The Journal of Immunology

RESEARCH ARTICLE | AUGUST 01 2007

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J Immunol (2007) 179 (3): 1913–1925.
https://doi.org/10.4049/jimmunol.179.3.1913

Nonclassical IL-1β Secretion Stimulated by P2X7 Receptors Is Dependent on Inflammasome Activation and Correlated with Exosome Release in Murine Macrophages[1]

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Several mechanistically distinct models of nonclassical secretion, including exocytosis of secretory lysosomes, shedding of plasma membrane microvesicles, and direct efflux through plasma membrane transporters, have been proposed to explain the rapid export of caspase-1-processed IL-1β from monocytes/macrophages in response to activation of P2X7 receptors (P2X7R) by extracellular ATP. We compared the contribution of these mechanisms to P2X7R-stimulated IL-1β secretion in primary bone marrow-derived macrophages isolated from wild-type, P2X7R knockout, or apoptosis-associated speck-like protein containing a C-terminal CARD knockout mice. Our experiments revealed the following: 1) a novel correlation between IL-1β secretion and the release of the MHC-II membrane protein, which is a marker of plasma membranes, recycling endosomes, multivesicular bodies, and released exosomes; 2) a common and absolute requirement for inflammasome assembly and active caspase-1 in triggering the cotemporal export of IL-1β and MHC-II; and 3) mechanistic dissociation of IL-1β export from either secretory lysosome exocytosis or plasma membrane microvesicle shedding on the basis of different requirements for extracellular Ca^{2+} and differential sensitivity to pharmacological agents that block activation of caspase-1 inflammasomes. Thus, neither secretory lysosome exocytosis nor microvesicle shedding models constitute the major pathways for nonclassical IL-1β secretion from ATP-stimulated murine macrophages. Our findings suggest an alternative model of IL-1β release that may involve the P2X7R-induced formation of multivesicular bodies that contain exosomes with entrapped IL-1β, caspase-1, and other inflammasome components. *The Journal of Immunology,* **2007, 179: 1913–1925.**

Interleukin-1β is a primary proinflammatory cytokine whose local and circulating levels are tightly regulated to prevent aberrant activation of pathways that can lead to chronic inflammatory diseases (1). In response to various pathogen-associated molecular pattern (PAMP)[3] molecules that target TLR, IL-1β accumulates as a biologically inactive 33-kDa procytokine (pro-IL-1β) in the cytoplasm of monocytes and macrophages. Conversion to the biologically active 17-kDa form requires proteolytic maturation by caspase-1 (2, 3), which itself is regulated by the assembly of multiprotein complexes termed inflammasomes (4, 5). The adapter or scaffolding proteins for inflammasome activation include apoptosis-associated speck-like protein containing a C-terminal CARD (ASC)/Pycard (6), which contains a PYRIN domain in addition to its CARD site, and members of the nucleoside oligomerization domain (NOD)/NACHT/CATERPILLER family (7, 8) that contain a PYRIN domain, a central nucleotide-binding oligomerization (NACHT/NOD) domain, and a leucine-rich repeat segment. This family includes Nacht, LRR, and PYRIN-domain containing proteins (Nalp)1, Nalp2, and Nalp3/cryopyrin. Other inflammasome adapter proteins, such as Ipaf, contain NACHT/NOD and CARD domains, but lack PYRIN domains (9).

Recent studies of inflammasome regulation have used macrophages from knockout mice with targeted deletions of these scaffolding proteins to compare caspase-1 activation in response to different PAMPs, including LPS (10, 11), lipopeptides, CpG-DNA, flagellin (12–14), bacterial RNA (15), viral RNA (16), and the antiviral imidazoquinoline analogues R837 and R846 (15). These studies have demonstrated that ASC is essential for activation of caspase-1 and production of mature IL-1β in response to all of these inflammatory stimuli (10, 11, 16, 17). Macrophages from Ipaf-null mice are specifically deficient in caspase-1 activation and IL-1β secretion in response to flagellin (12–14), whereas knockout of Nalp3 prevents inflammasome activation in response to bacterial RNA, viral RNA, or R837 (15, 16).

Flagellin, bacterial RNA, or R837 rapidly induces activation of caspase-1 inflammasome complexes in macrophages via mechanisms that can be dissociated from the parallel actions of these PAMPs as ligands for various TLRs. In contrast, other PAMPs, such as LPS and lipopeptides, stimulate a rapid and TLR-dependent transcription and translation of pro-IL-1β, but elicit only modest production of mature IL-1β due to inefficient stimulation of active caspase-1 inflammasomes (11, 18). However, when LPS- or lipopeptide-primed macrophages are additionally stimulated with extracellular ATP, they rapidly release large amounts of mature IL-1β at rates that are up to 100 times faster than with PAMP

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Received for publication February 9, 2007. Accepted for publication May 16, 2007.

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[1] This work was supported by National Institutes of Health Grants GM36387 (to G.R.D.) and AI063331/AI064748 (to G.N.).

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[3] Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; AMC, aminomethylcoumarin; BEL, bromoenol lactone; BMDM, bone marrow-derived macrophage; BSS, basal saline solution; ASC, apoptosis-associated speck-like protein containing a C-terminal CARD; NOD, nucleoside oligomerization domain; NALP, Nacht, LRR, and PYRIN domain containing proteins; LAMP, lysosome-associated membrane protein; MVB, multivesicular body; P2X7R, P2X7 receptor; Px1, pannexin-1.

exposure alone (19–21). This costimulatory action of ATP is mediated by the P2X7 receptor (P2X7R), which acts as a nondesensitizing cation channel to rapidly induce a complete collapse of normal ionic gradients (22). An essential role for K^+ release in the activation of caspase-1 is supported by the robust IL-1β processing induced by stimuli other than ATP that also cause a rapid lowering of cytoplasmic K^+ (19, 23–26). Significantly, the ability of ATP/P2X7R to massively accelerate caspase-1 activation and IL-1β secretion is eliminated in either ASC-null (10) or Nalp3-null (11, 18) macrophages primed with LPS.

A major unresolved issue is how inflammasome activation is linked to the rapid export of biologically active IL-1β. Because IL-1β lacks signal sequences for compartmentation within the Golgi and classical secretory vesicles, release of the mature cytokine to extracellular compartments requires nonclassical mechanisms of secretion (27–29). Moreover, P2X7R activation additionally stimulates the rapid corelease of active caspase-1, a 60-kDa tetrameric complex (10, 11, 21, 30), as well as the ASC inflammasome scaffolding protein. This suggests a mechanism that involves a coordinated mass transport of the IL-1β together with the caspase-1 inflammasome processing complex. Although P2X7R stimulation can trigger cytolysis (31), the early stages of IL-1β release from ATP-activated macrophages can be unequivocally dissociated from ATP-induced cytolysis (32).

Three nonclassical IL-1β secretion models with distinct mechanisms have been proposed to date. One is based on the observation that immunoreactive IL-1β and caspase-1 can be localized within lysosomes of human monocytes. This model posits that pro-IL-1β and caspase-1 are transported from the cytosol into a subset of secretory lysosomes (33–35). IL-1β processing occurs inside these secretory lysosomes during their trafficking to the plasma membrane, whereas ATP stimulation of P2X7R drives IL-1β release from these lysosomes via exocytotic fusion regulated by a Ca^{2+} and phospholipase-dependent mechanism. In the second model, based on studies with human THP-1 monocytes (36), murine microglial cells (37), or human dendritic cells (38), P2X7R stimulation initiates local accumulation of caspase-1 and IL-1β within the microdomain of the subplasma membrane cytosol and also the evagination of plasma membrane blebs that rapidly scission away from the cell surface. The shed blebs, which are phosphatidylserine-enriched microvesicles, contain entrapped IL-1β and caspase-1. Finally, Brough and Rothwell (39) have recently used immunofluorescence imaging of IL-1β trafficking within murine macrophages to propose that mature IL-1β protein may directly pass through the plasma membrane via molecularly undefined protein transporters.

In this study, we directly assessed the contribution of secretory lysosome exocytosis vs microvesicle shedding as IL-1β release mechanisms in an experimental model of primary murine bone marrow-derived macrophages (BMDM). A major advantage of this experimental system is that inflammasome activation, IL-1β export, and secretory lysosome exocytosis can be directly compared in macrophages with targeted deletion of the key regulatory proteins, P2X7R and ASC. Based on both genetic and pharmacologic approaches, we now report that the ability of activated P2X7R to trigger both IL-1β release and exocytosis of secretory lysosomes occurs via closely related, but completely dissociable, signaling mechanisms in primary murine macrophages. Likewise, ATP-stimulated microvesicle shedding was unaffected by manipulations that markedly attenuate ATP-activated IL-1β export. Significantly, accumulation of cytosolic active caspase-1 was found to be a necessary signal for the trafficking of ASC-based inflammasome complexes, as well as IL-1β itself, to the cellular compartment(s) that mediates export of the mature cytokine. Finally, we present data that support a possible role for endosome-derived recycling multivesicular bodies (MVB) and released exosomes as the major mechanism for the rapid corelease of IL-β and caspase-1 inflammasome complexes from primary murine macrophages.

Materials and Methods

Reagents

Key reagents and their sources were as follows: *Escherichia coli* LPS serotype O1101:B4 (List Biological Laboratories); ATP, cycloheximide, and carbenoxolone (Sigma-Aldrich); Bay 11-7085 and MG132 (BIOMOL); bromoenol lactone (BEL; Cayman Chemical); maitotoxin (Wako Bioproducts); and YVAD-cmk and Z-Arg-Arg-aminomethylcoumarin (AMC) (Bachem). Murine rIFN-γ was from Boehringer Mannheim. Abs for ELISA of murine IL-1β were from Pierce Endogen. Anti-P2X7R was from Alamone Laboratories. Anti-caspase-1 p10 rabbit polyclonal, anti-cathepsin B goat polyclonal, anti-lysozyme C goat polyclonal, anti-lysosome-associated membrane protein (LAMP-1) rat monoclonal, and all HRP-conjugated secondary Abs were from Santa Cruz Biotechnology. The 3ZD anti-IL-1β mAb, which recognizes both 33-kDa pro-IL-1β and 17-kDa mature IL-1β in Western blot analysis, was provided by the Biological Resources Branch, National Cancer Institute, Frederick Cancer Research and Development Center. Mouse mAb KL295 against MHC-II was a gift from C. Harding (Case Western Reserve University, Cleveland, OH). The rat anti-mouse ASC mAb has been previously described.

