Caspase-1-dependent processing of pro-interleukin-1β is cytosolic and precedes cell death

David Brough* and Nancy J. Rothwell

Faculty of Life Sciences, Michael Smith Building, University of Manchester, Oxford Road, Manchester, M13 9PT, UK

*Author for correspondence (e-mail: david.brough@manchester.ac.uk)

Accepted 12 December 2006
Journal of Cell Science 120, 772-781 Published by The Company of Biologists 2007
doi:10.1242/jcs.03377

Summary

The pro-inflammatory cytokine interleukin-1β is a key mediator of inflammation and is implicated in the pathogenesis of diverse disease states. Despite its biological importance, the mechanisms of its processing to an active form and its trafficking to the extracellular compartment remain poorly understood. Interleukin-1β secretion is proposed to occur via several distinct mechanisms including microvesicle shedding and the regulated secretion of lysosomes. In this study, we report for the first time that caspase-1-dependent processing of pro-interleukin-1β can occur in the cytosol following activation of P2X7-receptor. We also provide evidence that the pathway of secretion in this model is independent of the lysosomal trafficking regulator, a protein involved in lysosome secretion. Although release of interleukin-1β occurred before the appearance of significant levels of lactate dehydrogenase in the supernatant, the cells ultimately died. It is clear that structural changes preceding cell death, occurring after caspase-1 activation, promote the cellular release of interleukin-1β. We investigated the involvement of lipid rafts in this process and discovered that depleting the plasma membrane of cholesterol did not adversely affect interleukin-1β secretion in response to ATP. We propose that, in macrophages, ATP-induced interleukin-1β processing occurs in the cytosol by a mechanism that resembles pyroptosis.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/5/772/DC1

Key words: Interleukin-1, Cathepsin D, Caspase-1, Pyroptosis, Membrane fluidity

Introduction

Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine, central to the pathogenesis of local and systemic, acute and chronic inflammatory diseases of the central nervous system (CNS) and peripheral organs (Allan and Rothwell, 2003; Dinarello, 2005). Understanding the mechanisms of bioactive IL-1β generation may therefore identify new targets for anti-IL-1β therapies and contribute to the understanding of inflammatory processes during disease.

IL-1β is produced by cells of haematopoietic lineage in response to pro-inflammatory signals, such as bacterial lipopolysaccharide (LPS), like an inactive 31 kDa precursor termed pro-IL-1β (Matsushima et al., 1986). In freshly isolated human monocytes and rat microglia, LPS is sufficient to promote the maturation and externalisation of a fraction of cellular IL-1β (Perregaux et al., 1996; Chauvet et al., 2001), and in monocytes this is enhanced by the secretory stimulus ATP acting via the P2X7 receptor (Perregaux et al., 1996; Solle et al., 2001). In murine macrophages and microglia, LPS does not promote the release of mature IL-1β, and the cells require a secondary stimulus such as ATP (Perregaux and Gabel, 1994; Ferrari et al., 1997; Brough et al., 2002).

Pro-IL-1β is synthesized in the cytosol of activated cells without a signal sequence, thus precluding secretion via the classical ER-Golgi route (Rubartelli et al., 1990). Processing of pro-IL-1β to its active form requires caspase-1 (Thornberry et al., 1992), itself activated by a molecular scaffold termed the inflammasome (Martinon et al., 2002). Processing and release of IL-1β are thought to be linked closely, because mature IL-1β is only found inside inflammatory cells just prior to release (Brough et al., 2003). It has been reported that in monocytes, a fraction of cellular IL-1β is released by the regulated secretion of late endosomes and early lysosomes, and that this may represent a cellular compartment where caspase-1 processing of pro-IL-1β takes place (Andrei et al., 1999; Andrei et al., 2004). Shedding of microvesicles from the plasma membrane has also been proposed as a mechanism of secretion (MacKenzie et al., 2001). These proposals superseded previous models in which non-specific release due to cell lysis (Hogquist et al., 1991) and passage through a plasma membrane pore (Singer et al., 1995) was considered. However, there is evidence in the literature that can support or explain all of these mechanisms and there is, therefore, still controversy over how IL-1β makes its cellular exit.

The aim of this study was to identify where the processing of pro-IL-1β to mature IL-1β takes place within the cell, and what this means with regard to its secretion and the caspase-1-dependent death of the cell. We report that in primary cultured macrophages, pro-IL-1β is processed in the cytosol and suggest that the majority may not harness a lysosomal pathway for its cellular release. In addition, release of IL-1β by these cells precedes an inevitable cell death.

Results and Discussion

ATP-induced processing and release of IL-1β from macrophages

LPS-primed (1 μg/ml, 2 hours) murine macrophages were incubated with ATP (5 mM) for 5, 10, 15 and 20 minutes.

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Immunoblot and ELISA analysis indicated that after 5 minutes of ATP stimulation mature IL-1β (17 kDa) was generated. This IL-1β was largely intracellular, although significant levels were detected in the supernatant by ELISA (317±17 pg/ml, 5 minutes ATP, vs 29±8 pg/ml LPS alone, *P*=0.002, Fig. 1). From this point, the level of mature IL-1β in the cell decreased and correlated with an increase in mature IL-1β levels in the supernatant (Fig. 1Ai,Bi,Cii). Extracellular mature IL-1β was detected by immunoblotting 10 minutes after ATP stimulation and represented a significant increase when compared with its release after 5 minutes of ATP (3533±1157 pg/ml, *P*=0.04, Fig. 1Bi,Cii). There was a further, significant increase in the release of IL-1β at 20 minutes of ATP stimulation (7403±963 pg/ml, *P*=0.02 vs release following 10 minutes of ATP, Fig. 1Cii). The addition of the caspase-1 inhibitor Ac-YVAD-cmk (50 μM) simultaneously with ATP confirmed that the generation of the 17-kDa IL-1β species and its release was dependent upon the activity of caspase-1 (Fig. 1Aii,Bii).

