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Externalization of the Leaderless Cytokine IL-1F6 Occurs in Response to Lipopolysaccharide/ATP Activation of Transduced Bone Marrow Macrophages

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and Christopher A. Gabel[1]*

An interesting trait shared by many members of the IL-1 cytokine family is the absence of a signal sequence that can direct the newly synthesized polypeptides to the endoplasmic reticulum. As a result, these cytokines accumulate intracellularly. Recent studies investigating IL-1β export established that its release is facilitated via activation of an intracellular multiprotein complex termed the inflammasome. The purpose of the current study was to explore the mechanism by which murine IL-1F6 is released from bone marrow-derived macrophages (BMDMs) and to compare this mechanism to that used by IL-1β. BMDMs were engineered to overexpress IL-1F6 by retroviral transduction; cells overexpressing GFP also were generated to provide a noncytokine comparator. The transduced cells constitutively expressed IL-1F6 and GFP, but they did not constitutively release these polypeptides to the medium. Enhanced release of IL-1F6 was achieved by treating with LPS followed by ATP-induced activation of the P2X$_7$ receptor; GFP also was released under these conditions. No obvious proteolytic cleavage of IL-1F6 was noted following P2X$_7$ receptor-induced release. Stimulus-induced release of IL-1F6 and GFP demonstrated comparable susceptibility to pharmacological modulation. Therefore, transduced IL-1F6 is released in parallel with endogenous mature IL-1β from LPS/ATP-treated BMDMs, but this externalization process is not selective for cytokines as a noncytokine (GFP) shows similar behavior. These findings suggest that IL-1F6 can be externalized via a stimulus-coupled mechanism comparable to that used by IL-1β, and they provide additional insight into the complex cellular processes controlling posttranslational processing of the IL-1 cytokine family. *The Journal of Immunology,* 2009, 183: 4021–4030.

The IL-1 superfamily of cytokines includes the two original family members, IL-1α (IL-1F1) and IL-1β (IL-1F2), the natural receptor antagonist, IL-1ra (IL-1F3), and the more recently identified members IL-33 (IL-1F11), IL-18 (IL-1F4), IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9, and IL-1F10 (1). IL-1α and IL-1β bind to the same cell surface IL-1 receptors despite sharing only 30% amino acid identity (2, 3). Signaling mediated via these two cytokines occurs as a result of binding to the type I IL-1 receptor (4). The type 1 receptor-ligand pair, in turn, complexes with a second membrane-associated receptor, IL-1 receptor accessory protein (IL-1RAcP), to initiate signaling (5). A second form of the IL-1R, the type II receptor, contains a truncated cytoplasmic tail relative to that found in the type I receptor which does not support signaling (6); the type 2 receptor has been proposed to serve as a decoy receptor (7). IL-1α and IL-1β initially are produced as procytokines that lack signal sequences (8, 9). ProIL-1β is incapable of binding to the type I IL-1 receptor (10) and must be proteolytically processed by caspase-1 to generate a mature 17 kDa cytokine species capable of binding with high affinity to both IL-1 receptors (6, 11, 12). ProIL-1α, in contrast, binds to the type 1 IL-1 receptor with high affinity and mediates signaling (10).

Nonetheless, proIL-1α is thought to undergo proteolytic maturation leading to a biologically active 17 kDa species. The protease that processes proIL-1α is not caspase-1, and may correspond to a calpain-like enzyme (13). IL-18 also is produced as a procytokine and must be processed by caspase-1 to generate a biologically active cytokine species (14). IL-18 binds to its own receptor, IL-18R (15), and the IL-18-IL-18R complex subsequently recruits an additional membrane receptor to initiate signaling (AcPL; 16).

IL-1F6, 8, and 9 are reported to act as agonists by signaling through IL-1Rrp2, a receptor distinct from IL-1 and IL-18 receptors (17). As in the case of the IL-1 receptor-ligand complex, the IL-1Rrp2-ligand complex must recruit an additional membrane receptor, IL-1RAcP, to initiate signaling within target cells (17). When recombinant versions of the IL-F6, 8, and 9 are used as ligands, relatively high concentrations are required to elicit a cellular response. For example, a concentration of 1 μg/ml IL-1F8 is required to activate IL-1Rrp2-transfected Jurkat cells (17), while concentrations of IL-1α/β and other proinflammatory cytokines needed in vitro for activation of their cognate receptors are typically <10 ng/ml. It remains to be established whether IL-1F6, 8, or 9 undergo posttranslational processing in vivo resulting in increased biological activities.

Like IL-1α, IL-1β, and IL-18, IL-1Fs 5–9 are translated from mRNAs that do not encode signal sequences (18, 19). As a result, these mRNAs are expected to be translated on polysome complexes located within the cytosol and to generate newly synthesized polypeptides that accumulate intracellularly. To date, few studies have been conducted to delineate cellular mechanisms responsible for externalization and/or posttranslational processing of the newly identified IL-1 family members. IL-1F5 is reported to be secreted by trophoblastic JEG-3 cells (20), but the efficiency of

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secretion was not established. Expression of IL-1F7 (splice variant b) is up-regulated in LPS-activated human monocytes and the newly synthesized polypeptide colocalized intracellularly with IL-18 (21). IL-1F7b is reported to contain both a prodomain and a caspase-1 cleavage site analogous to those found on proIL-1β and proIL-18 (22). IL-1F6, 8, and 9, in contrast, contain no obvious prodomains or caspase cleavage sites.

Cellular processes that control posttranslational processing and export of mature IL-1β and IL-18 are complex and highly regulated. For example, human monocytes constitutively produce proIL-18, but release little of the mature cytokine species to the medium (23). These same cells do not constitutively produce IL-1β but when stimulated with a TLR ligand, such as LPS, they initiate production of proIL-1β. Despite producing copious quantities of the procytokine, release of mature IL-1β is inefficient (24). However, mature forms of IL-1β and IL-18 are rapidly released from LPS-stimulated monocytes following treatment with a secondary effector that promotes assembly of an inflammasome complex and, in turn, activation of procaspase-1 (25–27). Effectors that engage this posttranslational activation mechanism include various bacterial toxins and/or components (28–30), uric acid crystals (31), and ATP (32–34). The nucleotide-stimulated process is mediated via the $P2X_7$ receptor, a ligand-gated ion channel expressed by monocytes, macrophages, and lymphocytes (35–37). Activation of the $P2X_7$ receptor leads to loss of intracellular K^+, a requirement for activation of procaspase-1 (33, 38, 39). $P2X_7$ receptor activation also can lead to dramatic changes in monocyte/macrophage morphology and ultimately to cell death (40–42). Once processed by caspase-1, mature IL-1β polypeptides are reported to be externalized via specific transport mechanisms that operate in the absence of cell death (43–46); in many systems, however, release of the mature cytokine species coincides with release of lactate dehydrogenase (32, 47, 48). This correspondence suggests that cytokine release may be accompanied by loss of plasma membrane integrity.

In this report, bone marrow-derived macrophages (BMDMs)[2] are used to compare posttranslational processing of murine IL-1F6 and proIL-1β. BMDMs were selected as the model system because they express the $P2X_7$ receptor (49), and because they can be engineered via retroviral transduction to express genes of interest (50). Stable populations of BMDMs transduced with retroviral vectors encoding full length IL-1F6 or a noncytokine comparator GFP constitutively expressed the transduced polypeptides and remained competent to produce mature IL-1β in response to LPS/ ATP stimulation. Our findings confirm that IL-1F6 is not exported constitutively, and provide evidence that this polypeptide can be externalized rapidly but nonselectively in response to sequential LPS and ATP activation. Therefore, externalization of IL-1F6 from BMDMs proceeds via a stimulus-induced mechanism similar to that used by IL-1β and IL-18.

