulation in response to MV or LPS (Fig. 7A). Direct PKC inhibition through use of 50 μM BIM significantly decreased MV-mediated CD86 upregulation (Fig. 7B). In contrast, PKC inhibition enhanced LPS-mediated CD86 upregulation. MDL-12330A was used to inhibit adenylate cyclase, which generates cAMP, resulting in a significant decrease in expression from MV, but not LPS-treated BMDM (Fig. 7C). Inhibition of PKA using 10 μM H-89 HCl also resulted in a significant decrease (Fig. 7D). These results support that MV-mediated activation requires PKA and PKC pathways, which is in contrast to the observed activation response induced by LPS.

The stimulatory agent from MV consists of one or more phospholipids

To characterize the stimulatory agent(s) from MV, biochemical fractionation was used to separate lipids and proteins, as described in Materials and Methods. Only the lipid fraction, not protein, significantly activated BMDM, as measured by CD86 upregulation (Fig. 8A). Lipids were further separated according to polarity. The phospholipid fraction provided significant upregulation of CD86 as compared with nontreated control. Only minimal activity was recovered from the flow through, the neutral lipids fraction, or the glycolipid/sulpholipid fraction (Fig. 8B). Mock elution from the column for a phospholipid fraction also did not recover any stimulatory material, demonstrating that the columns themselves did not contain contaminants that could activate BMDM.

To test the TLR4 dependence of the phospholipid-containing fraction from the MV, TLR4−/− or WT BMDM were given equal amounts of phospholipid fraction. In agreement with the partial TLR4 dependence for MV-mediated activation, as observed in Fig. 4, the phospholipid fraction from MV had a significant difference (Fig. 8D). These results suggest that MV-mediated activation requires TLR4.

FIGURE 5. MV contain HMGB1, but CD86 upregulation is HMGB1 independent. A, J774A.1 cells were primed with 1 μg/ml LPS or left unprimed for 4 h, and then were treated with 3 mM ATP and 10 μM A23187 for 30 min or left untreated. Supernatants were collected, concentrated, and analyzed for HMGB1 by Western blot. Data shown are representative of repeat experiments. B, 25 μg protein equivalents from a reference lysate or 25 μg protein equivalents of MV were compared for HMGB1 expression via Western blot. C, WT and RAGE−/− BMDM were treated with 1 μg/ml LPS or 75 μg protein equivalents of MV with or without 30-min preincubation with 1 μg/ml soluble RAGE, or were left nontreated for 18 h. The histogram indicates TNF-α means ± SEM of n = 3. Statistical comparison is made to WT BMDM treated with 25 μg MV. D, WT or RAGE−/− BMDM were treated with 1 μg/ml LPS or 25 μg protein equivalents of MV, or were left untreated for 18 h. The histogram indicates surface CD86 MFI means ± SEM of n = 3. E, 25 μg protein equivalents of MV were pretreated with 100 ng/ml, 500 ng/ml, or 1000 ng/ml soluble RAGE for 30 min prior to addition to BMDM. No treatment, LPS, and 25 μg protein equivalents of MV were included as controls. Surface CD86 MFI ± SEM of n = 3 are shown. The statistical comparison is made to 25 μg MV-alone–treated BMDM. *p < 0.05; **p < 0.01; ***pp < 0.001, n.s., not significant; NT, nontreated.

FIGURE 6. MV activate p38 MAPK and NF-κB pathways. A, BMDM were left nontreated or were treated with 1 μg/ml LPS or 25 μg protein equivalents of MV for 2, 10, 30, or 60 min. Expression of phosphorylated p38 (p-p38) and total p38 (p38) was evaluated via Western blot. Data are representative of repeat experiments. B, BMDM were treated with 25 μg protein equivalents of MV with or without additional treatment of the phosphorylated p38 inhibitor SB203580 at either 1, 10, or 50 μM, or were left nontreated for 18 h. The histogram indicates surface CD86 MFI means ± SEM of n = 3. The statistical comparison is made to 25 μg MV-alone–treated BMDM. C, RAW264.7 macrophages expressing luciferase under control of a NF-κB promoter were treated with 1 μg/ml LPS or 2.5, 25, or 75 μg protein equivalents of MV for 1, 4, or 18 h. The fold change over nontreated cells is shown. The histogram indicates fold change means ± SEM of n = 3. The statistical comparison is made to the 1-h fold change value for each respective treatment. *p < 0.05; **p < 0.01; ***pp < 0.001, n.s., not significant; NT, nontreated.
inhibited cells did not decrease phosphatidic acid levels of treated BMDM treatment with MV from mock-treated J774A.1 (Fig. 9A). PLD1- and PLD2-inhibited J774A.1 with MV from mock-treated or from PLD1- or PLD2-inhibited J774A.1 producer cells were unable to stimulate CD86 upregulation (Fig. 9A). PLD inhibitors used to inhibit PLD during MV generation did not directly impact MV-mediated CD86 upregulation of recipient BMDM as inclusion of MV from PLD1- and PLD2-inhibited J774A.1 with MV from mock-treated J774A.1 did not result in any significant differences from BMDM treatment with MV from mock-treated J774A.1 (Fig. 9B). Furthermore, MV from mock-treated or from PLD1- or PLD2-inhibited cells did not decrease phosphatidic acid levels of treated J774A.1 recipient cells relative to nontreated J774A.1 (data not shown). In contrast to PLD, inhibition of iPLA2 (Fig. 9C) or cPLA2 (Fig. 9D) in MV producer cells did not decrease the stimulatory capacity of the MV generated, which were at least as potent as those from producer cells not pretreated with inhibitors. It should be noted that MV were obtained from producer cells treated with these inhibitors in amounts approximately equal to mock-treated producer cells, suggesting that the stimulatory phospholipid is not required for MV structural integrity or secretion from cells. Furthermore, PLD1, PLD2, iPLA2, and cPLA2 inhibitors were not toxic at the tried concentrations; cell viability was >85% (data not shown).

