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

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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: 3740–3749.
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 macrophage 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 spleen derived immature dendritic cell (DC) line (a gift of L. Kane, University of Pittsburgh, Pittsburgh, PA), were maintained in DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA), 1% additional t-glutamine (Lonza, Basel, Switzerland), and 1% penicillin and streptomycin (Lonza; hereafter called DMEM complete). J774A.1 and other myeloid cell lines (American Type Culture Collection) were maintained in IMDM (Lonza) supplemented with 10% FBS, 1% additional t-glutamine, and 1% penicillin and streptomycin (hereafter called DMEM 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-kB reporter plasmid pNF-kB-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 bone marrow (gift of L. Borghesi, University of Pittsburgh) and were differentiated in DMEM complete with 10% FBS, 1% additional t-glutamine, and 1% penicillin and streptomycin (Lonza; hereafter called DMEM 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-kB reporter plasmid pNF-kB-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 bone marrow (gift of L. Borghesi, 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). RAW264.7 macrophages stably transfected with pNF-kB-MetLuc Vector (BD Clontech, Mountain View, CA) encoding inducible Metridia luciferase protein expression and secretion was a gift of T. Dury (University of Pittsburgh). All knockout mouse bone marrow described were of the C57BL/6/6 background.

Other reagents include ATP (Thermo Fisher Scientific, Waltham, MA), A23187 (Sigma-Aldrich, St. Louis, MO), A38079 (Tocris Bioscience, Ellisville, MO), A 74003 (Tocris Bioscience), brefeldin A (Sigma-Aldrich), SB203580 (EMD Chemicals, Gibbstown, NJ), Wortmannin (AM) (BD Chemicals), tetrade 1 HCI (BIM; EMK Chemicals), MDL-123320 (Enzo Life Sciences, Farmingdale, NY), H-89 2HCl (EMD Chemicals), CAY10593 (Enzo Life Sciences), CAY10594 (Enzo Life Sciences), bromoe nol lactone (BEL; Enzo Life Sciences), LPS from Escherichia coli 026:B6 (Sigma-Aldrich), poly(I:C) (Sigma-Aldrich), synthetic monophosphoryl lipid A (MPLA; InvivoGen, San Diego, CA), Limulus amebocyte lysate QCL-1000 (Lonza; used according to manufacturer’s protocol to test endotoxin levels), and soluble RAGE (24) (gift of T. Dury).

**Yo-Pro-1 uptake by cells**

To measure P2X7-induced large pore formation, 1 × 10^6 J774A.1 were pretreated with or without 100 μM A 74003 for 15 min before exposure to ATP for 30 min, and then were stained with 5 μM Yo-Pro-1 (Invitrogen, Carlsbad, CA). Cells were kept on ice until flow cytometric analysis, which was performed with a BD Biosciences LSR II, and results were analyzed using FlowJo software (Tree Star, Ashland, OR).

**MV generation and harvest**

J774A.1 and other myeloid cell types were plated in T225 cm² flasks in duplicate per treatment. Cells were washed twice with PBS before adding inhibitors 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 centrifuged 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 gel (Sigma-Aldrich) column (1 mg silicic acid/1 ml vol lipid extract). The pass through was collected as a sample. Bed layer volumes of chloroform/methanol, and then hexane were passed to sequentially MV separate 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.

**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 ultracentrifuged 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 × 10^5 cells/ml in IMDM complete (unless described differently) in petri dishes. Inhibitors where indicated were applied for at least 30 min prior to sequential MV or compound treatments, 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 manufacturers’ protocols.