Murine macrophage models and cell culture

BALB/c and C57BL/6 mice were purchased from Taconic Farms; $P2X7R^{-/-}$ mice (C57BL/6 background) were provided by Pfizer Global Research and Development; ASC $^{-/-}$ mice (C57BL/6 background) were generated as described previously. All experiments and procedures involving mice were approved by the Institutional Animal Use and Care Committees of either Case Western Reserve University or University of Michigan. BMDM were isolated by previously described protocols (40). Mice were euthanized by CO_2 inhalation. Femurs and tibia were removed and briefly sterilized in 70% ethanol, and PBS was used to wash out the marrow cavity plugs. The bone marrow cells were resuspended in DMEM (Sigma-Aldrich) supplemented with 25% L cell-conditioned medium, 15% calf serum (HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies), plated onto 150-mm dishes, cultured in the presence of 10% CO_2. After 5–9 days, the resulting BMDM were detached with PBS containing 5 mM EDTA and 4 mg/ml lidocaine (40), replated into 6-well or 12-well plates, and used within 10 days.

Western blot analyses for caspase-1 activation and release, IL-1β processing and secretion, exocytosis of secretory lysosomes, and plasma membrane microvesicle shedding

BMDM were routinely seeded in 6-well plates to a cell density of ~2×10^6/well. In some experiments (Fig. 9), the BMDM were stimulated with 2 ng/ml IFN-γ for 16–18 h before LPS priming and ATP stimulation. Culture medium was replaced with fresh medium supplemented with or without 1 μg/ml LPS. In some experiments, cells were pretreated with various pharmacological inhibitors before and during LPS priming. Cells were primed with LPS for up to 4 h at 37°C, followed by washing once with PBS and transfer to 1 ml of basal saline solution (BSS) assay medium containing 130 mM sodium gluconate, 5 mM KCl, 20 mM Na HEPES, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$ (pH 7.5) supplemented with 5 mM glucose, 5 mM glycine, and 0.01% BSA. Experiments testing effects of extracellular Ca^{2+} on IL-1β secretion used BSS with no added $CaCl_2$. Cells were then equilibrated for an additional 5 min at 37°C before stimulation with ATP or maitotoxin. To terminate the release reaction, the entire 1 ml of extracellular medium was transferred to a tube on ice, whereas the cell monolayer was rapidly washed once with 1 ml of ice-cold PBS, and then lysed by addition of 150 μl of radioimmunoprecipitation assay extraction buffer (1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS (pH 7.4) in PBS) containing PMSF, leupeptin, and aprotinin. For processing of extracellular medium, the collected 1-ml samples were centrifuged at $10,000 \times g$ for 10 s to remove any detached cells, followed by transfer of the supernatant to a fresh tube. The supernatant was concentrated by TCA precipitation using 72 μl of 100% TCA and 15 μl of 10% cholic acid/1 ml extracellular medium. The precipitated pellets were washed three times with 1 ml of acetone, dissolved in 10 μl of 0.2 M NaOH, diluted with 35 μl of H_2O, supplemented with 15 μl of 4× SDS-PAGE sample buffer, and boiled for 5 min. For processing of cell lysates, the 150 μl radioimmunoprecipitation assay extracts were combined with any detached cells from the extracellular medium and recentrifuged, and supernatants were transferred to fresh

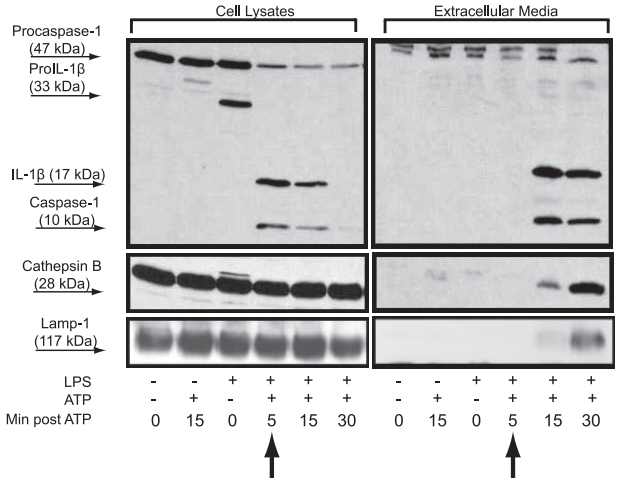

FIGURE 1. P2X7R-dependent IL-1β processing and secretion are strongly correlated with release of lysosomal marker proteins in murine BMDM. C57BL/6 BMDM were pretreated with or without LPS (1 μg/ml) for 4 h. Cells were transferred to BSS and stimulated with 5 mM ATP for the indicated times. The extracellular medium and cell lysates were separately collected and processed for Western blot analysis. The blot membrane was first probed by simultaneous treatment with anti-caspase-1 and anti-IL-1β, then stripped and sequentially probed with Abs against cathepsin B and LAMP-1. These data are representative of similar time course studies from seven separate experiments with C57BL/6 BMDM and five experiments using BALB/c BMDM.

tubes. A total of 45 μl of cell lysate was supplemented with 15 μl of 4× SDS-PAGE sample buffer and boiled for 5 min. Extracellular medium and cell lysates were processed by SDS-PAGE, electrophoretic transfer polyvinylidene difluoride membranes, and standard Western blot protocols, as described previously (30). Primary Abs were used at the following concentrations: 5 μg/ml for IL-1β, 1 μg/ml for caspase-1, 0.3 μg/ml for P2X7R, 0.04 μg/ml for LAMP-1, 1 μg/ml for cathepsin B, 1 μg/ml for lysozyme C, and 0.8 μg/ml for MHC-II. HRP-conjugated secondary Abs were used at a final concentration of 0.13 μg/ml.

Intracellular Ca^{2+} concentration measurements

BMDM were detached with lidocaine-EDTA buffer and resuspended in NaCl BSS (130 mM NaCl, 5 mM KCl, 20 mM Na HEPES, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$ (pH 7.5)) supplemented with 5 mM glucose and 0.01% BSA. Macrophage suspensions were loaded with 1 μM fura 2-AM and assayed for changes in cytosolic Ca^{2+}, as described previously (41).

Online assay of cathepsin activity released from intact cells

Release of cathepsin activity into the extracellular medium bathing intact macrophages was measured using a nonfluorescent cathepsin substrate, Z-Arg-Arg-AMC, which upon hydrolysis liberates AMC as a fluorescent product. We adapted a previously described fluorometric protocol (360 nm excitation and 450 nm emission) that allows this enzyme assay to be performed online using intact cell monolayers grown on plastic coverslips (42). The assay buffer was standard BSS (or Ca^{2+}-free BSS). For measurement of cathepsin release, a coverslip with adherent BMDM was placed diagonally into the cuvette with 2 ml of assay buffer, followed by 50 μM fluorogenic substrate Z-Arg-Arg-AMC.

ELISA analysis of IL-1β release

Aliquots (25–50 μl) of extracellular medium samples were assayed for IL-1β content by sandwich ELISA, exactly as described previously (43).

Results

P2X7R-dependent caspase-1 activation and IL-1β secretion are strongly correlated with release of endolysosomal marker proteins

Fig. 1 illustrates the very early kinetics of caspase-1 inflammasome activation and IL-1β processing and release in LPS-primed murine BMDM acutely stimulated with 5 mM ATP, a concentration that maximally activates P2X7R. At 5 min post-ATP, most of the intracellular pro-IL-1β was processed into mature IL-1β and ~50% of the procaspase-1 was converted to active tetramer (as indicated by accumulation of p10 subunit). Significantly, none of this processed IL-1β or caspase-1 was released to the extracellular compartment at this 5-min time point (compare lanes indicated by linked arrows). Over the next 10 min, most of this mature IL-1β and active caspase-1 was cleared from the cells into the extracellular medium. The 5-min lag before any appearance of extracellular caspase-1 or IL-1β indicates that P2X7R-induced inflammasome activation and maturation of IL-1β occurred in an intracellular compartment before any secretion. This finding of very efficient IL-1β maturation before export is similar to that recently reported by Brough and Rothwell (39) using a murine peritoneal macrophage system. A similar 5-min lag preceded the extracellular accumulation of cathepsin B, a soluble cysteine protease normally packaged within lysosomes, as well as LAMP-1, an intrinsic membrane protein associated with lysosomes and late endosomes. Following this 5-min lag period, all four proteins (caspase-1, IL-1β, cathepsin B, and LAMP-1) accumulated in the extracellular compartment with similar kinetics. These results indicate that ATP stimulation of mature IL-1β secretion in murine macrophages is strongly correlated with the exocytosis of secretory lysosomes, as previously reported for human monocytes (33–35). It is also important to note that all IL-1β release experiments presented in this study were performed in the presence of 5 mM extracellular glycine, which we (32) have shown can attenuate the pyroptotic lysis of ATP-stimulated, LPS-primed macrophages characterized by Brough and Rothwell (39). Moreover, no release of pro-IL-1β as an indicator of nonspecific lysis was observed under any experimental conditions that we used.