Materials and Methods
Reagents

Tissue culture medium, FBS, and penicillin-streptomycin were obtained from Life Technologies Invitrogen Cell Culture. *Escherichia coli* LPS serotype 055:B5, poly(I:C) sodium salt, ATP disodium salt, and cycloheximide were purchased from Sigma-Aldrich. Yo-Pro Yellow and Mito-Tracker Red 588 were obtained from Molecular Probes/Invitrogen, the caspase-1 inhibitor Ac-YVAD-CMK was obtained from Calbiochem/EMD Biosciences, recombinant murine IL-1β, IL-1F6, and IL-6 were obtained from R&D Systems, and recombinant GFP was purchased from BD Biosciences. Anti-mouse IL-1F6 and anti-mouse IL-β goat polyclonal Abs

were purchased from R&D Systems, anti-GFP mouse monoclonal from Clontech, and anti-His mouse monoclonal from Roche. Caspase-1 FLICA kit was from Immunochemistry Technologies. Recombinant mcsf-1 and CP-456773 were generated at Amgen.

Isolation of murine macrophages

C57BL/6 mice were euthanized by CO_2 inhalation. Femurs and tibias were excised, and the bone marrow flushed out with DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells from multiple animals were pooled, resuspended, and filtered through a cell strainer (BD Biosciences). Contaminating RBC were removed by incubation in RBC lysing solution (Sigma-Aldrich), after which surviving bone marrow cells were collected by centrifugation, resuspended in growth medium (αMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 ng/ml mcsf-1), and seeded into tissue culture flasks. Cells were cultured overnight in the presence of 5% CO_2 at 37°C. The following day the cells were either transduced or not and then cultured for an additional 7–8 days to allow differentiation into BMDMs.

Generation of retroviral expression vectors and transduction of BMDMs

The complete open reading frame of murine IL-1F6 (GenBank accession number AF206697 http://www.ncbi.nlm.nih.gov/GenBank/) plus a C-terminal flag/polyhis (fph) tag and the *Bam*HI–*Not*I fragment from pEGFP-N1 (Clontech) were subcloned into pENTR1A (Invitrogen). The entry clones then were recombined with the destination vector pRV137G via LR clonase. pRV137G was a Gateway-adapted pMSCV vector that also encoded for puromycin resistance. Packaged pRV137G IL-1F6 and eGFP ecotropic viral particles were obtained by transfecting into the complete packaging cell line Plat-E (51) using Lipofectamine 2000 (Invitrogen). The transfected cells were cultured for 24 h after which the transfection medium was replaced with fresh DMEM, 10% FBS, 15 mM HEPES (pH 7). Conditioned medium containing packaged retroviral particles was harvested 2 days later. The medium were subjected to an initial filtration through 0.22-μm surfactant-free cellulose acetate units (Nalge Nunc) followed by a 4-fold concentration in Centricon Plus-80 centrifugal units (Millipore) and a final filtration through 0.22 μm cellulose acetate (Corning). The concentrated virus was used immediately to transduce BMDMs. Cells had been seeded into T175 culture flasks the day before transduction; growth medium was removed and 10 ml of the concentrated virus-containing solution supplemented with 10 μg/ml DEAE-dextran (Sigma-Aldrich) was added. Cells were transduced over a 2-day period in a 5% CO_2 environment at 37°C. The transduction medium subsequently was replaced with fresh growth medium containing 2 μg/ml puromycin to allow selection of positive transductants over a 3-day period.

Two-step activation protocol

All incubations were conducted at 37°C in a 5% CO_2 incubator. Transduced and control BMDMs were seeded in 6-well plates at 8×10^5 cells/well and cultured for 2 days. The first step of the 2-step activation protocol involved priming cells with a TLR agonist. For this, growth medium was replaced with 2 ml/well of fresh medium supplemented (or not) with a TLR4 or TLR3 agonist (1 μg/ml LPS or 100 μg/ml poly(I:C), respectively) and the plates incubated for 3–4 h. In some cases, cycloheximide was added to the medium during the TLR agonist priming step. The second step involved treatment with a pulse of ATP. The TLR-primed BMDMs were rinsed once with assay medium (NaHCO$_3$-free αMEM, containing 1% FBS, 25 mM HEPES (pH 7), 5 mM NaHCO$_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin) and then 1 ml of assay medium, with or without 5 mM ATP, was added to each well. (Note: ATP was first prepared as a 500 mM stock in 100 mM HEPES and neutralized with NaOH.) The cells were pulsed for 20 min with ATP after which medium was replaced with 1 ml of fresh ATP-free assay medium and the cultures were incubated for an additional 1 h period. In some cases, the caspase-1 inhibitor Ac-YVAD-CMK or the cytokine release inhibitor CP-456773 was present during the ATP pulse and chase. Conditioned media (CM) subsequently were harvested and added to tubes containing a 25-fold concentrate of protease inhibitors (Roche, complete protease inhibitor mixture tablet). BMDM monolayers were washed with cold PBS and then lysed in 500 μl of a lysis buffer containing 1% NP40, 50 mM HEPES (pH 7.8), 150 mM NaCl, and complete protease inhibitor mix. All samples were incubated on ice for 30 min and then clarified by centrifugation at 45,000 rpm for 30 min at 4°C in a tabletop ultracentrifuge with a TLA-55 rotor (Beckman Coulter). In some experiments, samples were stored at -80°C before centrifugation. Where indicated, the ATP pulse and subsequent chase were conducted using a minimal medium composed of 20 mM HEPES (pH 7), 0.9 mM
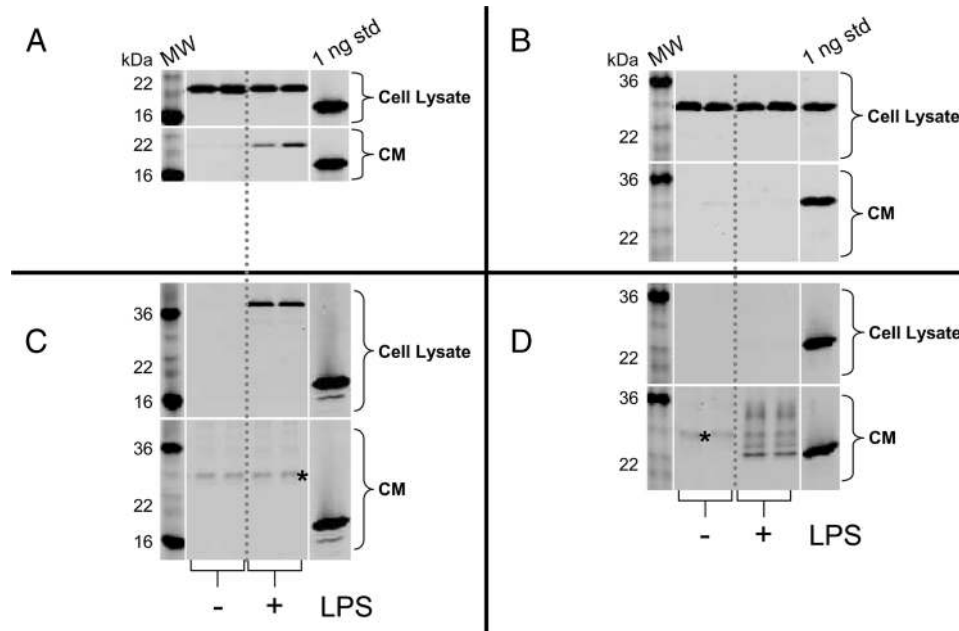