It has been reported that a fraction of cellular IL-1β is released via secretory lysosomes that are immuno-positive for cathepsin D (CD) (Andrei et al., 2004). Mice carrying a mutation in the *Lyst* gene have impaired lysosomal secretion from cytotoxic T-Lymphocytes (CTLs) and fibroblasts (Baetz et al., 1995; Huynh et al., 2004). The product of the *Lyst* gene, the 400 kDa lysosomal trafficking regulator, is thought to regulate lysosomal fusion to the plasma membrane through an interaction with SNARE proteins (Tchernev et al., 2002). We investigated whether the lysosomal trafficking regulator was involved in the secretion of IL-1β, by stimulating macrophages isolated from mice with a defective *Lyst* gene (*beige* mice) with LPS and ATP, and by comparing their response with that of wild-type (WT) macrophages (Fig. 1C). In response to ATP, cell death and IL-1β release were indistinguishable between wild-type and *beige* mice. This suggests that the lysosomal trafficking regulator protein is not important for the release of IL-1β from macrophages.

ATP-induced processing and release of IL-1β is reported to occur with significant cell death (Le Feuvre et al., 2002), and cell lysis has been suggested as a mechanism of release (Hogquist et al., 1991). Over the duration of the experiment, LDH release increased progressively (Fig. 1Ci), and after 10 minutes (5 minutes after the appearance of IL-1β in the supernatant) reached statistical significance when compared with LPS alone (25±4% 10 minutes ATP vs 18±6% LPS alone, *P*=0.02, Fig. 1Ci). This finding argues against a non-specific release due to complete cell lysis as the only mechanism of IL-1β secretion by these cells, but does suggest that IL-1β is released by dying cells. We have shown previously that