For flow cytometry studies to determine viability and expression of costimulatory receptors, cells were blocked with 1.5% normal goat serum diluted in PBS for 10 min, in PBS supplemented with allophycocyanin anti-CD80 (BD Biosciences, San Jose, CA), PE anti-CD83 (BD Biosciences), FITC anti-CD86 (BD Biosciences), or PE anti-IκBα (BD Biosciences) Abs for 40 min. Cells were stained with 1 μg/ml DAPI (Sigma-Aldrich) viability dye. Flow cytometry was performed with 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-kB reporter assay**

RAW264.7 macrophages stably transfected with pNF-kB-MetLuc Vector (BD Clontech) under G418 selection were plated as 1 × 10^4 cells/ml DMEM complete for each treatment. Supernatant following treatment was centrifuged at 10,000 × g for 10 min and then stored at −20°C until used to assay for luciferase activity, according to manufacturer’s protocol (BD Clontech). Luminescence readings from each sample were read with an Orion microplate luminometer (Berthold Detection Systems, Huntsville, AL) in duplicate, and the average was taken using Simplicity version 2.1 software (Berthold Detection Systems). Average readings and SEM were calculated according to the ratio of fold change over nontreated cells at each respective time point of the time course.

**Western blotting**

For whole-cell culture supernatant studies, 2 × 10^6 J774A.1 were plated in 1 ml DMEM complete with or without 1 μg/ml LPS for 4 h. Cells were washed twice with 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 centrifuged 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.
protease inhibition mixture (Sigma-Aldrich) and phosphatase inhibition mixture (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-HMGBl Ab (Abcam, Cambridge, MA), 6 µg/ml mouse anti–IL-1β Ab (32D; 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-p58 (Poly6224; 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). Signals were developed using Western blotting luminol reagent (Santa Cruz Biotechnology). Imaging was performed with KODAK Image Station 4000MM and its accompanying KODAK MI 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 Fisher Scientific), according to manufacturer’s protocols.

**Biotinylation and Cy5 labeling of MV and uptake by BMDM**

MV were labeled with biotin or Cy5 using EZ-Link Sulfo-NHS-SS-Biotin 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-(n-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 Olympus Fluoview 1000 (Inverted) and accompanying software (Olympus America, Center Valley, PA). Laser excitation 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 nM 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 1 740003 inhibitor. Both P2X7 inhibitors either completely abolished or significantly decreased MV shedding, as determined through protein concentration determination by Bradford assay (Fig. 1B). These results demonstrate that MV shedding induced by ATP is dependent on P2X7 activity, as others have shown (2, 3, 11, 12). MV isolated from culture supernatants by ultracentrifugation were analyzed by flow cytometry and range in size from 0.5 to 1 µm (Supplemental Fig. 1A).

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-Ab (Fig. 2A). In contrast, CD86 expression increased steadily, reaching an observed maximum at 18 h. Similar kinetics were seen with TNF-α secretion (Fig. 3A, 3B). In contrast, CD86 expression increased steadily, reaching an observed maximum at 18 h. Similar kinetics were seen with TNF-α secretion (Fig. 3C). Confocal microscopy demonstrated that CD86 and MV were not colocalized at the cell surface, and so by 4 h some MV congregated within lysosomes, whereas most of the MV remained at the plasma membrane (Supplemental Fig. 2).
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<sup>−/−</sup> mice. TNF-α induction by MV was reduced in TLR4<sup>−/−</sup> BMDM, which produced 35–81% less TNF-α than WT BMDM; however, the decrease was not statistically significant (Fig. 4A). TLR4<sup>−/−</sup> 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.

These data suggest that MV contain a stimulatory component that activates BMDM via TLR4, but that additional means of activation that are not dependent on TLR4 may also be present.