FIGURE 1. IL-1F6 is not efficiently released from BMDMs maintained in culture. BMDMs transduced with retroviral integrants expressing either IL-1F6-fph (*A*, *C*, and *D*) or GFP (*B*) were maintained in culture for a 7.5-h period in the absence or presence of 1 μg/ml LPS. CM and cell lysates were harvested separately, and equal portions of each were analyzed by Western blotting for the presence of IL-1F6 (*A*; 0.1% of total loaded), GFP (*B*; 0.1%), IL-1β (*C*; 0.2%), and IL-6 (*D*; 0.2%). Each blot contained 1 ng of an appropriate recombinant standard (1 ng std) as well as a lane containing m.w. standards (MW). In the case of IL-1F6, the recombinant polypeptide standard lacked the fph tag, accounting for its faster mobility relative to the BMDM-derived product. In the case of IL-6, the BMDM secreted product appeared as multiple bands; these are presumed to represent glycosylation variants. Bands denoted with an asterisk are considered nonspecific resulting from cross reactivity the Abs with nonrelevant Ags. Each culture condition was performed in duplicate.

CaCl$_2$, 0.5 mM MgCl$_2$, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 5 mM glucose, 1% FBS; this base solution then was adjusted to contain 137 mM NaCl or KCl.

Western analysis

Disaggregated CM and lysates samples were loaded onto 4–20% Tris-glycine gradient gels (Novex/Invitrogen) and electrophoresed under reducing conditions. The separated polypeptides were transferred to nitrocellulose after which the blots were blocked in Odyssey Blocking Buffer (Li-Cor). The blots were incubated overnight at 4°C with primary Ab in dilution buffer (Odyssey Blocking Buffer containing 0.1% Tween 20), washed with PBS, 0.1% Tween 20 (four washes, 5 min each), and then incubated 45 min at room temperature with Alexa Fluor 680 conjugated secondary Ab (Invitrogen) in dilution buffer. Blots were again washed as described above, rinsed with PBS, and imaged with an Odyssey Infrared Imaging System (Li-Cor).

FLICA and MitoTracker Red staining

BMDMs were subjected to the 2-step LPS/ATP activation protocol; where indicated, FLICA^{Casp1} and/or MitoTracker Red was added to the pulse and/or chase medium using dilutions recommended in the product instructions. The cells subsequently were washed and phase and fluorescence images were captured.

YoPro Yellow uptake

BMDM monolayers were incubated with 1 μM YoPro Yellow in the presence or absence of 5 mM ATP for 30 min. Phase and fluorescence images (20× objective) then were collected to assess cellular accumulation.

ELISAs

Aliquots of CM were analyzed for IL-1β and IL-1F6 content by sandwich ELISA; the former with a commercially available kit (R&D Systems) and the latter with an assay developed at Amgen. For the latter, ELISA plates (Nunc MaxiSorp) were coated with an anti-mouse IL-1F6 goat polyclonal Ab (R&D Systems) at 1 μg/ml in PBS. Plates were sealed, incubated overnight at room temperature, washed, and then blocked for 1 h with PBS containing 1% BSA. Recombinant murine IL-1F6 standards or CM samples in diluent (PBS, 0.5% Tween 20, 0.1% BSA) were added to individual wells and incubated for 1 h at room temperature. Plates were washed and incubated with biotinylated anti-mouse IL-1F6 goat polyclonal Ab for 1 h

at room temperature. Plates subsequently were washed and then incubated with streptavidin:poly80-HRP (Research Diagnostics) for 30 min at room temperature. After a final wash, 0.1 ml of TMB substrate was added (R&D Systems); the reaction was stopped by addition of 0.1 ml of 1 M sulfuric acid, and product measured at 450 nm with correction wavelength set at 540 nm.

Results

IL-1F6 does not behave as an endoplasmic reticulum-derived secretory protein

Expression of IL-1F6 message has been observed in a murine macrophage cell line (19), but our preliminary studies with BMDMs indicated that IL-1F6 protein expression, with or without LPS stimulation, was not discernable by Western blot analysis. Therefore, we developed a methodology allowing for overexpression of IL-1F6 in these cells. Separate retroviral expression vectors were constructed encoding GFP or IL-1F6 in conjunction with the murine stem cell virus promoter. The IL-1F6 construct also encoded a C-terminal flag/polyhis tag (IL-1F6-fph), and both vector constructs carried the puromycin resistance gene allowing selection of stable transductants. BMDMs were transduced with the viral constructs and then subjected to puromycin selection for 3 days. Surviving cells subsequently were allowed to recover in puromycin-free medium for up to 5 days; during this time the cells actively proliferated. At this point, the majority of cells within the GFP-transduced population appeared fluorescent when viewed under UV illumination, signifying that the viral transduction and selection strategies were successful (data not shown).

To assess the secretory potential of IL-1F6, transduced BMDMs were cultured for 7.5 h in the absence or presence of LPS after which CM and cell lysates were examined by Western blot analysis for proteins of interest. IL-1F6-fph was produced constitutively by the BMDMs and detected readily within the cell lysates, but this cytokine was not detected in the CM (Fig. 1*A*). Addition of LPS to the culture medium did not enhance levels of intracellular IL-1F6-fph but did
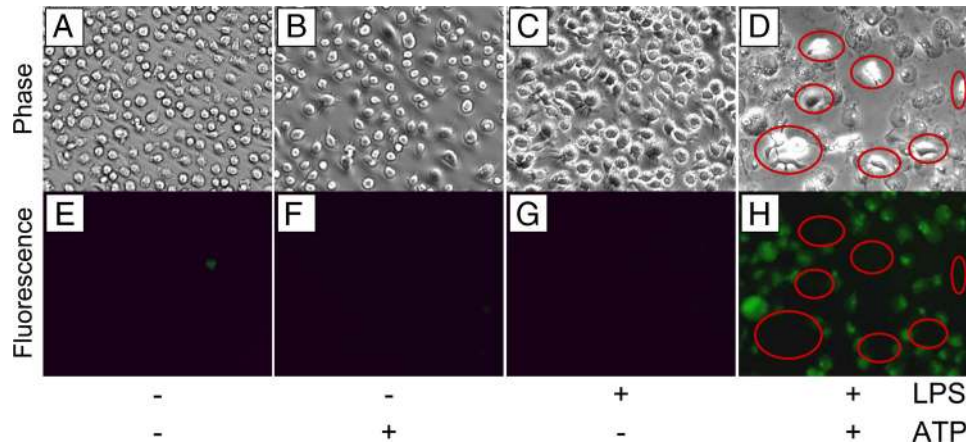
FIGURE 2. Both LPS and ATP are required for activation of caspase-1 in transduced BMDMs. Cells transduced with a retroviral integrant expressing murine IL-1F6-fph were subjected to a two-step LPS/ATP activation protocol. Transduced BMDMs were either not treated during the initial priming stage (*A*, *B*, *E*, and *F*) or primed with 1 μg/ml LPS (*C*, *D*, *G*, and *H*) for 3 h. The cells then were exposed (*B*, *D*, *F*, and *H*) or not (*A*, *C*, *E*, and *G*) to 5 mM ATP for 20 min. The medium then was replaced with fresh medium devoid of LPS and ATP but containing the fluorescent caspase-1 substrate FLICA, and the cultures were incubated for an additional 60 min. The cultures subsequently were photographed (20×) by phase (*A–D*) and fluorescence (*E–H*) microscopy. Cells circled in *D* do not show evidence of cytoplasmic clearing and do not appear FLICA-positive in *H*.