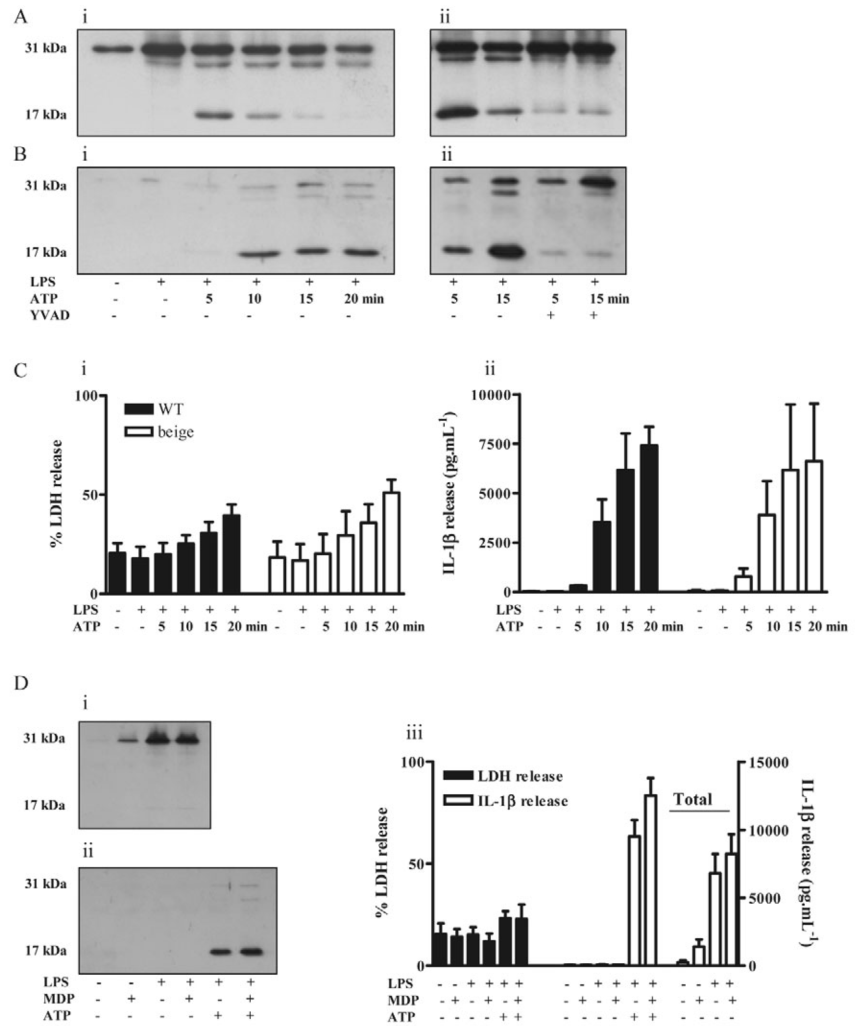

Fig. 1. ATP-induced IL-1β release and cell death. (A) IL-1β immunoblot of murine macrophage cell lysates after LPS treatment (1 μg/ml), and stimulation with ATP (5 mM) for the indicated time in minutes (min). Shown are blots of lysates from experiments conducted in the absence (i) or presence (ii) of the caspase-1 inhibitor Ac-YVAD-fmk (50 μM). (B) IL-1β immunoblot of murine macrophage supernatant after the LPS and ATP treatments described in A. Blots reflect experiments conducted in the absence (i) or presence (ii) of the caspase-1 inhibitor Ac-YVAD-fmk (50 μM). (C) Effects of LPS (1 μg/ml) and ATP (5 mM, for the indicated time in minutes on cell death (LDH release, i) and IL-1β release (ii). The responses of macrophages isolated from wild-type (WT, C57BL/6J, black bars) and *beige* mice (white bars) were compared. (D) Effects of MDP (5 μg/ml, 2 hours) on IL-1β expression and release and cell death. Shown are immunoblots for IL-1β of macrophage lysate after incubation without MDP or LPS, with MDP, with LPS (1 μg/ml, 2 hours) or with MDP and LPS together (i); in addition, macrophages treated with LPS or LPS and MDP were incubated with ATP (5 mM, 10 minutes) and the supernatants were analyzed for IL-1β by immunoblot (ii). The effects of MDP on LPS and ATP-induced cell death (LDH release, black bars, left *y*-axis, iii) and IL-1β release and production as measured by ELISA (white bars, right *y*-axis, iii) are also shown. Data are presented as the mean ± s.d. of duplicate and triplicate wells from at least three independent cultures. Immunoblots are representative of three independent experiments.

macrophage cell death and IL-1β release induced by P2X7-receptor activation (even a brief 5-minute ATP stimulation) are completely dependent upon caspase-1, because they are abolished in macrophages isolated from caspase-1-deficient mice or by using a caspase-1 inhibitor (Le Feuvre et al., 2002; Brough et al., 2003). The biochemical characteristics of the IL-1β release and cell death observed in our model bear remarkable similarity to those induced by infection of macrophages by the enterobacteria of *Salmonella* and *Shigella* genus (Brennan and Cookson, 2000; Hilbi et al., 1997). The caspase-1-dependent processing and release of IL-1β as well as a related cytokine IL-18 during caspase-1-dependent macrophage cell death are crucial for resistance to *Salmonella typhimurium* infection (Raupach et al., 2006; Lara-Tejero et al., 2006). The caspase-1-dependent execution of the macrophage coupled to an inflammatory response has recently been termed pyroptosis (Fink and Cookson, 2005).

P2X7-dependent activation of caspase-1 occurs through a cryopyrin (NALP3) containing inflammasome (Mariathasan et al., 2006). In order to examine the role of a cryopyrin-containing inflammasome in a pyroptotic-like mechanism, but in the absence of permeabilizing concentrations of ATP, we investigated the effects of muramyl dipeptide (MDP). In a human monocyte cell line, MDP promotes the activation of caspase-1 and processing of pro-IL-1β by a cryopyrin-dependent mechanism (Martinon et al., 2004). However, recent reports suggest that MDP does not activate a cryopyrin inflammasome in murine macrophages (Sutterwala et al., 2006; Mariathasan et al., 2006). This is in agreement with our findings, where 2-hour incubation with MDP (5 μg/ml), in the absence or presence of LPS, failed to promote processing or release of IL-1β, or induce cell death (Fig. 1D). Treatment of LPS- and MDP-stimulated macrophages with ATP induced a greater release of IL-1β compared with macrophages treated with LPS and ATP alone (12532±1263 pg/ml vs 9523±1208 pg/ml, P=0.004, Fig. 1Dii,iii, white bars, right y-axis), although the extent of cell death was unaffected (23±7% vs 23±3% LDH release, Fig. 1Diii, black bars, left y-axis). Analysis of total IL-1β cell content showed that MDP alone induced a significant increase in IL-1β production compared with control (1379±552 pg/ml vs 225±164 pg/ml respectively, P=0.001), and that it had an additive effect on IL-1β production in the presence of LPS (6796±1422 pg/ml (LPS) vs 8230±1444 pg/ml (LPS + MDP), P=0.1, Fig. 1Di,iii, white bars, right y-axis). Thus, it appears likely that the increased secretion of IL-1β in response to ATP, from an MDP- and LPS-treated macrophage is due to increased cellular IL-1β content.

Distribution of cellular IL-1β

We investigated the sub-cellular distribution of IL-1β, and whether it colocalized with markers of endosomal/lysosomal organelles. High-resolution images of LPS-primed macrophages stained for IL-1β (Fig. 2A, green) and co-stained for the early endosome marker, early endosomal antigen 1 (EEA1, Fig. 2A, red), or the late endosome/early lysosome marker, cathepsin D (CD, Fig. 2B, red), suggested no colocalization, although the widespread distribution of IL-1β throughout the cytoplasm made such a conclusion difficult. No signal was detected from macrophages stained for IL-1β without LPS treatment (data not shown).

To overcome this problem we adapted a recently described

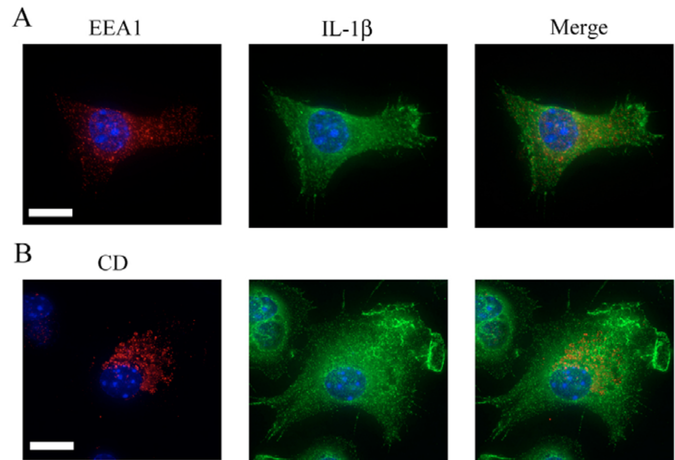

Fig. 2. Distribution of cellular IL-1β. (A) High-resolution, deconvolved image of a LPS-treated (1 μg/ml, 2 hours) murine macrophage immunostained for IL-1β (green) and EEA1 (red). Bar, 5 μm. (B) High-resolution, deconvolved image of a LPS-treated (1 μg/ml, 2 hours) murine macrophage immunostained for IL-1β (green) and cathepsin D (CD, red). Bar, 5 μm. Images are representative of three independent experiments.