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 macrophage (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-

FIGURE 1. P2X<sub>7</sub>-induced MV elicit TNF-α secretion and upregulate CD86. A, J774A.1 were treated with or without 100 μM A 740003 and then given further indicated treatment for 30 min. Cells were analyzed for Yo-Pro-1 association by flow cytometry. The histogram indicates median fluorescent intensities means ± SEM of n = 3. Statistical comparisons are made to nontreated J774A.1, except where indicated with the inclusion bars. B. J774A.1 were treated with or without 100 μM A 740003 or 10 μM A 438079 for 15 min prior to MV generation. Harvested MV were quantified for their protein concentration by Bradford assay. The histogram indicates protein concentration means ± SEM of n = 3. Statistical comparisons are made to MV harvested from nondrug-treated J774A.1. ND indicates that the protein concentration was lower than the lower limit of detection, which is marked with the dotted line. C. BMDM were treated with 1 μg/ml LPS or 75 μg protein equivalents of MV, or were left nontreated for 18 h. The histogram indicates TNF-α means ± SEM of n = 3. The statistical comparison is made to nontreated BMDM. D. BMDM were treated with 0.25, 2.5, or 25 μg protein equivalents of MV; 25 μg protein equivalents of cell homogenate; the volume equivalent of 25 μg protein equivalent from ultracentrifugat following generation of MV pellets; or 1 μg/ml LPS; or were left nontreated for 18 h. The histogram indicates TNF-α means ± SEM of n = 3. The statistical comparison is made to nontreated BMDM. E. BMDM from D were analyzed for surface CD86 MFI means ± SEM of n = 3. The statistical comparison is made to nontreated BMDM. F. BMDM were treated with 1 μg/ml LPS or 25 μg protein equivalents of MV, or were left nontreated with or without 10 μg/ml brefeldin A for 18 h. The histogram indicates surface CD86 MFI means ± SEM of n = 3. G. MV harvested from PMA-differentiated THP-1, FSDC, BMDM, or D2SC-1 (25 μg protein equivalents) were incubated with 1 × 10<sup>6</sup> BMDM for 18 h. CD86 expression induced by MV from each indicated cell (solid line) is compared with CD86 expression induced by an equivalent amount of J774A.1 MV (dotted line) and with nontreated BMDM (black filled). Each flow cytometry histogram is representative of n = 2. *p < 0.05; **p < 0.01; ***p < 0.001. BFA, brefeldin A; Hom., homogenate; n.s., not significant; NT, nontreated; Sup., supernatant.
nontreated for 18 h. The histogram indicates IL-12p70 or IL-23 means ± SEM of CD86 upregulation between WT and RAGE−/− BMDM (Fig. 5B). Additionally, there were no significant differences in TNF-α release between WT and RAGE−/− BMDM (Fig. 5C) following stimulation with either LPS or MV. To determine more broadly whether HMGB1 is a stimulatory agent in MV-induced activation, BMDM were incubated with MV in the presence of soluble RAGE, which through competitive inhibition should block the binding of HMGB1 to all of its potential receptors. Soluble RAGE coincubated with MV did not diminish MV-mediated TNF-α release (Fig. 5C). Additionally, there were no significant differences in CD86 upregulation between WT and RAGE−/− BMDM, however, demonstrating that RAGE does not participate in MV-induced activation (Fig. 5D). Furthermore, soluble RAGE did not block MV-induced CD86 upregulation even at high concentrations, suggesting that HMGB1 does not mediate MV activation (Fig. 5E).

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-Ab 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 adenylyl 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 and PKA and PKC inhibition (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 in activation between the TLR4−/− and WT BMDM. Addi-
phosphatidic acid levels of treated BMDM. BMDM were left nontreated or treated with 1 μg/ml LPS or 25 μg MV with or without additional treatment of 10 μM intracellular calcium chelator BAPTA/AM in A, 50 μM PKC inhibitor BIM in B, 10 μM adenylate cyclase inhibitor MDL-12330A in C, or 10 μM PKA inhibitor H-89 in D, for 18 h. Surface CD86 MFI ± SEM of n = 3 are shown. *p < 0.05; **p < 0.01; ***p < 0.001. n.s., not significant; NT, nontreated.