Obligatory role for P2X7R in the ATP-induced corelease of mature IL-1β, active caspase-1, cathepsin B, and LAMP-1

Multiple studies have indicated that secretory lysosomes can be exocytotically released from immune effector cells subjected to various stimuli that increase cytosolic Ca^{2+} (44–46). Because murine macrophages express multiple Ca^{2+}-mobilizing P2 receptors (47, 48), it is possible that the observed release of lysosomal marker proteins in Fig. 1 was due to ATP receptors other than P2X7. This is significant because the P2X7R-null macrophages, like wild-type macrophages, do express three G protein-coupled P2 receptors (P2Y1, P2Y2, and P2Y6) that trigger strong Ca^{2+} mobilization responses to ATP, UTP, or UDP (Fig. 2*B*). Consistent with previous reports (22, 31, 49), we observed that macrophages from P2X7R-null mice exhibited no caspase-1 activation or IL-1β secretion when stimulated with 5 mM ATP, a concentration that will maximally activate P2X7R as well as P2Y1 and P2Y2 receptors (Fig. 2*A*). Significantly, the ATP-induced exocytosis of secretory lysosomes was also completely repressed in these knockout cells. This indicates that increased cytosolic Ca^{2+} per se, which is one of the downstream responses to P2X7R, is not a sufficient signal for either the release of mature IL-1β, as reported previously (50), or the coincident exocytosis of secretory lysosomes observed in ATP-stimulated wild-type macrophages. This further suggests a requirement for other signals, such as a rapid loss of cytosolic K^+, which are not triggered by G protein-coupled ATP receptors, but are elicited by P2X7R (51). IL-1β processing and release from wild-type macrophages can be stimulated by maitotoxin (11, 26), which bypasses the P2X7R to activate other nonselective cation channels that facilitate both K^+ efflux and Ca^{2+} influx. Fig. 2, *C* and *D*, shows that maitotoxin also coordinately induced secretory

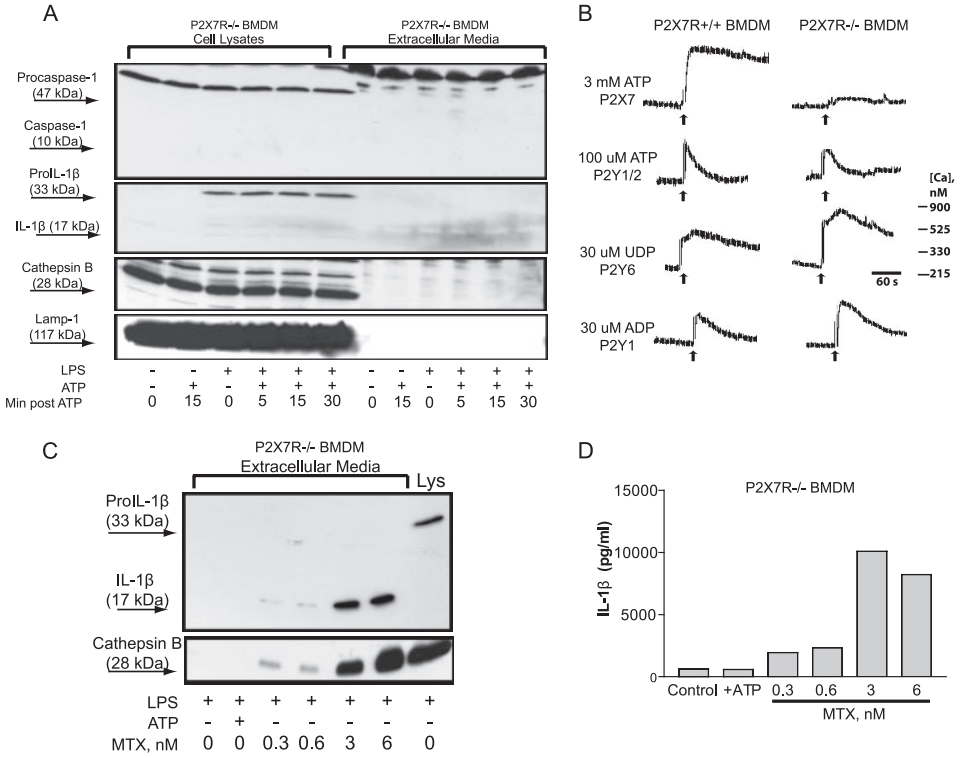

FIGURE 2. Obligatory role of P2X7R in ATP-induced release of mature IL-1β, active caspase-1, cathepsin B, and LAMP-1. *A*, BMDM from P2X7R-null mice were primed with or without LPS (1 μg/ml) for 4 h, stimulated with 5 mM ATP, and processed for Western blot analysis of cell lysates or extracellular medium. These data are representative of results from five experiments. *B*, BMDM from P2X7R$^{+/+}$ or P2X7R$^{-/-}$ mice (both in C57BL/6 background) were assayed for changes in cytosolic-free Ca^{2+} in response to the indicated nucleotides. For analysis of P2X7R-mediated Ca influx, cells were first stimulated with a mixture of 30 μM each of ATP, ADP, and UDP to activate and desensitize P2Y receptors before P2X7R stimulation by 3 mM ATP. *C* and *D*, BMDM from P2X7R-null mice were primed with LPS (1 μg/ml) for 4 h, and then stimulated with 5 mM ATP or the indicated concentrations of maitotoxin for 30 min. The extracellular medium was collected and assayed for IL-1β and cathepsin B release by Western blot (*C*) or IL-1β ELISA (*D*). *C*, "Lys" indicates whole cell lysates from unstimulated LPS-primed BMDM. Similar results were obtained in three experiments.

lysosome exocytosis as well as mature IL-1β release in the P2X7R-null BMDM.

P2X7R-induced exocytosis of secretory lysosomes is LPS dependent and requires protein synthesis, proteasome activity, and NF-κB signaling

Given our previous finding (30) that LPS priming of BMDM was required for efficient coupling of P2X7R to inflammasome activation and IL-1β export, we tested whether a similar requirement for LPS priming characterized the activation of secretory lysosome exocytosis by ATP. Notably, non-LPS-primed BMDM secreted only a minimal amount of cathepsin B and no active caspase-1 in response to ATP stimulation (Fig. 3*A*). The coupling of P2X7R to inflammasome activation could be observed within 30 min of LPS priming, as indicated by the ATP-stimulated accumulation of intracellular caspase-1 p10. Similarly, an increase in ATP-elicited cathepsin B release was apparent within 30 min after initiation of LPS priming. In cells primed with LPS for longer durations (1 or 4 h), the ATP-stimulated release of both active caspase-1 and cathepsin B was further increased in a coordinated manner. In the absence of ATP stimulation, even 4 h of LPS priming alone did not result in inflammasome activation, IL-1β secretion, or cathepsin B release. Furthermore, the ability of LPS to potentiate ATP-dependent caspase-1 activation and secretory lysosome exocytosis occurred before significant expression of pro-IL-1β protein. These observations indicate that LPS priming provides a necessary and common signal(s) for efficient coupling of P2X7R to exocytosis of secretory lysosomes as well as inflammasome activation.

Other experiments tested whether de novo protein synthesis or maintained synthesis of rapid turnover proteins was necessary for P2X7R-dependent inflammasome activation and P2X7R-activated lysosome exocytosis. Pretreatment of cells with the protein synthesis inhibitor cycloheximide, the proteasome inhibitor MG132, or the Iκ-B kinase inhibitor Bay 11-7085 before LPS priming completely repressed not only intracellular pro-IL-1β accumulation, but the P2X7R-induced activation of the caspase-1 inflammasome. As indicated in Fig. 3, *B* and *C*, these three inhibitors similarly repressed the correlated lysosomal protein secretion, but did not change the expression levels of procaspase-1 or cathepsin B. The expression of P2X7R and LAMP-1 protein, as well as P2X7R channel function, was not altered in cells treated with these pharmacological reagents (data not shown).

ASC, active caspase-1, and pannexin-1 (Px1) are required for IL-1β secretion, but not secretory lysosome exocytosis stimulated by P2X7R

The adaptor protein ASC, which is essential for assembly of caspase-1 inflammasome complexes, is also coreleased with caspase-1 and IL-1β (10) (Fig. 8*B*). This suggests that the entire inflammasome complex is copackaged and released as a unit with its IL-1β product from ATP-stimulated cells. We used BMDM from ASC-null mice to test whether ASC was also required for the P2X7R-regulated exocytosis of secretory lysosomes, which coincided with IL-1β secretion. Fig. 4*A* shows that ASC$^{-/-}$ BMDM were completely deficient in ATP-induced activation of inflammasome complexes as well as the coupled release of mature IL-1β and active caspase-1. However, release of the soluble

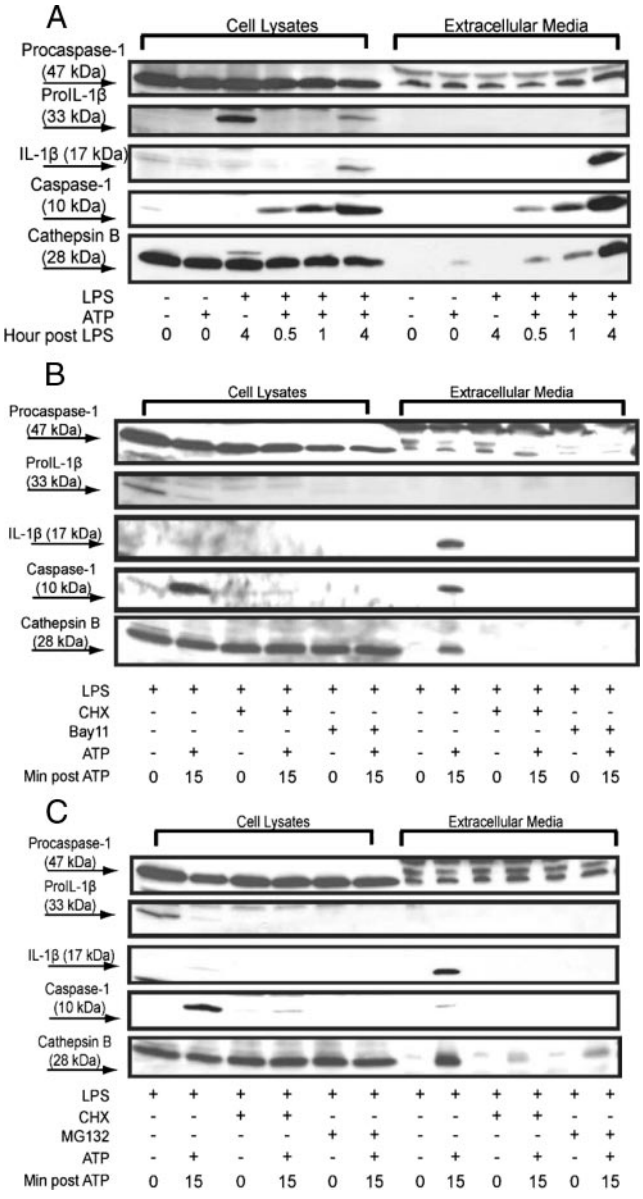

FIGURE 3. P2X7R-induced caspase-1 activation, IL-1β secretion, and exocytosis of secretory lysosmes are coordinately dependent on LPS priming, de novo protein synthesis, proteasome activity, and NF-κB signaling. *A*, BALB/c BMDM were pretreated with or without LPS (1 μg/ml) for the indicated times, transferred to BSS, and stimulated with 1 mM ATP for 15 min. The extracellular medium and cell lysates were collected and processed for Western blot analysis. These data are representative of results from two experiments. *B* and *C*, BALB/c BMDM were pretreated with or without 50 μM cycloheximide or 20 μM Bay 11-7085 (*B*), 50 μM cycloheximide, or 50 μM MG132 (*C*), for 30 min before and during LPS (1 μg/ml) priming for 4 h. Cells were transferred to BSS and stimulated with 1 mM ATP for 15 min. The extracellular medium and cell lysates were processed for Western blot analysis. *B*, Representative of results from six experiments. *C*, Representative of results from two experiments.