strategy (Lorenz et al., 2006) to selectively permeabilize the plasma membrane and extract cytosolic IL-1β using the cholesterol binding drug digitonin while leaving lysosomal cargo intact. As CD is a luminal lysosomal hydrolase it made an excellent control to identify selective plasma membrane permeabilization without compromising lysosomal membrane integrity. In a given experiment, the concentration of digitonin required varied, but was always 50-100 μM. Macrophages were incubated for increasing times with digitonin, after which the supernatant was removed and the cells were fixed, permeabilized with Triton X-100 and stained for IL-1β and CD. Fig. 3 shows a typical experiment. Following 30-second and 60-second incubations with 100 μM digitonin, the IL-1β signal was lost from the cytosol in the majority of cells but the CD signal remained (Fig. 3A). 90 seconds after incubation with digitonin, the CD signal was lost in some cells examined, although this was not detectable by immunoblot (Fig. 3Bii). At all time points, in some cells that had lost cytosolic IL-1β, there was a strong nuclear signal (Fig. 3A). At no stage was colocalization of IL-1β and CD observed. Under these conditions digitonin extracted pro-IL-1β, but not CD, into the supernatant of treated cells (Fig. 3Bi,ii). The increased levels of IL-1β in the supernatant correlated with an increase in measurable activity of the normally cytosolic LDH (Fig. 3Biii). Thus, in LPS-primed macrophages pro-IL-1β exists as a cytosolic protein.

It is possible, however, that pro-IL-1β traffics to a lysosomal compartment to be processed into the mature form (Andrei et al., 1999). We therefore stimulated LPS-primed macrophages with ATP for 5 minutes prior to treatment with digitonin. At this time, processing of pro- IL-1β to mature IL-1β had occurred and was largely intracellular (Fig. 1). Following digitonin extraction, any colocalization should be obvious. However, we could completely extract the IL-1β from cells that remained CD-positive (Fig. 4A). This, and the identification of

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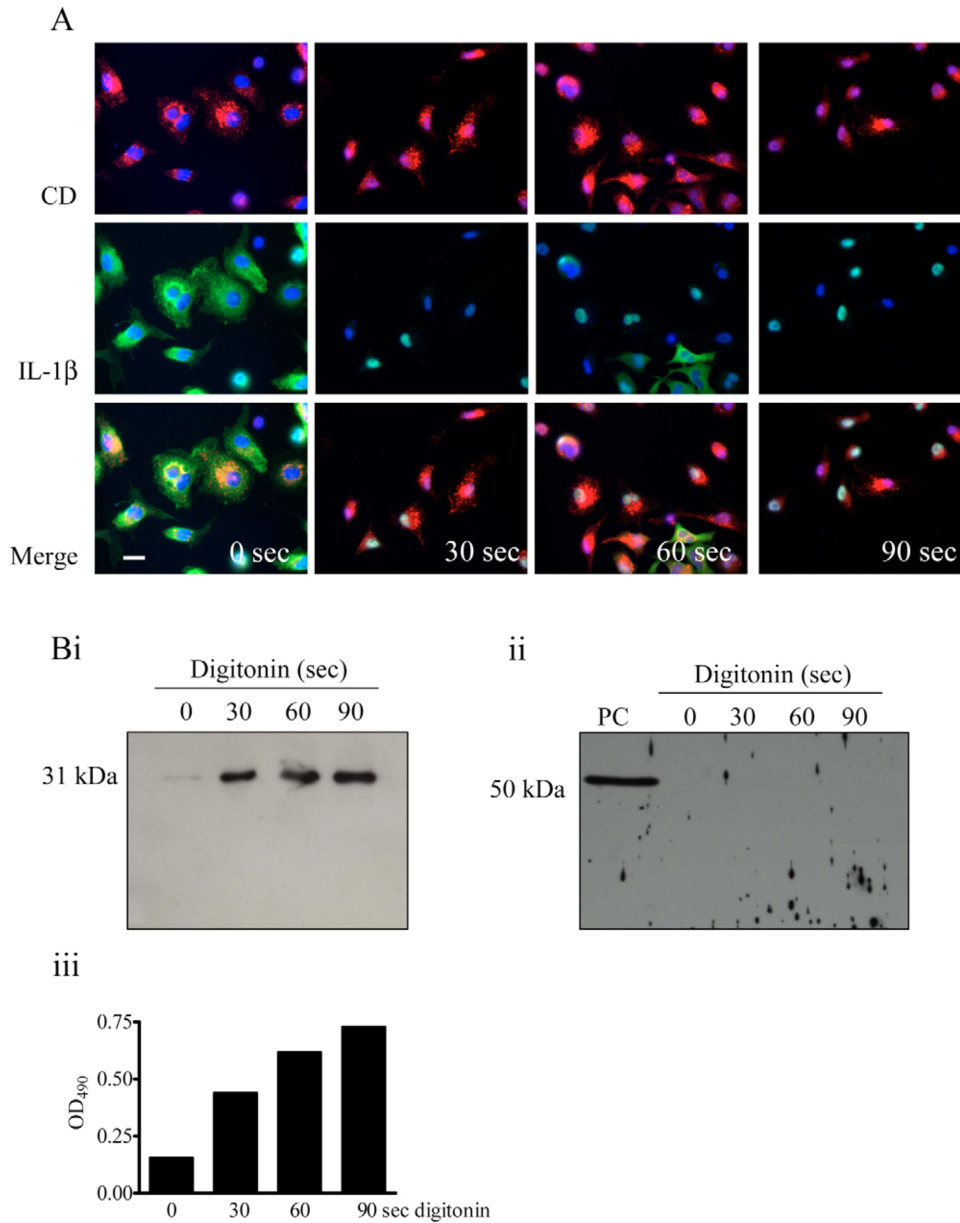
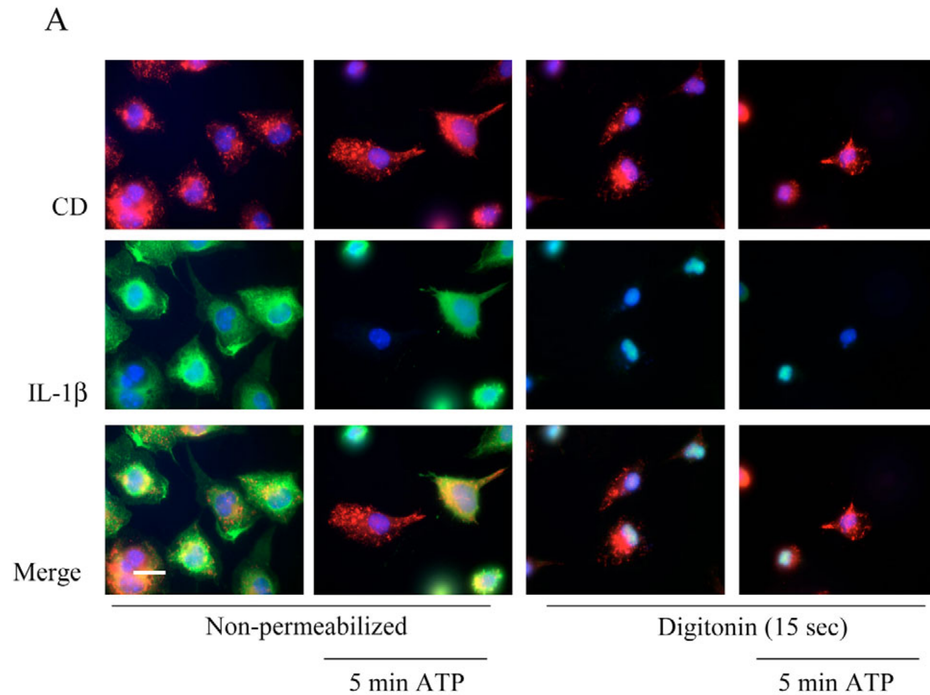