cause a small amount of the cytokine to be externalized. Likewise, GFP was produced constitutively and not externalized in the absence or presence of LPS (Fig. 1*B*). In contrast, IL-1β was not constitutively produced by the transduced BMDMs and proIL-1β was only detected in the lysates after treatment with LPS. LPS-treated cells did not release IL-1β to the medium (Fig. 1*C*). Similarly, IL-6 was not detected in the absence of LPS but was produced upon activation. In this case, however, IL-6 levels in the CM were greater than those detected intracellularly (Fig. 1*D*). Lack of accumulation of IL-6 within the lysates is consistent with this polypeptide being secreted via the traditional secretory apparatus involving the endoplasmic reticulum and Golgi apparatus. BMDM-produced IL-1F6-fph thus behaves more like the atypical secretory cytokine IL-1β than an endoplasmic reticulum/Golgi-processed secreted cytokine (e.g., IL-6).

Baseline analysis of ATP-induced IL-1β output from BMDMs

After growth in the presence of mcsf-1 to induce differentiation, BMDMs demonstrated a rounded morphology (Fig. 2*A*). When treated with LPS, these cells flattened and extended processes as expected of activated macrophages (Fig. 2*C*). Treatment with ATP (in the absence of LPS) caused a subtle change in appearance (Fig. 2*B*). Conversely, sequential treatment with LPS followed by ATP caused many BMDMs to detach from the plates and to assume a swollen state accompanied by clearing of the cytoplasm (Fig. 2*D*). To assess the activation state of caspase-1, these same cultures were treated with a fluorescent probe that reacts irreversibly with the active form of the protease (52). Active caspase-1 was readily detected within the LPS/ATP-treated macrophages but not within cells treated individually with LPS or ATP (Fig. 1, *E–H*). It should be noted that the ATP treatment paradigm used in this experiment (and those that follow) consisted of a 20-min exposure to 5 mM ATP after which the nucleotide was removed from the medium and the cells were incubated for an additional 60 min. This type of pulse format was previously shown to promote release of mature IL-1β from LPS-activated murine peritoneal macrophages while minimizing release of the procytokine species (47).

Not all LPS/ATP-activated macrophages stained positive for active caspase-1, and the FLICA-negative cells appeared to possess a more normal morphological appearance relative to the marked cytoplasmic clearing associated with FLICA-positive cells (Fig. 2, *D* and *H*). To determine whether this differential appearance correlated with cell vi-

ability, macrophages were primed with LPS, treated with ATP for 20 min, and then chased in ATP-free medium containing the active caspase-1 probe as well as the cell viability probe MitoTracker Red. Active caspase again was detected in a subpopulation of the treated macrophages (Fig. 3*D*). Caspase-positive cells stained less intensely with MitoTracker Red than did caspase-negative cells (Fig. 3*E*); an overlay of the images highlights the distinct nature of the two populations (Fig. 3*F*). BMDM cultures treated with LPS alone contained few FLICA-positive cells (Fig. 3*A*) but uniformly accumulated MitoTracker Red (Fig. 3*B*). Therefore, activation of caspase-1 appears to be associated with loss of cell viability.

The failure of ATP on its own to promote morphological changes comparable to those observed in cells treated sequentially with LPS and ATP may indicate that LPS is needed to induce expression of the P2X$_7$ receptor. However, when nontransduced BMDMs were treated with ATP in the presence of YoPro yellow, a fluorescent dye that passes through pores formed in response to P2X$_7$ receptor activation (53, 54), robust accumulation of the probe
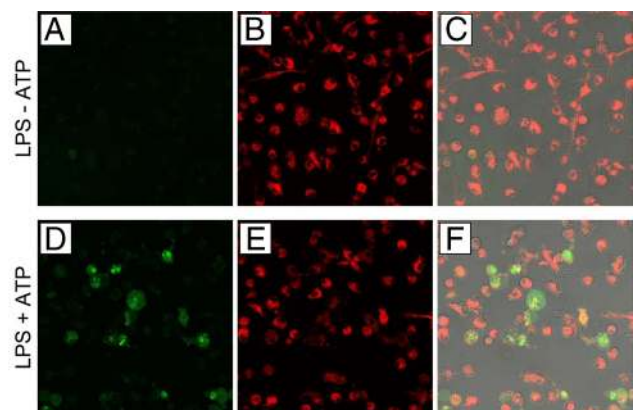

FIGURE 3. Activation of caspase-1 in BMDMs is accompanied by reduced mitochondrial function. Nontransduced BMDMs were primed with LPS for 3 h and then treated with (*D–F*) or without (*A–C*) ATP for 20 min. The cells subsequently were placed in ATP-free medium containing both FLICA and MitoTracker Red and subsequently photographed (within 30 min) by confocal microscopy using 488 nm and 546 nm laser line excitations, respectively. *C* and *F* are overlays of *A* and *B* and *D* and *E*, respectively.
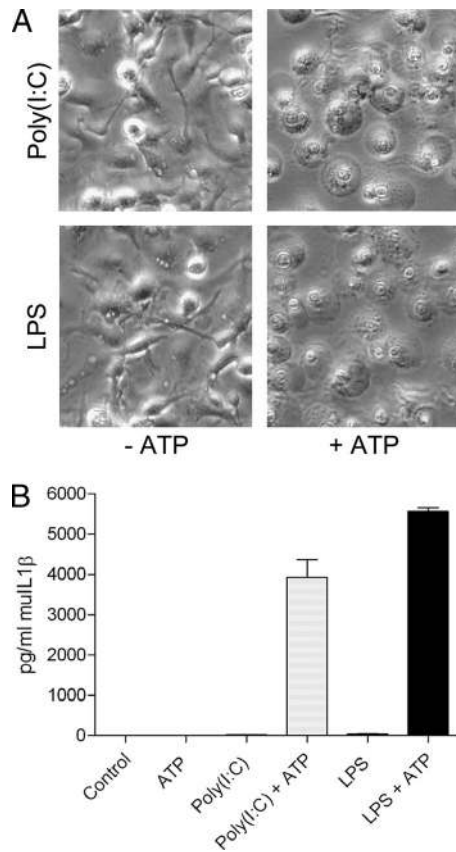
FIGURE 4. The requirement for a secondary secretion stimulus extends to BMDMs primed with a TLR3 agonist. *A*, BMDMs were primed for 4 h with the TLR3 agonist poly(I:C) (100 μg/ml; *upper panels*) or with LPS (1 μg/ml; *lower panels*). Where indicated, 5 mM ATP subsequently was introduced for 20 min after which the cells were incubated for an additional 60 min in stimulus-free medium. Phase contrast images (20×) were captured to demonstrate the cytoplasmic clearing promoted by ATP. *B*, Media harvested from the cultures shown in panel A (as well as additional control cultures containing no effector (control) or ATP only (ATP)) were analyzed for IL-1β content by ELISA. Quantities of IL-1β are indicated as a function of treatment. Each condition was conducted in duplicate and plotted as mean and range.

was observed. In contrast, few cells accumulated the fluorescent dye in the absence of ATP (supplemental Fig. 1).[3] Thus, non-LPS-activated BMDMs appear to constitutively express the P2X$_7$ receptor.