We lastly considered whether lysophosphatidic acid (LPA) could be identified as a potential stimulatory phospholipid from the MV. LPA is a product of P2X7 activity (45), and it can activate macrophages to promote cytokine production (46) and cAMP synthesis (47). Pretreatment of BMDM with Ki16425, a lysophosphatidic acid receptor inhibitor selective for LPA1 and LPA3 receptors, did not diminish CD86 upregulation by MV (Fig. 9E). This suggests LPA does not participate in MV-induced activation of macrophages, although it does not exclude the possibility that LPA could mediate effects through other receptors.

Discussion
MV released by cells can potently influence immune responses in a number of ways. Many cell types release exosomes constitutively, and, depending on the cell of origin, may transfer Ags or other cargo to DCs that can initiate immune responses. A specialized type of MV release exists for myeloid cells that express P2X7 receptors, which, when exposed to receptor agonists, such as ATP, shed MV from the cell surface as well as release them from intracellular stores. Characterizing MV generation induced by P2X7 activation on macrophages is important for understanding inflammatory processes, because tissue damage has been shown to release intracellular con-
sufficient for P2X7 activation, we observe enhanced P2X7 activity for their ability to activate macrophages. Whereas 3 mM ATP alone is not iPLA2 or cPLA2, are required for generating stimulatory MV that can induce CD86 expression. J774A.1 were pretreated with 50 μM PLD1 inhibitor CAY10593 or 50 μM PLD2 inhibitor CAY10594 in A and B, 10 μM iPLA2 inhibitor BEL1; in C, 10 μM AACOF3; or in D, DMSO vehicle control for 30 min prior to and during MV generation. A total of 25 μg protein equivalents from the generated MV was incubated with BMDM for 18 h. B, 25 μg protein equivalents from MV from mock-treated J774A.1 were incubated with or without 25 μg protein equivalents from MV from PLD1– or PLD2-inhibited J774A.1. E, BMDM were treated with or without 10 μM Ki61425 prior to MV treatment. Nontreated BMDM are included as controls. Surface CD86 MFI means ± SEM of n = 3 are shown. The statistical comparison is made to mock-treated BMDM. *p < 0.05; **p < 0.01; ***p < 0.001. n.s., not significant; NT, nontreated.

FIGURE 9. Activities of lipid-modifying enzymes PLD1 and PLD2, but not iPLA2 or cPLA2, are required for generating stimulatory MV that can induce CD86 expression. J774A.1 were pretreated with 50 μM PLD1 inhibitor CAY10593 or 50 μM PLD2 inhibitor CAY10594 in A and B, 10 μM iPLA2 inhibitor BEL1; in C, 10 μM AACOF3; or in D, DMSO vehicle control for 30 min prior to and during MV generation. A total of 25 μg protein equivalents from the generated MV was incubated with BMDM for 18 h. B, 25 μg protein equivalents from MV from mock-treated J774A.1 were incubated with or without 25 μg protein equivalents from MV from PLD1– or PLD2-inhibited J774A.1. E, BMDM were treated with or without 10 μM Ki61425 prior to MV treatment. Nontreated BMDM are included as controls. Surface CD86 MFI means ± SEM of n = 3 are shown. The statistical comparison is made to mock-treated BMDM. *p < 0.05; **p < 0.01; ***p < 0.001. n.s., not significant; NT, nontreated.