Fig. 3. Sub-cellular localization of pro-IL-1β. (A) Wide-field fluorescence images of LPS-treated (1 μg/ml, 2 hours) murine macrophages treated with digitonin for the indicated time seconds in (sec) after which they were immunostained for IL-1β (green) and cathepsin D (CD, red). Bar, 10 μm. (B) Immunoblot of the supernatants isolated from the experiment described in A for IL-1β (i) and cathepsin D (ii). The corresponding supernatants were also assayed for activity of the cytosolic LDH protein (iii). Images and blots are representative of three independent experiments.

mature IL-1β in the digitonin extract (Fig. 4B), suggests that the majority of processing of pro- to mature IL-1β in macrophages is a cytosolic event. These data are consistent with studies reporting the cellular location of apoptosis-associated speck-like protein (ASC) (Masumoto et al., 2001; Moriya et al., 2005; Yu et al., 2006), an adaptor protein involved in caspase recruitment to the inflammasome, the molecular scaffold that is central to caspase-1 activation and pro-IL-1β processing (Srinivasula et al., 2002) – although see also Johnston et al., for indirect evidence for an ASC endosomal localization (Johnston et al., 2005) – and also consistent with a report on the localization of caspase-1 in Jurkat cells (Shikama et al., 2001). Our data are also consistent with the mechanisms that occur during pyroptosis. Activation of caspase-1 in macrophages infected by *Salmonella* species depends upon secretion of the *Salmonella* invasion protein SipB into the cytosol where it activates caspase-1 by associating with components of the inflammasome, such as Ipaf (Hersh et al., 1999; Lara-Tejero et al., 2006; Franchi et al., 2006). Likewise, following the phagosomal escape of *Shigella* species, the secreted invasion plasmid antigen IpaB localizes to caspase-1 in the cytosol of macrophages (Thirumalai et al., 1997). The differences in localization described may be explained by the existence of distinct mechanisms for IL-1β



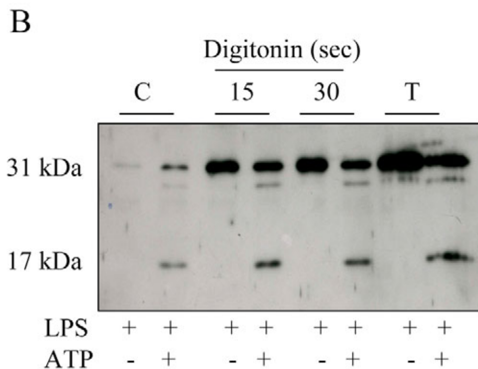


Fig. 4. Sub-cellular localization of mature IL-1β. (A) Wide-field fluorescence images of LPS-treated (1 μg/ml, 2 hours) murine macrophages in the absence or presence of ATP (5 mM, 5 minutes). Cells were immunostained for IL-1β (green) and cathepsin D (CD, red), before (non-permeabilized), and after digitonin treatment. Bar, 10 μm. (B) Immunoblot for IL-1β in supernatants isolated from the experiment described in A. C, control; T, supernatants isolated from macrophages permeabilized with Triton X-100. Images and blots are representative of three independent experiments.

release that differ between monocytes and macrophages, although such possibilities remain to be explored.