Mcsf-1-treated BMDMs displayed the expected requirements with respect to output of IL-1β. Thus, in the absence of a challenge, no IL-1β was detected within cells or in CM, and ATP treatment alone did not promote cytokine synthesis. BMDMs stimulated with LPS, in contrast, generated high levels of cell-associated 35 kDa proIL-1β but no detectable IL-1β was recovered extracellularly. However, following a short exposure to ATP and subsequent chase, LPS-treated BMDMs released mature IL-1β to the medium; lysates of these cells still contained proIL-1β but little of the procytokine was released (supplemental Fig. 2).

To determine whether the requirement for a separate secretion stimulus extends to other TLR agonists, nontransduced BMDMs were treated with the TLR3 agonist poly(I:C) (55). Poly(I:C)-treated BMDMs assumed a morphology similar to that displayed by LPS-treated cells, and after brief exposure to ATP demonstrated morphological changes characteristic of LPS/ATP-treated cells including rounding and cytoplasmic clearing (Fig. 4*A*). Poly(I:C)-
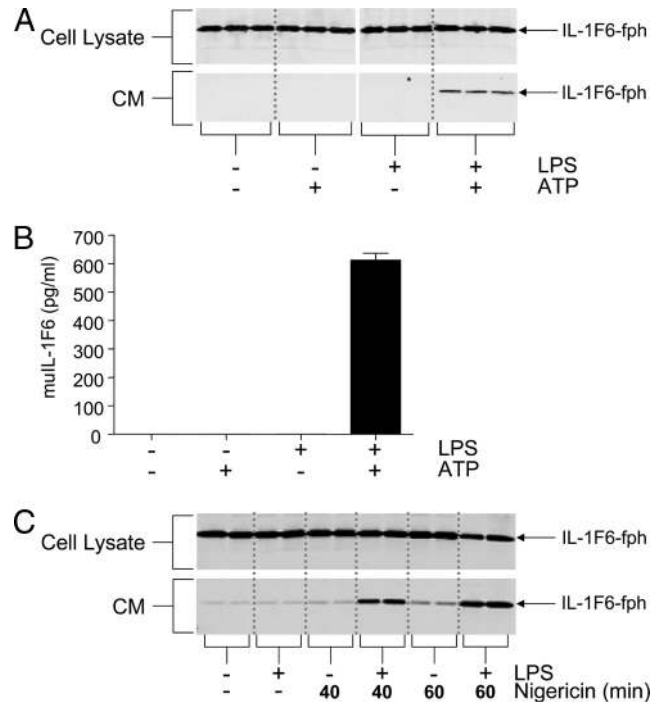
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[3] The online version of this article contains supplementary material.

FIGURE 5. BMDMs retrovirally transduced with IL-1F6-fph constitutively express the recombinant polypeptide but require a secondary stimulus to facilitate export to the medium. *A*, Transduced BMDMs were either untreated or primed with LPS (1 μg/ml) for 3 h and then subjected (or not) to a 20 min pulse of ATP (5 mM). Following a subsequent 60 min incubation in ATP-free medium, CM and cell lysates were harvested separately and analyzed by Western blotting for the presence of IL-1F6-fph (anti-His primary Ab). *B*, The same CM samples generated in *A* also were analyzed for IL-1F6 content by ELISA. Each condition was analyzed in triplicate. *C*, IL-1F6-fph-transduced BMDMs were treated with LPS and nigericin (20 μM) as indicated after which CM were harvested and analyzed by Western blotting (anti-mouse IL-1F6 goat polyclonal primary antibody). Each condition was performed in duplicate. Non-nigericin treated cultures were harvested after 40 min.

treated BMDMs accumulated proIL-1β intracellularly (detected by Western blot; data not shown) but failed to export ELISA-detectable cytokine (Fig. 4*B*). Sequential treatment of BMDMs with poly(I:C) and ATP promoted release of IL-1β to the medium (Fig. 4*B*). The amount of IL-1β externalized in the presence of the poly(I:C)/ATP combination was similar to that produced by LPS/ATP-treated BMDMs (Fig. 4*B*). Thus, both LPS- and poly(I:C)-primed BMDMs require a secondary effector such as ATP to promote efficient caspase-1 activation and release of mature IL-1β.

Characterization of IL-1F6 release from LPS/ATP-treated BMDMs

BMDMs transduced with IL-1F6-fph remained competent to produce endogenous proIL-1β in response to LPS activation and to release mature IL-1β following subsequent ATP challenge (see below). Likewise, the virally transduced BMDMs rapidly accumulated YoPro Yellow and demonstrated a profound change in morphology when incubated with LPS and ATP, signifying the continued presence of functional P2X$_7$ receptors (supplemental Fig. 3). Thus, the retroviral transduction and puromycin selection processes did not adversely impact cellular behavior.

IL-1F6-fph transduced BMDMs were subjected to the two-step LPS/ATP activation protocol to engage IL-1β posttranslational processing after which aliquots of the medium and cellular lysates were analyzed by Western blotting (probed with an anti-His Ab). In the absence of a stimulus, IL-1F6-fph was readily detected intracellularly as a 22 kDa polypeptide species (Fig. 5*A*). No
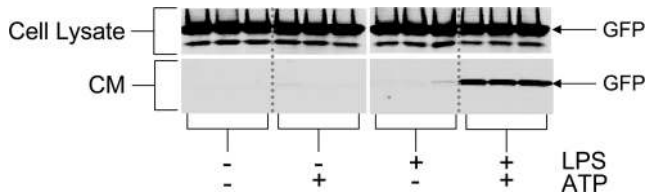
FIGURE 6. BMDMs constitutively expressing GFP release this polypeptide in response to LPS/ATP challenge. Transduced BMDMs were either untreated or primed with LPS (1 μg/ml) for 3 h and then subjected (or not) to an additional 20 min pulse of ATP (5 mM). Following a 60-min chase in ATP-free medium, CM and cell lysates were harvested separately and analyzed by Western blotting for the presence of GFP. Each condition was conducted in triplicate.

IL-1F6-fph was recovered in CM derived from these cells. Stimulation of the IL-1F6-fph-transduced BMDMs with LPS or ATP individually resulted in no additional synthesis of the polypeptide nor in partitioning of this cytokine to the medium (Fig. 5*A*). In contrast, the sequential addition of LPS and ATP promoted externalization of IL-1F6-fph (Fig. 5*A*). IL-1F6-fph released to the medium migrated on the Western blot with the same mobility as the cell-associated species, suggesting that proteolytic cleavage of the cytokine did not occur during release. To confirm that the combination of LPS and ATP was required for efficient IL-1F6-fph externalization, the same CM samples were analyzed by ELISA (detection via IL-1F6 epitopes). As shown in Fig. 5*B*, only IL-1F6-fph-expressing BMDMs subjected to the two-step LPS/ATP activation protocol released ELISA-positive material to the medium. Thus, IL-1F6-fph is constitutively expressed by the retrovirally transduced BMDMs but release of the cytokine to the medium is minimal in the absence of the sequential LPS/ATP activation protocol.