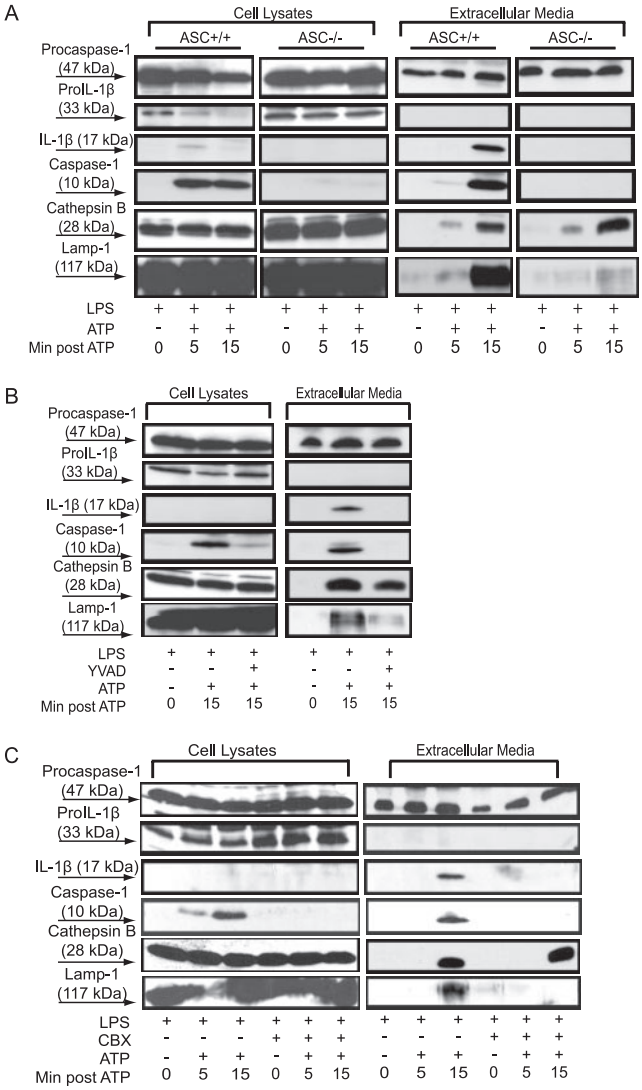

FIGURE 4. ASC, active caspase-1, and Px1 are required for IL-1β secretion, but not secretory lysosome exocytosis stimulated by P2X7R. *A*, BMDM from ASC$^{+/+}$ or ASC$^{-/-}$ mice (both in C57BL/6 background) were primed with or without LPS (1 μg/ml) for 4 h, transferred to BSS, stimulated with or without 5 mM ATP for the indicated times, and processed for Western blot analysis of cell lysates or extracellular medium. These data are representative of results from five experiments. *B*, BALB/c BMDM were pretreated with or without LPS (1 μg/ml) for 4 h, transferred to BSS, and preincubated with 10 μM YVAD for 30 min before stimulation with 1 mM ATP for 15 min. The extracellular medium and cell lysates were collected and processed for Western blot analysis. These data are representative of results from six experiments performed in BALB/c BMDM and one experiment in C57BL/6 BMDM. *C*, BALB/c BMDM were pretreated with or without LPS (1 μg/ml) for 4 h, transferred to BSS, and preincubated with 50 μM carbenoxolone (CBX) for 30 min before stimulation with 1 mM ATP for 5 or 15 min. The cell lysates and extracellular medium were processed for Western blot analysis. The data are representative of results from three experiments.

lysosomal marker protein, cathepsin B, was intact and exhibited secretion kinetics comparable to that observed in ASC$^{+/+}$ BMDM. In contrast, release of the endosomal/lysosomal membrane marker protein, LAMP-1, was strongly inhibited in the ASC-deficient BMDM.

The preceding data indicate that ASC is essential for the trafficking of active caspase-1 and mature IL-1β to the P2X7R-regulated IL-1β secretory machinery. Previous studies indicated that macrophages from caspase-1-null mice are unable to release pro-IL-1β in response to P2X7R activation (31, 50). Given the critical role for ASC in regulating accumulation of active caspase-1, this suggests that active caspase-1 per se is involved in trafficking or packaging inflammasome complexes within the machinery used for secretion of mature IL-1β and inflammasome proteins. This was tested by inclusion of YVAD-cmk, an inhibitor of active caspase-1, during ATP stimulation of ASC$^{+/+}$ BMDM (Fig. 4*B*).

YVAD treatment reduced, but did not eliminate the accumulation of intracellular active caspase-1; this is consistent with an expected inhibitory action downstream of inflammasome assembly and caspase-1 activation per se (52). However, similar to the results observed in ASC$^{-/-}$ BMDM, the YVAD-treated wild-type BMDM exhibited a marked repression of ATP-induced export of mature IL-1β and active caspase-1. As with ASC knockout, YVAD treatment also inhibited the release of the endosomal/lysosomal membrane marker LAMP-1, but not the lysosomal soluble marker cathepsin B (Fig. 4*B*). Pelegrin and Surprenant (53, 54) recently identified Px1 as a plasma membrane protein that couples P2X7R to activation of the caspase-1 inflammasome based on the repressive actions of Px1 siRNA or pharmacological Px1 inhibitors, such as carbenoxolone. We observed that carbenoxolone coordinately inhibited the ATP-stimulated release of IL-1β, active caspase-1, and LAMP-1, but had no effect on secretion of cathepsin B (Fig. 4*C*).

Different ATP concentration-response relationships describe IL-1β release vs secretory lysosome exocytosis

The preceding data indicated that ATP-stimulated exocytosis of secretory lysosomes can be functionally dissociated from inflammasome activation and IL-1β secretion. Analysis of ATP concentration-response relationships provided additional evidence for a dissociation of these two P2X7R-triggered responses. Fig. 5*A* compares the effects of 1, 3, and 5 mM ATP stimulation (15 min) on the release of mature IL-1β, active caspase-1, cathepsin B, and LAMP-1 in BMDM. Consistent with our previously reported data (32), 1 mM ATP was submaximal for caspase-1 activation as well as release of mature IL-1β and active caspase-1. ATP, at 1 mM, was similarly submaximal for LAMP-1 externalization, but produced the greatest magnitude of cathepsin B secretion of the three ATP concentrations tested. In contrast, 3 or 5 mM ATP resulted in less cathepsin B release, but greater secretion of mature IL-1β, active caspase-1, and LAMP-1. This indicated an atypical biphasic relationship between ATP stimulus dose and the activation of secretory lysosome exocytosis.

As an alternative approach for measurement of P2X7R-dependent lysosome exocytosis, we adapted an online fluorometric assay (42) using BMDM grown on plastic coverslips that allowed a continuous monitoring of cathepsin activity released into the extracellular medium of intact macrophages. The steady generation of fluorescent AMC upon the placement of the coverslip (but before ATP stimulation) represents the ambient cathepsin, which may result from damaged cells present at time zero. Any changes in the rate of AMC production, as indicated by the instantaneous slope of fluorescence/time, represent an increased release of cathepsin from the intact cells into the extracellular medium. Fig. 5*B* compares the basal- vs ATP (1 mM)-stimulated cathepsin release from nonprimed vs LPS-primed BMDM. Although basal cathepsin release was modestly higher in the LPS-primed cells, the ATP-sensitive cathepsin secretion from these primed cells was greatly increased relative to that observed in the nonprimed macrophages. Consistent with the Western blot data (Fig. 1), there was a 5-min lag between the initial addition of ATP and the rapid phase of cathepsin extracellular appearance. Upon lysis with detergent, similar amounts of cathepsin release were observed in the nonprimed vs LPS-primed monolayers. Fig. 5*C* shows that with increased concentrations of ATP as the stimulus for LPS-primed BMDM, less cathepsin was released, which was consistent with the biphasic ATP dose-response relationship indicated by the Western blot data (Fig. 5*A*).