We sought to further test our hypothesis that pro-IL-1β is a cytosolic protein that is processed to the mature form in the cytosol of activated macrophages. Processing may reveal some cryptic targeting motif that, once uncovered, would allow the transit of mature IL-1β from the cytosol to the lysosome. HEK 293 cells provide an amenable transfection system and are reported to provide a model of IL-1β secretion when transfected with plasmids expressing IL-1β, P2X7 and caspase-1 (Gudipaty et al., 2003). We transfected HEK 293 cells with plasmids expressing the three forms of IL-1β (all carrying a C-terminal FLAG epitope) that had been detected in our samples: the 31-kDa pro-form and the caspase-1-generated 27-kDa and 17-kDa cleavage products. At 24 hours after transfection, cells were co-stained for IL-1β and for CD. All three IL-1β proteins localized to the cytosol and showed no colocalization with CD (Fig. 5). This provides support for our hypothesis that mature IL-1β lacks a lysosomal targeting motif and can exist in the cytosol.

Physical properties of IL-1β secretion

Immunocytochemical analysis of macrophage cell preparations suggested that IL-1β was a cytosolic protein with a diffuse pattern of staining (Fig. 2). At all time points investigated in the processing and release experiment described above (Fig. 1), the cells retaining IL-1β for the duration of the experiment exhibited a blebbing phenotype in response to ATP (Fig. 6A,B). Blebbing has been reported previously for HEK cells in response to P2X7-receptor stimulation (MacKenzie et al., 2001), and is suggested to be an early apoptotic event (Nusbaum et al., 2004). As stated above, P2X7-receptor-induced cell death in macrophages is, in addition to IL-1β release, caspase-1 dependent (Le Feuvre et al., 2002).

We therefore hypothesized that blebbing macrophages retain their IL-1β for the duration of the experiment owing to an absence of caspase-1 activity. This was tested by analyzing bright-field movie sequences of macrophages treated with ATP, plus or minus Ac-YVAD-cmk (50 μM). Control movies showed that for the duration of the experiment the cells were unchallenged or damaged by the imaging protocol (Fig. 7B, and supplementary material Movie 1). In response to ATP, the majority of the cells contracted, and then appeared to swell and round up until a 'halo' effect of the plasma membrane was observed (Fig. 7Ai,B, and supplementary material Movie 2). These cells did not retain IL-1β over the course of the experiment and therefore represent the IL-1β-secreting cells (90±5% of cells 20 minutes after ATP, Fig. 6A, Fig. 7B). They no longer appeared viable, as has been described previously, although membrane integrity was not compromised as suggested by the ability of the cells to retain significant levels of LDH (Fig. 1) (Perregaux and Gabel, 1994; Perregaux et al., 1996). We classified these cells as being dormant (Fig. 7B). However, not all cells showed this effect on ATP, and some