Release of the mature IL-1β from LPS-treated macrophages also can be achieved by treatment with the potassium ionophore nigericin in a P2X$_7$ receptor-independent process (33). To ascertain whether nigericin could facilitate release of IL-1F6-fph, transduced BMDMs were activated with LPS and nigericin individually, or the two agents sequentially (Fig. 5*C*). In combination with LPS activation, nigericin promoted externalization of IL-1F6-fph.

BMDMs transduced to express GFP were subjected to the two-step LPS/ATP activation protocol and aliquots of the medium and cellular lysates again were subjected to Western analysis (blot probed with an anti-GFP Ab). In the absence of a stimulus, GFP was abundantly present intracellularly as a 30 kDa polypeptide species but no GFP was recovered in CM (Fig. 6). Stimulation of the GFP-transduced BMDMs with LPS or ATP individually did not increase the levels of intracellular GFP nor did they cause the probe polypeptide to partition to the medium (Fig. 6). In contrast, sequential addition of LPS and ATP promoted export of GFP (Fig. 6). The externalized polypeptide possessed an apparent molecular mass comparable to that of the intracellular polypeptide (30 kDa).

The kinetics of IL-1F6 and GFP release were compared by subjecting separately transduced BMDM populations to the two-step LPS/ATP activation protocol in parallel. Following a 20-min ATP pulse and 15-min chase in ATP-free medium, both IL-1F6-fph and GFP were detected in the medium (Fig. 7). Increasing the chase time to 30 min led to greater levels of both polypeptides in the medium, and extracellular levels continued to increase slightly during an additional 30-min chase period (Fig. 7). Therefore, the kinetics of release of the cytokine IL-1F6-fph and the noncytokine GFP correlate temporally.
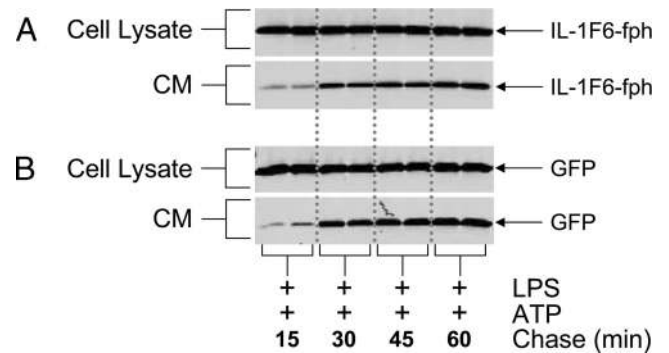

FIGURE 7. IL-1F6 (*A*) and GFP (*B*) are released from LPS/ATP-treated BMDMs with similar kinetics. Separate populations of IL-1F6-fph- and GFP-transduced BMDMs were primed with LPS for 4 h and then treated with ATP for 20 min. Fresh medium (devoid of ATP) subsequently was added and cultures were chased for 15, 30, 45, or 60 min. At the end of the chase, cells and CM were harvested and analyzed by Western blotting for IL-1F6 (anti-mouse IL-1F6 goat polyclonal primary Ab) and GFP. Each condition was performed in duplicate.

Comparison of the pharmacological sensitivity of IL-1F6, GFP, and IL-1β export

IL-1F6-fph-transduced BMDMs produced proIL-1β in response to LPS challenge and released mature IL-1β to the medium following ATP treatment. As noted with nontransduced BMDMs, release of the mature form was not accompanied by release of proIL-1β (Fig. 8*A*). Addition of the protein translational inhibitor cycloheximide (either 20 or 50 μM) to the medium prevented the intracellular accumulation of proIL-1β in response to the LPS challenge, confirming that this polypeptide's presence requires de novo protein
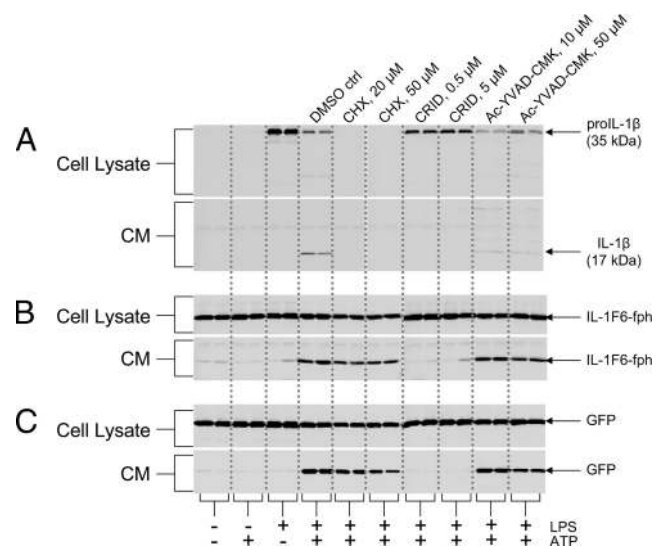

FIGURE 8. Pharmacological sensitivity of LPS/ATP-induced export. BMDMs were transduced with retroviral integrants expressing either IL-1F6-fph (*A* and *B*) or GFP (*C*). Where indicated, the cells were primed with LPS (1 μg/ml) for 3 h and treated with a 20-min pulse of ATP (5 mM). All cultures subsequently were incubated for 60 min in stimulus-free medium. Where indicated, cycloheximide (CHX; 20 or 50 μM) was present during the LPS priming phase and then removed. CP456773 (cytokine release inhibitory drugs; 0.5 and 5 μM), caspase-1 inhibitor Ac-YVAD-CMK (10 and 50 μM), and vehicle control (DMSO) were added the 20 min pulse with ATP and remained present during the 60-min chase. CM and cell lysates were harvested separately and analyzed by Western blotting for the presence of IL-1β (*A*), IL-1F6-fph (*B*), and GFP (*C*). Each condition was conducted in duplicate.

synthesis (Fig. 8A). As a result of the absence of cell-associated proIL-1β, no mature IL-1β was recovered in the medium following ATP activation of the cycloheximide-treated cells (Fig. 8A). In contrast, cycloheximide had no discernable impact on levels of cell-associated IL-1F6-fph within the timeframe of the experiment and did not inhibit LPS/ATP-induced IL-1F6-fph output (Fig. 8B). Likewise, cycloheximide did not alter cellular levels of GFP or export of this probe polypeptide in response to LPS/ATP stimulation (Fig. 8C).

Generation of mature IL-1β requires cleavage of proIL-1β by caspase-1 (11, 12), and ATP acting via the P2X$_7$ receptor promotes caspase-1 activation (see Fig. 2) and, in turn, processing of proIL-1β (32–34). Addition of the caspase-1 inhibitor Ac-YVAD-CMK (either 10 or 50 μM) to BMDM cultures blocked formation and release of mature 17 kDa mature IL-1β (Fig. 8A). In the presence of this caspase inhibitor, proIL-1β was detected in the CM samples but the quantities did not appear sufficient to compensate for the reduction in the mature species (a caveat being that sensitivity of the Ab to detect the mature and propolypeptide species may not be identical). In contrast, addition of Ac-YVAD-CMK to the medium did not impact the amount of IL-1F6-fph released in response to LPS/ATP activation (Fig. 8B). Similarly, Ac-YVAD-CMK did not affect release of GFP from LPS/ATP-treated cells (Fig. 8C).