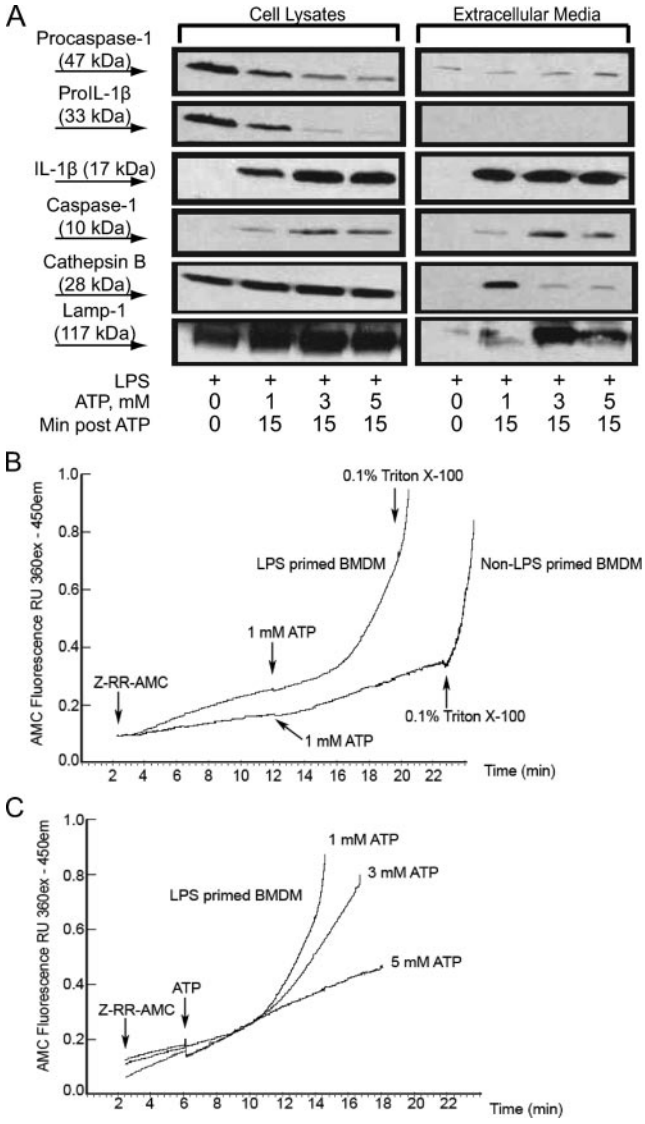

FIGURE 5. Different ATP concentration-response relationships describe IL-1β release vs secretory lysosome exocytosis. *A*, C57BL/6 BMDM were pretreated with LPS (1 μg/ml) for 4 h, transferred to BSS, and then stimulated with 1, 3, or 5 mM ATP stimulation for 15 min. The extracellular medium and cell lysates were collected and processed for Western blot analysis. These data are representative of results from two experiments. *B* and *C*, C57BL/6 BMDM grown on plastic coverslips were used for the online cathepsin activity assay, as described in *Materials and Methods*. *B*, Cell-loaded coverslips pretreated with or without LPS (1 μg/ml) for 4 h before assay. *C*, All cell-loaded coverslips were pretreated with LPS before assay. The 50 μM fluorogenic substrate Z-Arg-Arg-NHMec was added to the assay buffer at zero time. Cell monolayers were incubated for another 10–15 min to measure basally released cathepsin, followed by stimulation with 1 mM (*B* and *C*), 3 mM (*C*), or 5 mM (*C*) ATP for up to 15 min. *B*, Triton X-100 (0.1%) was added at the end of ATP stimulation to measure total content of cellular cathepsin. These results are representative of two experiments.

Extracellular Ca^{2+} is required for secretory lysosome exocytosis, but not IL-1β secretion stimulated by P2X7R

A possible explanation for the attenuated exocytosis of secretory lysosomes when extracellular ATP is raised to supramillimolar levels is the ability of tetrabasic ATP^{4-} to chelate extracellular Ca^{2+} and Mg^{2+}. Although experiments with P2X7R-null macrophages (Fig. 2) indicated that ATP-induced mobilization of intracellular Ca^{2+} stores was not sufficient for exocytosis of lysosomes,

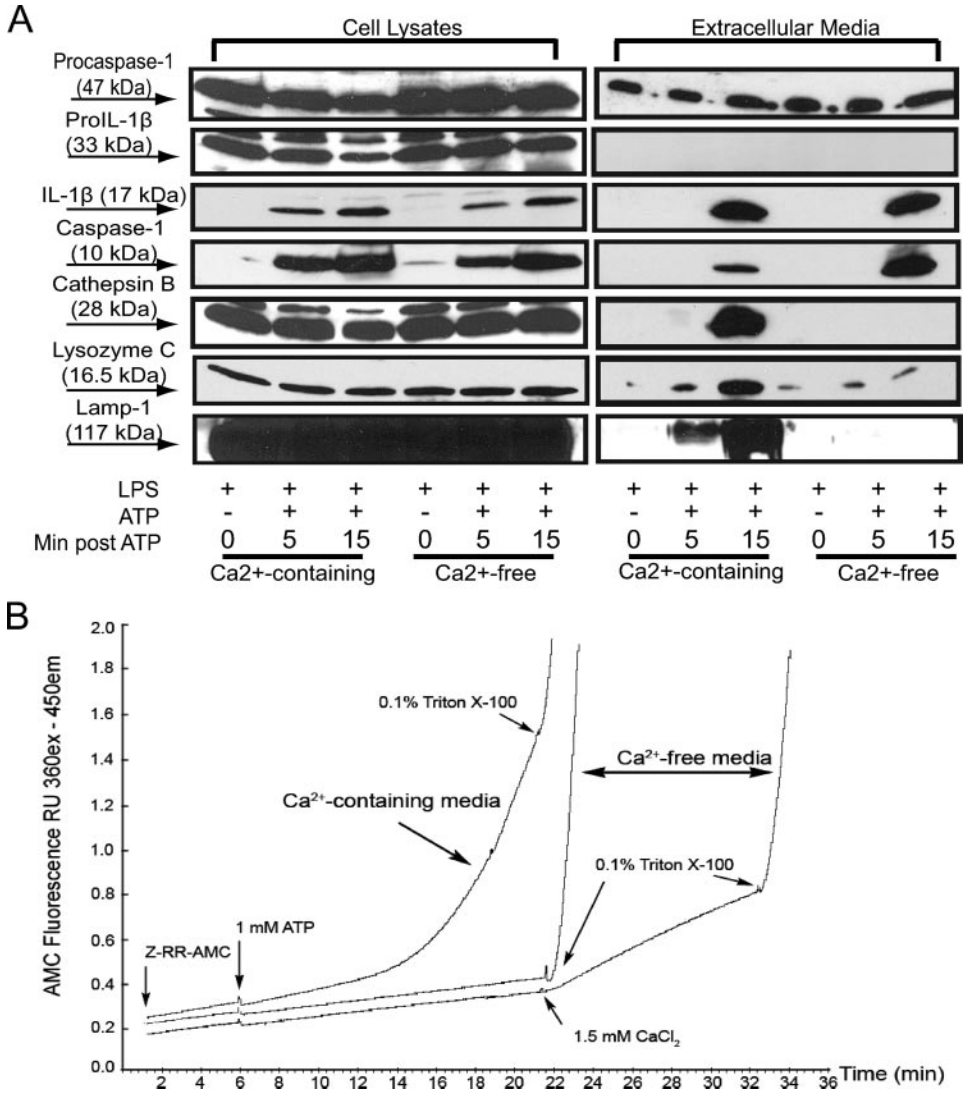

FIGURE 6. Extracellular Ca^{2+} is required for secretory lysosome exocytosis, but not IL-1β secretion stimulated by P2X7R. *A*, BALB/c BMDM were pretreated with or without LPS (1 μg/ml) for 4 h. Cells were transferred to either Ca^{2+}-containing BSS or Ca^{2+}-free BSS and then stimulated with 1 mM ATP stimulation for 5 or 15 min. The extracellular medium and cell lysates were collected and processed for Western blot analysis. These data are representative of results from two experiments performed in BALB/c BMDM and two experiments in C57BL/6 BMDM. *B*, C57BL/6 BMDM grown on plastic coverslips were used for the online cathepsin activity assay. Cell-loaded coverslips were pretreated with LPS (1 μg/ml) for 4 h before transfer to cuvets with Ca^{2+}-containing or Ca^{2+}-free BSS to measure basally released cathepsin, followed by stimulation with 1 mM ATP. Where indicated, 1.5 mM $CaCl_2$ was added 15 min post-ATP stimulation in an assay initiated in Ca^{2+}-free BSS. In all cases, 0.1% Triton X-100 was added at the end of experiments to release the total cellular content of cathepsin. These results are representative of two experiments.

this did not rule out a necessary role for a sustained influx of extracellular Ca^{2+}. This was tested by incubation of BMDM in medium lacking added extracellular $CaCl_2$ (normally 1.5 mM) during the ATP stimulus (Fig. 6*A*). Consistent with the previous findings of Brough et al. (50), the absence of extracellular Ca^{2+} had no effect on either the rapid intracellular accumulation of active caspase-1 and mature IL-1β at 5 min or the subsequent export of these processed proteins over the next 10 min. In contrast, the ability of P2X7R to trigger the coincident exocytosis of secretory lysosomes, as assayed by release of cathepsin B or lysozyme C (another soluble lysosomal marker), was completely suppressed in the Ca^{2+}-free medium. Release of the endosomal/lysosomal membrane marker LAMP-1 was also inhibited when BMDM were stimulated in the absence of extracellular Ca^{2+}. Online fluorescence-based measurements of cathepsin activity released from intact BMDM supported a critical role of Ca^{2+} influx in triggering secretory lysosome exocytosis in response to P2X7R activation (Fig. 6*B*). No increase in extracellular AMC accumulation rate was observed when BMDM were bathed in Ca^{2+}-free BSS during stimulation with 1 mM ATP. However, when 1.5 mM $CaCl_2$ was pulsed into the medium bathing ATP-activated monolayers, an immediate increase in cathepsin release was triggered. Thus, an absolute dependence on extracellular Ca^{2+} and Ca^{2+} influx distinguishes P2X7R-induced secretory lysosome exocytosis from the P2X7R-regulated mechanisms used to export mature IL-1β and inflammasome complexes.

K^+ efflux and BEL-sensitive enzyme(s) are common upstream signals required for both secretory lysosome exocytosis and IL-1β secretion stimulated by P2X7R

Rapid efflux of cytosolic K^+ is a necessary (but not sufficient) signal for P2X7R-dependent activation of ASC-caspase-1 inflammasome complexes (30, 50, 51). Thus, incubation of BMDM in high K^+ extracellular medium to prevent P2X7R-induced K^+ efflux also repressed the rapid processing and export of active caspase-1 and IL-1β (Fig. 7*A*). Furthermore, as with ASC knockout and YVAD treatment, high K^+ inhibited ATP-induced release of LAMP-1. However, unlike ASC knockout and YVAD treatment, high K^+ also markedly attenuated P2X7R-triggered exocytosis of the cathepsin B-containing secretory lysosomes.