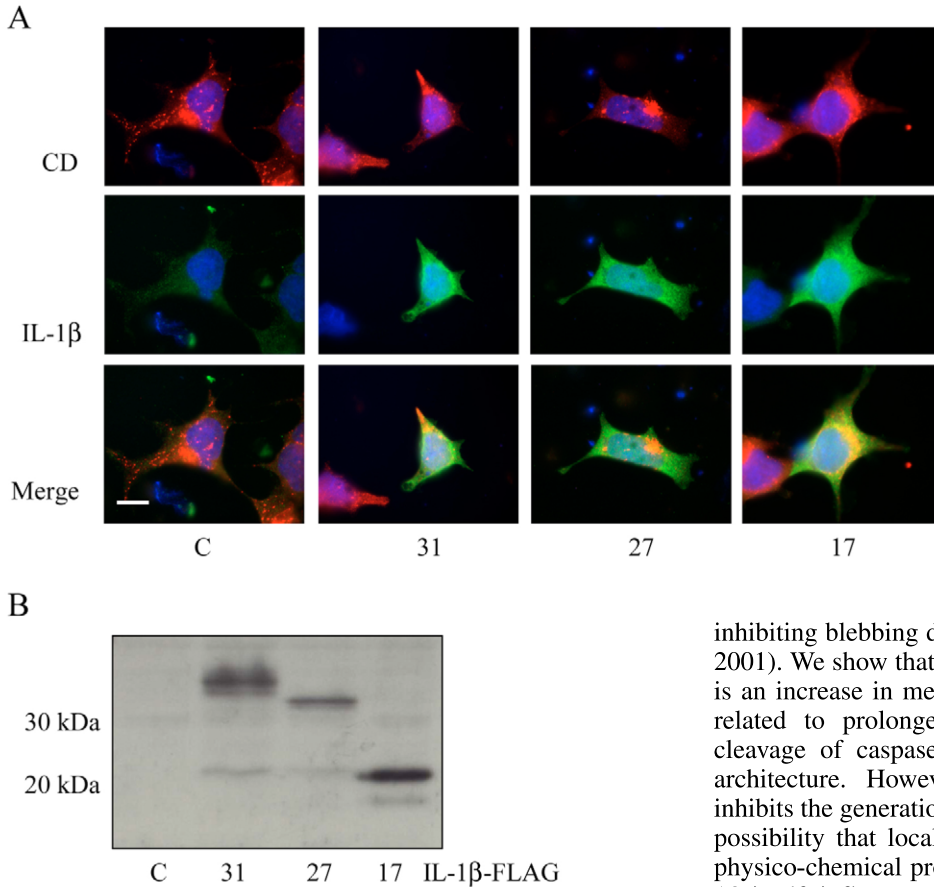


Fig. 5. Expression of IL-1β proteins in HEK 293 cells. (A) HEK 293 cells were transfected with empty vector (C), and constructs encoding Flag-labeled pro-IL-1β (31), Flag-labeled 27-kDa IL-1β (27) and Flag-labeled mature, 17-kDa IL-1β (17). Cells were immunostained for IL-1β (green) and cathepsin D (CD, red). Bar, 10 μm. (B) IL-1β immunoblot of HEK 293 cell lysates transfected with empty vector (C), and constructs encoding Flag-labeled pro-IL-1β (31), Flag-labeled 27-kDa IL-1β (27) and - Flag-labeled mature, 17-kDa IL-1β (17). Images and blots are representative of three independent experiments.

showed extensive blebbing for the duration of the experiment (7±5% of cells, Fig. 7Ai,B, arrows, and supplementary material Movie 2). The addition of Ac-YVAD-cmk just before the addition of ATP shifted the cellular phenotype dominated by cell death to a phenotype with extensive cell blebbing (35±9% of cells, P=0.01 vs ATP without Ac-YVAD-cmk at 20 minutes, Fig. 7Aii,B, and supplementary material Movie 3). This result suggests that there is a population of IL-1β-producing cells devoid of caspase-1 activity, but still responsive to ATP. The fact that it is the blebbing population of macrophages that retain their cellular IL-1β content and a non-blebbing population that secrete IL-1β, provides further evidence to support the previous literature dissociating IL-1β release from membrane blebbing (Verhoef et al., 2003).

Membrane blebbing may be driven by localized changes in hydrostatic pressure, with extension of the bleb a passive process driven by the pressure change, and bleb retraction an active process driven by actin and myosin II (Charras et al., 2005). Rho GTPase regulation of the actin skeleton can be regulated by the Rho-associated kinase ROCK I, inhibition of which impairs membrane blebbing without affecting the biochemical characteristics of apoptosis in fibroblasts (Coleman et al., 2001). Using Rho kinase inhibitors the process of membrane blebbing following P2X7-receptor activation and IL-1β secretion can be dissociated (Verhoef et al., 2003). However, as the process of blebbing is a physical consequence, inhibition of a blebbing phenotype in this way does not rule out the possibility that local changes in hydrostatic pressure can in some way affect IL-1β secretion, in the same way that inhibiting blebbing does not inhibit apoptosis (Coleman et al., 2001). We show that in the absence of caspase-1 activity there is an increase in membrane blebbing, and this is likely to be related to prolonged cellular survival and by preventing cleavage of caspase-1 substrates that maintain the cellular architecture. However, because caspase-1 inhibition also inhibits the generation of mature IL-1β, we cannot rule out the possibility that local changes in hydrostatic pressure, or the physico-chemical properties of the plasma membrane and IL-1β itself, influence IL-1β secretion.

Lipids and proteins in the plasma membrane have a heterogeneous distribution with confinement based on lipid properties to cholesterol and sphingolipid rich microdomains termed rafts (Munro, 2003), or by the actin cytoskeletal meshwork (Lenne et al., 2006). Stimulation of macrophages with ATP caused a massive structural reorganization of the plasma membrane when mature IL-1β was appearing in the supernatant (Figs 1, 6 and 7). Thus, the involvement of microdomains in IL-1β secretion confined by the actin meshwork appears unlikely. We therefore investigated whether lipid rafts could be involved in the secretion of IL-1β. It was recently reported that disruption of lipid rafts, by depleting cholesterol from the plasma membrane using the cholesterol-binding drug methyl-β-cyclodextrin (β-MCD), inhibits the secretion of tumor necrosis factor (TNF)-α from macrophages (Kay et al., 2006). Caspase-1-dependent cell death of macrophages infected with *Shigella flexneri* is cholesterol dependent and is attenuated following β-MCD-treatment, although the opposite effects are observed on ATP-induced cell death (Schroeder and Hilbi, 2007). We adopted this strategy to determine whether lipid rafts are important for the secretion of IL-1β. A 10-minute incubation with ATP was chosen because it is a time at which significant levels of IL-1β are released in the absence of widespread cell lysis (Fig. 1), and would thus ease interpretation of the effects on cholesterol depletion on IL-1β release. Stimulation of LPS-primed, β-MCD-treated macrophages (7.5 mM β-MCD in serum-free medium for 30 minutes prior to a medium change into serum-containing medium) with ATP for 10 minutes actually resulted in increased IL-1β secretion compared with cells treated in the