Compounds composed of a diarylsulfonylurea core structure (referred to as cytokine release inhibitory drugs) previously were reported to antagonize ATP-induced IL-1β posttranslational processing (56). These agents are not direct antagonists of the P2X$_7$ receptor; rather, they inhibit events downstream of the P2X$_7$ receptor that lead to activation of caspase-1 and processing of proIL-1β (57). A prototype of these agents, CP-456773, effectively blocked ATP-induced formation and release of mature IL-1β by retrovirally transduced BMDMs (Fig. 8A). In the presence of CP-456773, proIL-1β was not detected in CM and intracellular levels of the procytokine remained elevated (Fig. 8A). Likewise, CP-456773 (at both 0.5 and 5 μM) effectively blocked release of IL-1F6-fph and GFP from LPS/ATP-treated BMDMs expressing these polypeptides (Fig. 8, B and C).

To address the question of whether activation of an inflammasome platform is necessary for release of IL-1F6 and GFP, transduced BMDMs were subjected to the two-step LPS/ATP activation protocol in the absence or presence of high extracellular K$^+$. Previous studies have shown that elevation of extracellular K$^+$ blocks activation of the NALP3 inflammasome and, in turn, formation and release of mature IL-1β (58). BMDMs that were maintained in the presence of a minimal medium containing 137 mM NaCl during the ATP pulse and subsequent chase released quantities of both IL-1F6-fph and GFP comparable to those released from cells maintained in complete culture medium (Fig. 9). However, when the minimal medium contained 137 mM KCl rather than NaCl, the amounts of the two polypeptides released extracellularly were reduced to background levels. Thus, a K$^+$-sensitive inflammasome component appears to be required for release of IL-1F6-fph and GFP from LPS/ATP-treated BMDMs.

Discussion

Expression studies have indicated that monocytes and keratinocytes produce mRNA encoding IL-1F6, but studies documenting that this leaderless cytokine is produced and exported by these cells have not been reported (18, 19). The level of endogenous IL-1F6 production by murine BMDMs is below the level of detection of the ELISA and Western blotting reagents used in this study. As a result, stable populations of retrovirally transduced
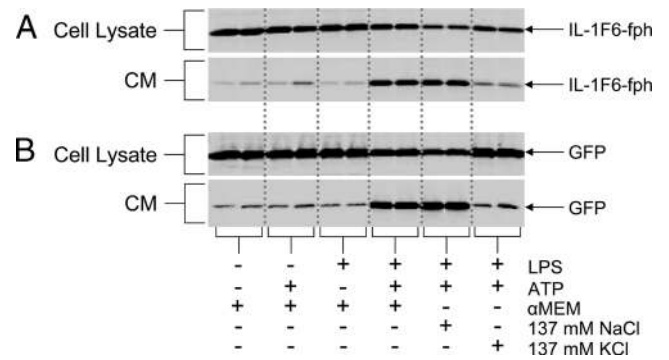

FIGURE 9. Elevated extracellular K$^+$ concentrations reduce ATP-induced output of IL-1F6 and GFP. Separate populations of IL-1F6-fph- and GFP-transduced BMDMs were activated with LPS (where indicated) for 4 h. The cultures then were placed in one of three different media: αMEM, a minimal medium containing 137 mM NaCl, or a minimal medium containing 137 mM KCl. The cells subsequently were treated with ATP (where indicated) for 20 min and then chased for an additional 60 min in the absence of ATP (composition of medium was the same during both the ATP pulse and chase phases). Cells and CM were harvested separately and analyzed by Western blotting for the presence of: A, IL-1F6-fph (anti-mouse IL-1F6 goat polyclonal primary Ab) and B, GFP.

BMDMs were generated that allowed robust expression of murine IL-1F6-fph (or GFP). These virally transduced cells constitutively produced IL-1F6-fph but the cytokine accumulated intracellularly and was not released to the medium. Treatment of IL-1F6-fph transductants with LPS did not augment production of the recombinant cytokine nor significantly induce its release to the medium. The lack of export of IL-1F6-fph was in stark contrast to the efficient secretion observed for a traditional secreted cytokine such as IL-6 but paralleled the atypical behavior of the leaderless cytokine IL-1β.

Studies that have explored export of other members of the IL-1 superfamily have established that a two-step activation mechanism is required. For example, murine macrophages do not constitutively express IL-1β. Following exposure to LPS these cells generate abundant quantities of proIL-1β but they still do not release this cytokine to the medium (32, 33). When LPS-primed cells subsequently are treated with a stimulus that promotes activation of an inflammasome complex, they rapidly release mature IL-1β to the medium (25, 32, 33). Agents demonstrated to promote mature IL-1β formation and release in vitro include ATP (32–35), bacteria and their toxins (28, 59–62), K$^+$ ionophores (32, 39, 63), and uric acid crystals (31). All of these agents promote assembly of an inflammasome platform (27–30, 64) and, in turn, activation of caspase-1 within the cytosol. Once activated, caspase-1 cleaves the 35 kDa proIL-1β species to generate the mature 17 kDa species; cleavage appears to occur within the cell followed by release of mature cytokine to the medium (48). IL-18 similarly requires a two-step mechanism for its release. Monocytes and macrophages produce proIL-18 constitutively, but the cell-associated polypeptides are neither processed by caspase-1 nor released to the medium (65). ATP treatment (in the absence of LPS) is not sufficient to promote caspase-1 activation and/or release of mature IL-18 (65). However, sequential treatment with LPS and ATP promotes release of mature IL-18 from human monocytes (65). Thus, to achieve export of mature IL-1β and IL-18, monocytes and macrophages must be treated sequentially with a priming stimulus (e.g., LPS) followed by a secretion stimulus (e.g., ATP) to promote inflammasome activation and posttranslational processing.

From the above observations it is apparent that LPS serves multiple roles. In the case of IL-1β, LPS promotes synthesis of the

procytokine and primes the cell such that a subsequent secretion stimulus can activate an inflammasome platform. In the case of IL-18, in contrast, LPS is not required to activate synthesis of proIL-18 but is still needed to sensitize the cell such that subsequent engagement of the $P2X_7$ receptor by ATP promotes activation of an inflammasome platform and release of mature IL-18. Currently, the mechanism by which LPS exposure enables $P2X_7$ receptor-mediated activation of an inflammasome complex is unknown. As shown previously (66) and confirmed in this study, mouse BMDMs express the $P2X_7$ receptor constitutively and the receptor is functionally competent in the absence of LPS. Thus, the priming requirement appears to be downstream of $P2X_7$ receptor. The priming step sensitizes macrophages to ATP-induced cell death, and is not dependent on the actions of IL-1β or IL-18 (67). Cycloheximide treatment is reported to inhibit LPS/ATP-induced caspase-1 activation, suggesting that protein synthesis is necessary for the priming step (66). Bacterially derived effector molecules such as LPS are reported to facilitate inflammasome activation via a TLR signaling-independent intracellular mechanism involving pannexin-1 (68, 69). In contrast to the uncertainty that exists with respect to how LPS primes a cell to respond to ATP, the role of LPS in promoting NFκB-dependent transcription and translation of proinflammatory cytokines is well understood (70). Importantly, the two-step mechanism required to promote production and release of IL-1β is not restricted to LPS-activated cells. As shown in this study, BMDMs activated with the TLR3 agonist poly(I:C) generate abundant quantities of proIL-1β but release little of this cytokine extracellularly. However, subsequent treatment with ATP promoted rapid and efficient release of the mature cytokine. Thus, BMDMs appear to maintain tight control over IL-1β output independent of the TLR priming stimulus.