We (30, 51) and others (55) have reported that pharmacological inhibitors of type VI Ca^{2+}-independent phospholipase A_2 enzymes, such as BEL, strongly inhibit the processing and release of IL-1β induced by P2X7R and other K^+-efflux stimuli. Similar to high K^+ treatment, the presence of BEL completely inhibited caspase-1 activation (Fig. 7*B*), as well as the release of mature IL-1β and active caspase-1. BEL also markedly attenuated the ATP-stimulated release of both cathepsin B (Fig. 7*B*) and LAMP-1

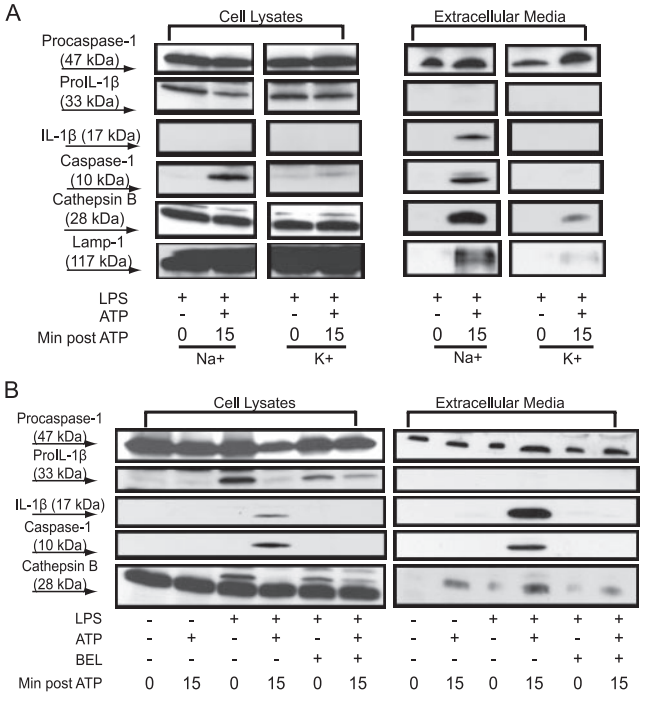

FIGURE 7. K^+ efflux and BEL-sensitive enzyme(s) are common upstream signals required for both secretory lysosome exocytosis and IL-1β secretion stimulated by P2X7R. *A*, BALB/c BMDM were pretreated with or without LPS (1 μg/ml) for 4 h and then transferred to either Na-gluconate BSS or K-gluconate BSS before stimulation with or without 1 mM ATP for 15 min. The extracellular medium and cell lysates were collected and processed for Western blot analysis. These data are representative of results from six experiments performed with BALB/c BMDM and one experiment with C57BL/6 BMDM. *B*, C57BL/6 BMDM were pretreated with or without LPS (1 μg/ml) for 4 h, transferred to BSS, and preincubated with 10 μM BEL before stimulation with or without 5 mM ATP for 15 min. The extracellular medium and cell lysates were collected and processed for Western blot analysis. These data are representative of results from six experiments.

(Fig. 8*C*). The similar ability of BEL to attenuate P2X7R-activated secretory lysosome exocytosis from ASC knockout BMDM indicated that this effect is independent of ATP-induced inflammasome assembly and accumulation of active caspase-1 (data not shown).

Differential sensitivity to LPS priming and BEL inhibition discriminates P2X7R-stimulated plasma membrane microvesicle release from P2X7R-stimulated IL-1β secretion

We measured the extracellular appearance of intact P2X7R protein as an index of ATP-stimulated release of plasma membrane-derived microvesicles that have also been mechanistically associated with IL-1β export. Time course studies (Fig. 8*A*) indicated that microvesicle shedding from these primary BMDM was observed even during the initial 5 min of ATP stimulation that comprises the lag phase preceding extracellular appearance of mature IL-1β, active caspase-1, and secretory lysosomal marker proteins. With longer ATP stimulation, progressively more P2X7R protein appeared in the extracellular medium together with mature IL-1β and active caspase-1. However, other data indicated that this microvesicle shedding could be discriminated from ATP-induced activation of caspase-1 and secretion of mature IL-1β. Fig. 8, *B* and *C*, shows that the microvesicle shedding, in contrast to the release of mature IL-1β, active caspase-1, ASC, and endosome/lysosome markers, was not potentiated by LPS priming of the BMDM. Finally, microvesicle shedding was not markedly inhibited by the

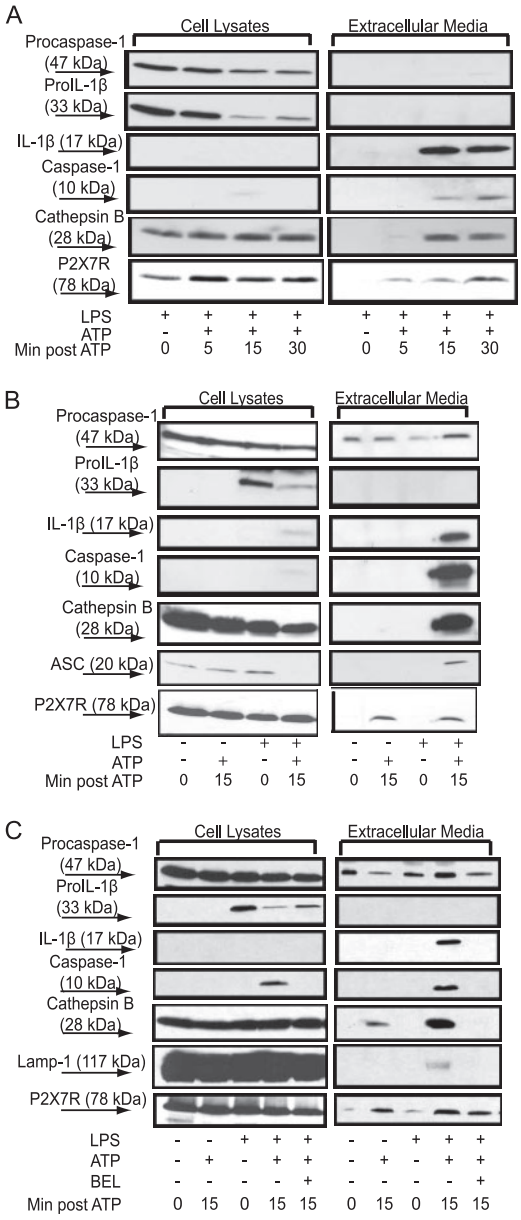

FIGURE 8. Differential sensitivity to LPS-priming and BEL inhibition discriminates P2X7R-stimulated plasma membrane microvesicle release from P2X7R-stimulated IL-1β secretion. *A* and *B*, BALB/c BMDM were pretreated with or without LPS (1 μg/ml) for 4 h, transferred to BSS, and stimulated with or without 1 mM ATP for the indicated times. The extracellular medium and cell lysates were collected and processed for Western blot analysis. These data are representative of results from one experiment for *A* or three experiments for *B*. *C*, BALB/c BMDM were primed with or without LPS (1 μg/ml) for 4 h, transferred to BSS, and preincubated with 10 μM BEL before stimulation with or without 1 mM ATP for 15 min. The cell lysates and extracellular medium were processed for Western blot analysis. The data are representative of results from four experiments.

BEL treatment that repressed inflammasome activation and the coincident export of mature IL-1β and secretory lysosome exocytosis (Fig. 8*C*).

P2X7R-stimulated release of MHC-II is strongly correlated with P2X7R-dependent export of IL-1β and inflammasome components

Our observation that ATP-stimulated LAMP-1 release, like ATP-induced IL-1β export, was suppressed by the deletion or inhibition

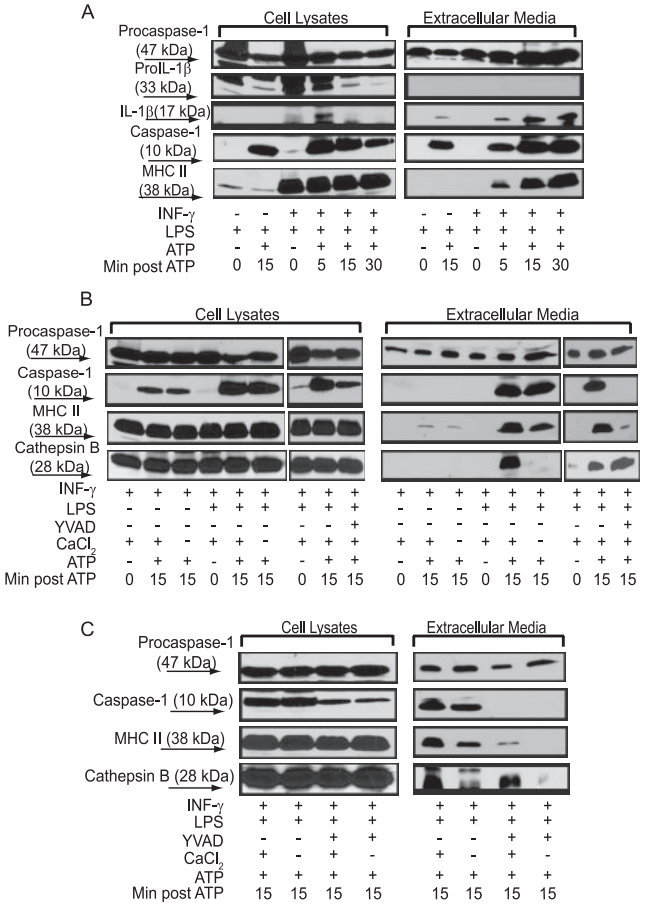

FIGURE 9. P2X7R-stimulated release of MHC-II is strongly correlated with P2X7R-dependent export of caspase-1. *A–C*, C57BL/6 BMDM were pretreated with or without 2 ng/ml IFN-γ for 16–18 h before a secondary incubation with or without 1 μg/ml LPS for an additional 4 h. The cells were then treated and stimulated before collection of extracellular medium and cell lysates for Western blot analysis of caspase-1, MHC-II, cathepsin-B, or IL-1β, as indicated. *A*, Cells were transferred to BSS and then stimulated with or without 5 mM ATP for 5, 15, or 30 min, as indicated. *B* and *C*, Cells were transferred to Ca^{2+}-containing BSS, Ca^{2+}-free BSS, or BSS ($\pm Ca^{2+}$) supplemented with 10 μM YVAD and then stimulated with or without 5 mM ATP for 15 min. The data are representative of results obtained from four experiments.