When macrophages are primed by exposure to TLR agonists, cytokines including IL-1β and TNF-α are synthesized and may be released from the cell following appropriate stimulation by a secondary signal (19). For IL-1β release, P2X7 engagement is followed by cleavage of pro–IL-1β into the bioactive form by caspase-1 in a NLRP3-dependent process. MV have been demonstrated to contain mature IL-1β and were first characterized as surface-derived vesicles (3, 14), and possibly including secreted lysosomes (7–9). The MV described in our study are predominantly 0.5–1.0 μm in diameter (Supplemental Fig. 1A), distinguishing them from larger 1- to 4-μm apoptotic blebs (53). We observed relatively few smaller MV that would be characteristic of exosomes as analyzed by electron microscopy (data not shown), and believe that our preparations were devoid of them for several reasons. Whereas our MV were obtained from ultracentrifugation at 100,000 × g, we also found that material obtained from 10,000 × g centrifugation exerts equivalent ability to activate macrophage (data not shown). Exosomes do not pellet at the low speed as they are only 50–100 nm in diameter. Thus, MV-induced macrophage activation seems to be exosome independent. Furthermore, the release of class II MHC-containing exosomes from macrophages requires apoptosis-associated speck-like protein containing a CARD (ASC)/NLRP3 inflammasome (12). Nonprimed myeloid cells, such as we have used, typically do not express high levels of NLRP3 (54), and thus, would not be expected to release class II MHC+ exosomes efficiently. In addition, we have purified MV from D2SC-1 cells, a murine splenic DC-derived cell line that lacks ASC (data not shown), and found that these MV are potent stimulators of macrophage activation (Fig. 1G). In this way, D2SC-1 act similarly to RAW264.7, which also lack ASC (27), yet also shed MV in response to P2X7 stimulation (55). These results support the conclusion that MV distinct from previously characterized class II MHC+ exosomes are shed by myeloid cells and stimulate primary macrophages through a TLR4-dependent process involving recognition of phospholipids contained within MV.

Endogenous phospholipids can activate macrophages through TLR4 activities. Recently, it was demonstrated that oxidized low density lipoprotein (oxLDL), which binds to the scavenger receptor B family member CD36, can promote sterile inflammation through activation of TLR4/6 heterodimer on macrophages (56). Both cell death (57, 58) and foam-cell formation (59) have also been shown to be induced by oxLDL through TLR4 in macrophages. Furthermore, oxidized phospholipids from minimally modified low density lipoprotein, which contain essentially the same phospholipids as oxLDL, stimulate macrophage reactive oxygen species generation (60), ERK activation (61), membrane spreading (62), and inhibition of phagocytic uptake of apoptotic bodies (62), through a partially TLR4-dependent pathway. Whether phospholipids in P2X7-induced MV are structurally similar to those in oxLDL or minimally modified low density lipoprotein will be addressed in future studies. Our study suggests that the stimulatory phospholipid is not lysophosphatidic acid (Fig. 9E). We were also unable to induce BMDM activation with commercially available lysophosphatidic acid, phosphatidic acid, or phosphatidylserine (data not shown).
Importantly, P2X7-induced MV from PLD1- and PLD2-inhibited MV producer cells were unable to activate macrophages (Fig. 9A); at the same time, MV yields were equivalent between drug-treated and nondrug-treated cells (data not shown). This dissociates MV formation from incorporation of the stimulatory phospholipid into vesicles, and suggests that generation of a bioactive phospholipid results from P2X7 activation, leading to downstream PLD activation. Whereas PLD (44) and iPLA2 (43) are activated following P2X7 activation (data not shown), blocking PLD activity, but not iPLA2, impaired the MV-activating capacity (Fig. 9). These results may explain why cell homogenates of producer cells were unable to stimulate macrophage activation (Fig. 1B, 1C), because PLD was not activated. Activated PLD produces phosphatidic acid to coordinate ADP-ribosylation factor-6, a known regulator of exocytosis, to sites of potential exocytosis (63). Future studies will be done to understand the specific PLD activities that enable stimulatory phospholipid loading into P2X7-induced MV.

It has been suggested that host cell-derived stimulators of TLR activity might contain microbial contaminants introduced during biochemical purification, a hypothesis described in detail recently in a thought-provoking review (64). Based on this, we considered whether MV preparations might contain endotoxin. When tested by Limulus amebocyte lysate assay, endotoxin was present within stimulatory MV preparations at low levels, typically ~0.25 EU/ml (data not shown). However, the same amount of endotoxin is observed in nonstimulatory MV from PLD-inhibited cells (data not shown), indicating that these low levels cannot explain the stimulation we observe. Furthermore, treatment of BMDM with equivalent amounts of LPS to that found in MV (~50 pg/ml LPS for 25 µg protein equivalents of MV) did not induce significant TNF-α release or CD86 upregulation (data not shown). It should also be noted that in addition to testing nonstimulatory MV, we observed that cell homogenates and ultracentrifugation supernatants were also devoid of BMDM-stimulating activity.

Our study suggests that MV derived from macrophages in an environment where there is tissue damage without infection could have potent biological activities that may further drive inflammation. In tumors and other settings with significant necrosis, infiltrating macrophages expressing P2X7 would be exposed to elevated levels of extracellular ATP, as previously shown in tumors (65–67). Macrophage-produced MV would then bind to adjacent cells, including macrophages and DCs, leading to their activation and resulting in secretion of TNF-α and potentially other pro-inflammatory mediators. The most novel aspect of this work, implicating phospholipids from MV as the stimulatory component, may explain why cell homogenates of producer cells were unable to stimulate macrophage activation (Fig. 1B, 1C), because PLD was not activated. Activated PLD produces phosphatidic acid to coordinate ADP-ribosylation factor-6, a known regulator of exocytosis, to sites of potential exocytosis (63). Future studies will be done to understand the specific PLD activities that enable stimulatory phospholipid loading into P2X7-induced MV.

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Disclosures

The authors have no financial conflicts of interest.

References