IL-1F6-fph (or GFP)-transduced BMDMs demonstrated the expected requirement for LPS and ATP to promote IL-1β synthesis and posttranslational processing; viral transduction, therefore, did not adversely affect this cellular pathway. IL-1F6-fph released from BMDMs in the presence of LPS and ATP migrated during SDS gel electrophoresis with the same apparent mobility as the cell-associated species suggesting that the IL-1F6-fph polypeptides did not undergo proteolytic maturation during their release. Murine IL-1F6 lacks a consensus caspase-1 cleavage sequence; as such, it is perhaps not surprising that the released IL-1F6-fph was not processed by caspase-1. Moreover, pharmacological inhibition of caspase-1 with YVAD-CMK did not inhibit LPS/ATP-induced release of IL-1F6-fph output. Thus, IL-1F6-fph release appears completely independent of caspase-1. In this regard, previous studies have reported that release of proIL-1β and proIL-18 from LPS/ATP-treated human monocytes occurs independently of caspase-1 (65); although caspase-1 inhibitors prevent proteolytic maturation of these cytokines, the proforms continue to be released extracellularly in the presence of these agents. In contrast, mouse peritoneal macrophages treated with a caspase-1 inhibitor release less IL-1β than nontreated cells (67). In our system, YVAD-CMK-treated BMDMs generated reduced quantities of the 17 kDa mature IL-1β species, but the quantity of proIL-1β released in the presence of the caspase antagonist did not appear to equate to the quantity of mature IL-1β exported in the absence of this agent. This may reflect that the Western blotting protocol does not provide an accurate quantitative assessment of the two distinct species. Alternatively, it has been observed previously that proIL-1β can distribute into a detergent-insoluble compartment when murine macrophages are treated with ATP in the presence of pharmacological antagonists (47). The nature of this compartment is unclear, but displacement of the procytokine species to this location may restrict its release extracellularly. In this regard, the quan-

tity of proIL-1β recovered from the YVAD-CMK-treated BMDM cell extracts was less than that recovered from cells treated with CP-456773 even though both agents blocked release of the mature cytokine; thus, the caspase inhibitor may cause some proIL-1β to partition into a detergent insoluble compartment that is lost during sample workup. The diarylsulfonylurea CP-456773 effectively inhibited release of both IL-1F6-fph and IL-1β from LPS/ATP treated BMDMs, indicating that the release mechanisms used by these two cytokines share sensitivity to this pharmacological agent. GST-Omega 1–1 was previously shown to bind compounds related to CP-456773 (57), and this interaction may contribute to their pharmacological effect via a mechanism that remains to be established.

GFP was produced constitutively by the retrovirally transduced BMDMs and was retained intracellularly. However, when these cells were treated sequentially with LPS and ATP, GFP polypeptides were released to the culture medium. This release was not impaired by cycloheximide or YVAD-CMK, but was antagonized by CP-456773. Thus, release of GFP from BMDM transductants paralleled that of IL-1F6-fph. It is interesting to note that intracellular pools of IL-F6-fph and GFP were not depleted following the LPS/ATP activation protocol, and this parallels the situation observed with IL-1β. Notable also is the differential state of IL-1 recovered from the intra and extracellular locations. IL-1β released from the LPS/ATP-treated cells was processed efficiently to the mature cytokine species while cytokine that remained cell associated persisted as the procytokine species. To account for these observations, we propose that the activation process is stochastic and results in two distinct cell populations. One population was altered during the 20-min pulse of ATP such that the cells activated caspase-1 and converted their entire content of newly synthesized proIL-1β to the mature cytokine species. These cells stained positive with the FLICA reagent, demonstrated cytoplasmic clearing, and displayed attenuated MitoTracker Red staining. The second population, in contrast, did not reach the same "committed state" during the ATP pulse period and therefore did not activate caspase-1. Failure to attain a committed state may reflect an inability of LPS to prime the cells effectively, from an insufficient number of $P2X_7$ receptors, and/or from insufficient K^+ efflux which is necessary to commit the cells along the posttranslational processing pathway. This latter population of cells retained proIL-1β intracellularly, did not react with the FLICA reagent, retained a normal morphological appearance, and possessed functional mitochondria as assessed by MitoTracker Red staining. Release of IL-1F6-fph and GFP from the LPS/ATP-treated BMDMs occurred independently of caspase-1 activity and new protein synthesis. Given that GFP is not expected to possess a motif or recognition marker that should facilitate release from cells, its presence in the CM is assumed to reflect a nonselective process, as would result from disruption of the plasma membrane. In this regard, LPS/ATP activated monocytes/macrophages also have been reported to release other nonsecretory polypeptides including LDH (32), caspase-1 (66, 71), cryopyrin (25), and the kinase p38 (49). BMDMs that released IL-1F6-fph or GFP may correspond to the population of cells that reached a committed state during the ATP pulse and, in turn, activated caspase-1 and released mature IL-1β. Although caspase-1 activity is not required for facilitating release of IL-1F6-fph and GFP, events that led to activation of the inflammasome and, in turn, caspase-1 may contribute to loss of plasma membrane integrity. Indeed, a cyropyrin-dependent but caspase-1 independent necrotic cell death process has been reported (72). Likewise, $P2X_7$ receptor-mediated release of proIL-1β from LPS-activated macrophages is reported to occur independently of

caspase-1 and pannexin-1 (73). Factors affecting the rate and extent of release of an individual polypeptide may include size, net charge, and physical association with various macromolecular structures, including cytoskeletal components.

Several mechanisms have been proposed to account for how IL-1β is released from ATP-treated monocytes/macrophages in vitro. Based on inhibition by glyburide, for example, it was postulated that that IL-1β may be actively transported across the plasma membrane of LPS/ATP-treated murine macrophages via ABC1, a member of the ATP cassette transporter superfamily (43, 74). In LPS-activated THP-1 cells, in contrast, it was observed that ATP promotes rapid shedding of microvesicles from the cell surface enriched in mature IL-1β (45). LPS-treated human monocytes also are reported to sequester proIL-1β and procaspase-1 into specialized secretory lysosomes that, following ATP activation, fuse with the plasma membrane via a phospholipase-dependent process resulting in release of mature IL-1β (44, 46). Similarly, in LPS-stimulated mouse macrophages, data have been presented recently suggesting that IL-1β, caspase-1, and inflammasome components are sequestered into exosomes and incorporated into multivesicular bodies that subsequently fuse with the plasma membrane in the presence of ATP (75). Finally, in the original report noting that ATP induced release of mature IL-1β from LPS-activated murine macrophages, it was observed that release of IL-1 paralleled release of the cytoplasmic enzyme LDH and by DNA fragmentation (32). On this basis, release was postulated to occur secondarily to apoptosis and loss of plasma membrane integrity. More recently, the death process has been designated as pyroptosis to differentiate it from a noninflammatory apoptotic process (76). Our findings demonstrating that mature IL-1β, IL-1F6-fph, and GFP are exported in parallel are consistent with the ultimate release step being accomplished via a nonselective mechanism as would occur with breakdown of the plasma membrane. It is important to note, however, that steps leading up to this compromised state, involving TLR-dependent signaling, TLR-independent priming, and ATP-dependent activation of P2X$_7$ receptors, constitute a complex, highly regulated biological process that ensures tight regulation of the release of proinflammatory cytokines of the IL-1 superfamily.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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