of ASC, Px1, and active caspase-1 suggested that active inflammasome complexes and IL-1β might be trafficked into a pool of MVB derived from recycling endosomal compartments (56), followed by exocytosis of these MVB to release exosomes containing mature IL-1β, active caspase-1, and other inflammasome proteins. That LAMP-1 release was greatly reduced when BMDM were stimulated in Ca^{2+}-free medium (Fig. 6) nominally argued against this model. However, this observed Ca^{2+} dependence of ATP-induced LAMP-1 release may reflect a kinetically complex pattern of LAMP-1 trafficking involving an initial transfer from secretory lysosomes to the plasma membrane before incorporation into recycling endosomes and MVB. To offset this potential complication, we analyzed the ability of P2X7R to induce release of MHC-II, another intrinsic membrane protein that is both highly expressed in macrophage plasma membranes and known to traffic into MVB and released exosomes (57). Because naive BMDM constitutively express only low levels of MHC-II, we pretreated these cells with IFN-γ to up-regulate MHC-II expression before the standard LPS-priming and ATP stimulation protocols (Fig. 9*A*). Activation of P2X7R in cells primed with both IFN-γ and LPS triggered a robust release of MHC-II into the extracellular medium that was temporally correlated with export of IL-1β and active caspase-1 (Fig. 9*A*). Conversely, in the absence of LPS priming, ATP elicited only minor release of MHC-II that matched the absence of secreted active caspase-1. We further compared the effects of Ca^{2+}-free medium vs inclusion of YVAD on the P2X7R-stimulated release of MHC-II in these IFN-γ- and LPS-primed murine macrophages. Fig. 9*B* shows that YVAD treatment markedly reduced the ATP-stimulated MHC-II release, whereas Ca^{2+}-free medium only slightly decreased MHC-II secretion in response to ATP. These patterns of MHC-II release correlated with the export of active caspase-1 and contrasted with the Ca^{2+}-sensitive, but YVAD-insensitive, secretion of cathepsin B. The combination of YVAD inclusion and removal of extracellular Ca^{2+} completely inhibited the ability of ATP to trigger release of all three marker proteins, as follows: active caspase-1, MHC-II, and cathepsin B (Fig. 9*C*).

Discussion

Three mechanistically distinct models of nonclassical secretion, exocytosis of secretory lysosomes, shedding of plasma membrane microvesicles, and direct efflux through plasma membrane transporters, have been proposed to explain the rapid export of mature IL-1β (and IL-18) from monocytes/macrophages/microglia following P2X7R activation of caspase-1 inflammasome complexes. We have directly compared the contribution of the first two mechanisms in an experimental model of primary murine BMDM. This experimental system facilitated a variety of genetic and pharmacologic perturbations for dissecting the tightly coupled signaling reactions that underlie inflammasome assembly, caspase-1 activation, and trafficking of IL-1β, plus its processing machinery into the nonclassical export pathways. The major finding is that P2X7R-regulated export of mature IL-1β can be mechanistically dissociated from the exocytosis of secretory lysosomes on the basis of markedly distinct requirements for extracellular Ca^{2+} and Ca^{2+} influx (Fig. 6). Several experimental approaches (Figs. 1–3 and 7) indicated that these two secretory responses represent parallel and cotemporal pathways that are coordinately regulated by multiple common signals entrained by P2X7R activation. Other data indicated that ATP-stimulated microvesicle shedding was unaffected by manipulations that markedly attenuate ATP-activated IL-1β export (Fig. 8). Thus, neither the secretory lysosome nor the microvesicle shedding models can account for key features that characterize nonclassical IL-1β secretion from primary murine macrophages in response to P2X7R activation. Our experiments also revealed the following: 1) a novel correlation between IL-1β secretion and the release of the MHC-II membrane protein that is a marker of plasma membranes, recycling endosomes, and multivesicular bodies; and 2) a common and absolute requirement for inflammasome assembly and active caspase-1 in this cotemporal export of IL-1β and MHC-II (Fig. 9). These latter findings suggest an alternative model of IL-1β release that may involve the P2X7R-induced formation of MVB that contain exosomes with entrapped IL-1β (Fig. 10).

Secretory lysosome exocytosis and IL-1β export in murine macrophages

A direct role for secretory lysosomes as an IL-1β release mechanism has been proposed on the basis of several observations and correlations. Overlap between the signaling pathways that regulate lysosome exocytosis and those required for IL-1β processing and release has provided a major body of support for secretory lysosomes as a nonclassical mechanism of IL-1β secretion (33, 58). ATP-induced release of lysosomal markers and mature IL-1 from human monocytes can be coordinately blocked by inhibitors that

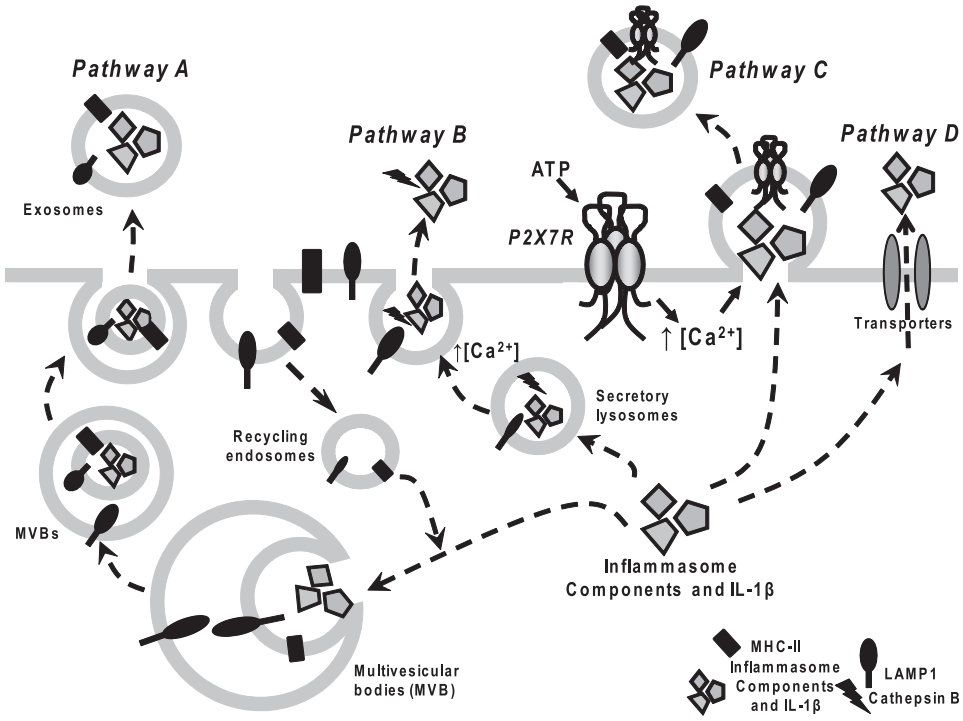

FIGURE 10. Four models of nonclassical IL-1β secretion. Pathway A describes the MVB/exosome model suggested by the results of this study. This may involve the P2X7R-induced concentration/assembly of caspase-1 inflammasomes and pro-IL-1β within the cytosolic vicinity of recycling endosomes, formation of MVB that contains exosomes with entrapped IL-1β, and fusion of these MVB with the plasma membrane to release exosomes containing IL-1β, other inflammasome components, and membrane marker proteins, such as MHC-II. Pathway B describes the Ca^{2+}-dependent secretory lysosome exocytosis model, in which pro-IL-1β and its processing enzyme caspase-1 are targeted to a subset of secretory lysosomes that may also deliver lysosomal membrane marker proteins such as LAMP-1 to the plasma membrane. Pathway C describes the microvesicular shedding model, in which cytosolic pro-IL-1β and caspase-1 inflammasome complexes are entrapped within Ca^{2+}-dependent evaginating blebs of plasma membrane that are then released as microvesicles containing IL-1β, caspase-1, and plasma membrane marker proteins such as P2X7R or MHC-II. Pathway D posits the direct efflux of cytosolic IL-1β (and perhaps other inflammasome components) through as-yet-uncharacterized transporters localized in the plasma membrane.

target both Ca^{2+}-dependent and Ca^{2+}-independent subtypes of phospholipase A_2 as well as phosphatidylcholine-specific phospholipase C (34). A recent study of P2X7R activation in human monocytes has indicated that several histone deactylase inhibitors attenuate both secretory lysosome exocytosis and IL-1β export via perturbation of microtubule-directed movements of lysosomes (35). Our studies have further extended the network of common upstream signaling pathways used by activated P2X7R to regulate lysosome mobilization and IL-1β release. Data from P2X7R knockout macrophages demonstrated that IP_3-dependent Ca^{2+} mobilization and protein kinase C activation by G protein-coupled P2Y-type purinergic receptors were not sufficient signals for either secretory lysosome exocytosis or IL-1β release (Fig. 2). The P2X7R-mimetic effects of maitotoxin (Fig. 2), as well as the coordinate repression by high extracellular K^+ or BEL (Fig. 7), on both responses indicate a common pathway involving rapid efflux of cytosolic K^+ and stimulation of a BEL-sensitive effector enzyme during the 5-min lag phase that precedes extracellular appearance of the mature cytokine or lysosomal markers. That secretory lysosome exocytosis was strongly potentiated by an LPS-dependent and cycloheximide-sensitive priming process further indicates that common set of rapid turnover signaling protein(s) appears to orchestrate the coupling of P2X7R to inflammasome activation, IL-1β export, and lysosome exocytosis (Fig. 3). Despite these multiple correlations between IL-1β secretion and secretory lysosome mobilization, the ability of Ca^{2+}-free medium to completely abrogate cathepsin B release, while exerting no effect on IL-1β maturation and export, unequivocally dissociates secretory lysosome exocytosis from the nonclassical IL-1β secretion machinery regulated by P2X7R (Fig. 6). Although mobilization of intracellular Ca^{2+} stores by G protein-coupled P2Y receptors was not a sufficient stimulus of either secretory lysosome exocytosis or IL-1β release, our direct online assays of extracellular cathepsin activity indicated that P2X7R-induced secretory lysosome exocytosis involves an absolute dependence on extracellular Ca^{2+}.