**FIGURE 7.** CD86 upregulation is cAMP, PKA, and PKC dependent, but Ca2+ independent. BMDM were left nontreated or treated with 1 μg/ml LPS or 25 μg MV with or without additional treatment of 10 μM intracellular calcium chelator BAPTA/AM in A, 50 μM PKC inhibitor BIM in B, 10 μM adenylate cyclase inhibitor MDL-12330A in C, or 10 μM PKA inhibitor H-89 in D, for 18 h. Surface CD86 MFI ± SEM of n = 3 are shown. *p < 0.05; **p < 0.01; ***p < 0.001. n.s., not significant; NT, nontreated.

**FIGURE 8.** The phospholipid, but not protein fraction from MV activates BMDM. A, Lipids and protein were isolated from 25 μg protein equivalents of MV and were applied to BMDM for 18 h. No treatment and nonfractionated 25 μg protein equivalents of MV were included as controls. Surface CD86 MFI means ± SEM of n = 3 are shown. The statistical comparison is made to nontreated BMDM. B, Total lipid fractions from 225 μg protein equivalents of MV were isolated and were further fractionated as follows: column flow through, neutral lipids, glycolipids and sphingolipids, and phospholipids. Mock methanol elution of the column without any loaded material was included as a control. Fractions were incubated with BMDM for 18 h. Nontreated BMDM and incubation of the phospholipid fraction with TLR4+/− BMDM are also shown. Surface CD86 MFI means ± SEM of n = 3 are shown. The statistical comparison is made to nontreated BMDM, except where indicated. *p < 0.05; **p < 0.01; ***p < 0.001. FT, column flow through; GL/SL, glycolipid and sphingolipid; NL, neutral lipid; n.s., not significant; NT, nontreated; PL, phospholipid.

**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-
The statistical comparison is made to mock MV-treated BMDM. Included as controls. Surface CD86 MFI means ± SEM of n = 3 are shown. The statistical comparison is made to mock MV-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), but later as exosomes (11). TNF-α–containing exosomes have been reported to be secreted from human melanoma cells (52). We have detected TNF-α within P2X7–induced MV from primed macrophages; however, TNF-α was also detected from cell culture supernatants devoid of MV (data not shown). Nevertheless, only primed macrophages would release pro-inflammatory cytokines, whose synthesis primarily depends on NF-κB activation (40), whether in MV or as soluble proteins.

Our study instead focuses on characterizing the biological effects of MV released by nonprimed, primary macrophages, a condition that may be seen during sterile inflammation. As reported by others, MV are released following P2X7 engagement in nonprimed macrophages (12), yet, to the best of our knowledge, have not been studied for their ability to activate macrophages. Whereas 3 mM ATP alone is sufficient for P2X7 activation, we observe enhanced P2X7 activity when coadministering 10 μM A23187 with 3 mM ATP. A23187 with 0.3 mM ATP was not able to induce P2X7 activity; thus, in this manner, A23187 is serving in some other function than for the autocrine release of endogenously stored ATP for P2X7 activation. We found that P2X7–induced MV from nonprimed myeloid cells can induce expression of CD86, CD80, CD83, and class II MHC (Fig. 2A), and also induce secretion of TNF-α from primary macrophages (Fig. 1A, 1B). We further characterized the contents of these MV and found that phospholipids were responsible for stimulating macrophage activation in a TLR4-dependent process (Fig. 8A). As separated phospholipids from MV activate macrophages, we can exclude a requirement for interaction of intact MV in this process. That being said, based on our observation that MV-mediated activation was only partially TLR4 dependent, we hypothesize that MV must contain components in addition to phospholipids that activate macrophages independently of TLR4, and that these activities may be contingent upon intact MV delivery into intracellular departments.

Stimulation of primed macrophages by P2X7 yields MV that are heterogeneous, consisting of exosomes (11, 12) and 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 macrophage 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 (63), 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-riboylation 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 proinflammatory 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-riboylation 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.

Disclosures

The authors have no financial conflicts of interest.

References