Electron microscopy studies based on immunogold labeling as well as conventional Western blot analyses have demonstrated that a significant fraction of intracellular pro-IL-1β and procaspase-1 colocalizes with lysosomal marker proteins in an endolysosomal fraction of LPS-activated human monocytes (33). However, it is possible that this colocalization reflects the normal housekeeping cycle of steady-state protein synthesis and degradation, rather than the acutely induced compartmentalization of the IL-1β pool destined for maturation by active inflammasomes and regulated export. Given the defined roles of ASC and Nalp3/cyropyrin in P2X7R-regulated inflammasome assembly and caspase-1 activation, it is unclear whether inflammasome components are transported into the lysosomal lumen followed by caspase-1 activation and IL-1β maturation or, vice versa, whether caspase-1 activation and IL-1β processing might precede their accumulation within lysosomes. Recent immunofluorescence analyses of intact murine peritoneal macrophages indicated that IL-1β did not colocalize with cathepsin B and LAMP-1 in lysosomal compartments (39). Rather, the IL-1β was predominantly cytosolic both before and during P2X7R-triggered maturation.

Given its strongly associated, but nonobligatory role in IL-1β secretion, what might be the possible function for the lysosome

exocytosis activated by P2X7R? P2X7R$^{-/-}$ mice are characterized by resistance to the development of anticollagen-induced arthritis (49) and abnormally low sensitivity to inflammatory pain stimuli (59). Using a CFA-injected paw model of inflammation, Chessel et al. (59) found that local IL-1β levels in the inflamed paws of P2X7R-null mice were markedly reduced despite an absence of global changes in serum IL-1β. Moreover, the hypersensitivity to thermal or mechanical stimuli (i.e., allodynia or inflammatory pain) that characterizes the inflamed paws of control mice was completely absent in the P2X7R knockout animals. These findings indicate that in vivo P2X7R activation must trigger the highly localized remodeling of extracellular matrix and accumulation of paracrine mediators, e.g., kinins, eicosanoids, and chemokines, that will stimulate both nociceptive neurons and newly arrived leukocytes within a discrete inflammatory locus (60, 61). The wide range of proteases, phospholipases, and heat shock proteins packaged within the secretory lysosomes (44, 62) of macrophages and other immune effector cells may be critical for facilitating P2X7R regulation of extracellular matrix remodeling and inflammatory mediator accumulation. Moreover, if IL-1β export mechanistically involves release of cytokine packaged within membrane-delimited vesicles (from either plasma membrane blebbing or MVB exosomes), secretory lysosome-derived lipases may play an important role in permeabilizing the extracellular microvesicles for delivery of the entrapped IL-1β into interstitial fluid.

Microvesicle shedding and IL-1β export in murine macrophages

A rapid P2X7-stimulated release of plasma membrane-derived microvesicles containing IL-1β, caspase-1, and external leaflet phosphatidylserine has been demonstrated in human THP-1 monocytes (36), murine microglial lines (37), and human dendritic cells (38). In these model systems, the IL-1β initially released into the extracellular compartment is entrapped within membrane vesicles that can be biochemically isolated by either direct ultracentrifugation (38) or a more gentle immunoaffinity method based on annexin V binding of the phosphatidylserine-containing membranes (36, 37). At early time points, much of this intravesicular IL-1β is in the form of pro-IL-1β; the caspase-1-dependent maturation of the cytokine subsequently occurs within the microvesicles after their shedding from the cell body. Significantly, Mackenzie et al. (36) found that the formation and release of such microvesicles were critically dependent on the presence of extracellular Ca^{2+}. We also have reported that P2X7R-stimulated IL-1β secretion is strongly dependent on extracellular Ca^{2+} ($\sim$10-fold decrease in Ca^{2+}-free medium) in several models, including THP-1 monocytes and HEK293 cells engineered to coexpress heterologous P2X7R and mature IL-1β (41). In contrast, removal of extracellular Ca^{2+} was less effective ($\sim$2-fold decrease) in reducing the ATP-stimulated release of IL-1β from the Bac1.2F5 murine macrophage line. Thus, the relative contribution of Ca^{2+}-sensitive IL-1β export pathways, presumably involving microvesicle shedding, varies markedly between different cell types.

In our murine BMDM model, we also observed a rapid ATP-induced shedding of intact P2X7R protein that was indicative of released plasma membrane microvesicles. However, several observations contraindicated a clear role for shed microvesicles as the major IL-1β export mechanism in murine macrophages. First, in contrast to the stimulated export of IL-1β and caspase-1, the induced microvesicle shedding from murine BMDM did not require LPS priming, nor was it inhibited by BEL (Fig. 8). Second, in contrast to an early release of pro-IL-1β and subsequent IL-1β maturation in an extracellular compartment, we observed a near-complete processing to mature IL-1β in an intracellular compartment before release and virtually no secretion of pro-IL-1β at any time point (Fig. 1). Third, removal of extracellular Ca^{2+} had no major effect on the rate or extent of IL-1β maturation and export (Fig. 6). Fourth, we have been unable to isolate IL-1β-containing vesicles from the extracellular medium of ATP-stimulated BMDM using ultracentrifugation or annexin V affinity beads (data not shown). Although membrane fractions containing LAMP-1 and P2X7R protein can be sedimented, mature IL-1β and caspase-1 are found only in the supernatant fractions. It remains possible that the integrity of membrane vesicles released from the ATP-stimulated macrophages is rapidly compromised by the following: 1) sustained P2X7-dependent ion and osmotic fluxes in the vesicles, and/or 2) the action of phospholipases and proteases released from the coincidently mobilized secretory lysosomes.

MVB-derived exosomes as an alternative model for IL-1β export

Our experiments (Fig. 4) indicated that ASC, a critical regulator of inflammasome assembly (10); Px1, a proposed intermediary in P2X7R-inflammasome coupling (53, 54); and the active caspase-1 generated as a consequence of inflammasome assembly are also required for the trafficking of this caspase-1 protein complex along with its IL-1β substrate into the cellular compartment that facilitates export from the macrophage. In contrast, ASC, Px1, and active caspase-1 were not required for exocytosis of the secretory lysosome pool that was coincidently mobilized by P2X7R activation. However, deletion or inhibition of these three factors coordinately suppressed the ATP-stimulated release of LAMP-1, a membrane protein marker of both lysosomal and endosomal compartments (63). The similar repressive effects of ASC deletion or a caspase-1 activity blocker suggested the intriguing possibility that active caspase-1 per se may regulate the formation of specialized MVB that accumulate cytosolic IL-1β and caspase-1 within the invaginating exosomes that define MVB (64). Such a role for active caspase-1 in membrane compartmentation may be related to the recent demonstration that caspase-1 can rapidly target the sterol regulatory element-binding proteins that regulate membrane biogenesis (65).

Although ASC deletion and caspase-1 inhibition greatly reduced ATP-induced release of LAMP-1, removal of extracellular Ca^{2+} also blocked the extracellular accumulation of this membrane protein. This suggested that a Ca^{2+}-dependent delivery of lysosomal LAMP-1 to the plasma membrane may precede the trafficking of this marker protein into MVB exosomes. In contrast, we found that P2X7R strongly stimulated the release of MHC-II, another intrinsic membrane protein that trafficks to MVB exosomes, regardless of the presence or absence of extracellular Ca^{2+} (Fig. 9, *B* and *C*). MHC-II, in contrast to LAMP-1, is abundantly expressed as a cell surface protein in IFN-γ-treated macrophages, and thus readily available for incorporation into recycling endosomes and MVB. Significantly, the ability of ATP to trigger robust MHC-II export was dependent on LPS priming (Fig. 9*A*), repressed by the YVAD caspase-1 inhibitor (Fig. 9*B*), and markedly reduced in ASC-null BMDM (data not shown). In the absence of LPS priming or in presence of YVAD (for LPS-primed cells), ATP elicited a much lower externalization of MHC-II that was blocked in Ca^{2+}-free medium. This Ca^{2+}-sensitive component may reflect the ATP-induced shedding of plasma membrane microvesicles that contain MHC-II.

The remarkable overlap between the signals that regulate co-temporal release of mature IL-1β, active caspase-1, and MHC-II in response to P2X7R stimulation supports, but does not prove, a role for MVB-derived exosomes in nonclassical IL-1β secretion. As noted previously, we have been unable to isolate IL-1β-containing vesicles from the extracellular medium of ATP-stimulated

BMDM. Although this may be due to a rapid loss of soluble IL-1β from released exosomes, it may indicate that exosome release, like exocytosis of secretory lysosomes, is another parallel response to P2X7R activation, but is not an IL-1β export pathway. Additional experiments are required to resolve these possibilities.

Regardless of its mechanistic relationship to IL-1β export, the P2X7R-induced release of MVB-derived exosomes represents a further addition to the remarkably diverse array of membrane trafficking responses that have already been associated with P2X7R activation in macrophages, monocytes, dendritic cells, or microglia. These include exocytosis of secretory lysosomes (34), phosphatidylserine flip to the outer leaflet of the plasma membrane (36, 66), dramatic blebbing (43, 67–69), shedding of plasma membrane-derived microvesicles (36–38), fusogenic formation of multinucleate giant cells (70, 71), and fusion of mycobacteria-loaded phagosomes with lysosomes (72). Furthermore, Kanneganti et al. (73) recently reported that P2X7R activation of Px1 is required for delivery of PAMP ligands, such as LPS, to the cytosolic compartments wherein Nalp/ASC inflammasomes are activated; this pathway may involve a P2X7R/Px1-dependent trafficking of extracellular PAMPs internalized within endosomes. Although only a subset of these membrane trafficking events may be directly involved in cellular export of IL-1β, all might contribute to the optimal propagation of cytokine-entrained paracrine signals or Ag presentation within inflamed and infected tissues.

Disclosures

The authors have no financial conflict of interest.

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