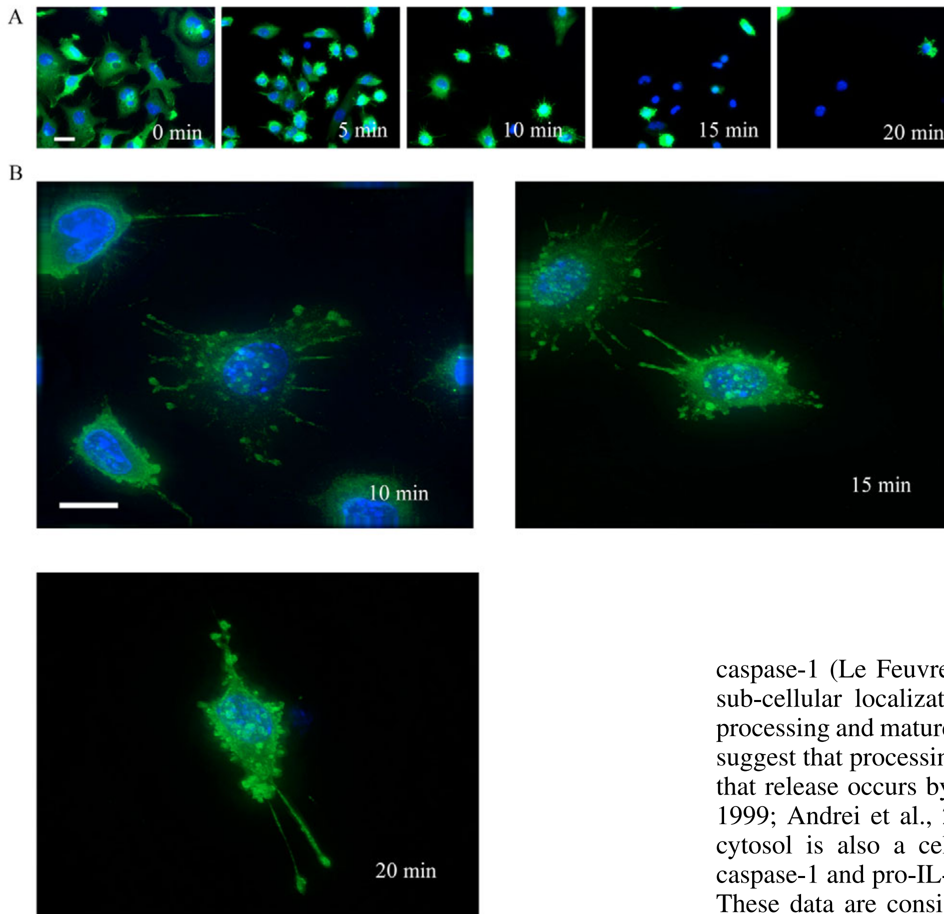


Fig. 6. Blebbing macrophages retain their IL-1β. (A) Wide-field fluorescence images showing IL-1β in LPS-treated (1 μg/ml, 2 hours) murine macrophages stimulated with ATP (5 mM) for the indicated times in minutes (min). Bar, 10 μm. (B) High-resolution, deconvolved images showing IL-1β in LPS-treated (1 μg/ml, 2 hours) murine macrophages stimulated with ATP (5 mM) for the indicated times. Bar, 5 μm. These images are representative of three independent experiments.

absence of β-MCD (incubated in serum-free medium alone for 30 minutes prior to medium change in to serum-containing medium) (5707±1513 pg/ml vs 4105±2069 pg/ml respectively, P=0.003, Fig. 7C). The enhanced IL-1β release cannot be accounted for by increased cell death because LDH release was the same in cultures treated with (23±9%) or without β-MCD in response to ATP (26±11%, Fig. 6D). This suggests that secretion of IL-1β in response to ATP occurs independently of lipid rafts. These findings raise an interesting issue regarding the specificity of IL-1β secretion and it is possible that, under these conditions, the membrane is permeable to mature IL-1β. Indeed, a related cytokine, IL-1α, is known to have membrane permeabilizing properties under certain conditions (Mandinova et al., 2003).

Conclusions

ATP, acting through the P2X7-receptor, activates caspase-1 via a cryopyrin-containing inflammasome (Mariathasan et al., 2006). This is in contrast to infection with *Salmonella typhimurium*, where inflammasome activation can occur in the absence of cryopyrin, but requires the adaptor protein Ipaf (Mariathasan et al., 2006). Regardless of this difference, the similarities in the IL-1β release and cell-death profile are striking. When activated, caspase-1 in our cells elicits a rapid cell death preceded by release of IL-1β that is analogous to the process of pyroptosis (Fink and Cookson, 2005). We have shown previously that this process is entirely dependent upon caspase-1 (Le Feuvre et al., 2002; Brough et al., 2003). The sub-cellular localization of the events leading to pro-IL-1β processing and mature IL-1β secretion is unclear. Some reports suggest that processing may occur in secretory lysosomes, and that release occurs by their regulated secretion (Andrei et al., 1999; Andrei et al., 2004). We propose that, in addition, the cytosol is also a cellular compartment where activation of caspase-1 and pro-IL-1β processing can take place (Figs 3, 4). These data are consistent with a previously described model suggesting a trans-membrane exit for IL-1β (Singer et al., 1995), and with the apparent cytosolic location of inflammasome components including caspase-1 (see Discussion). Together, with previously reported mechanisms, our data suggest that there are multiple mechanisms of IL-1β release.

Our data suggest that the majority of IL-1β is secreted directly across the plasma membrane in response to P2X7-receptor activation. The direct transport of a secretory protein across the plasma membrane has been reported for FGF-1, FGF-2 and for hydrophilic acylated surface proteins (HASPs) from *Leishmania* infection (Nickel, 2005), and has indeed been suggested for IL-1β (Singer et al., 1995) and IL-1α (Mandinova et al., 2003). The molecular nature of a transporter for IL-1β – should one exist – is unknown. Glyburide, an inhibitor of the ATP-binding-cassette-1 (ABCA1) transporter is an inhibitor of IL-1β export (Hamon et al., 1997). However, ABCA1 is involved in cholesterol transport and this has prompted researchers to question the specificity of the glyburide effect on IL-1β release owing to the completely unrelated nature and structure of the two molecules (Nickel, 2005). This is not to say that ABCA activity is not important for IL-1β secretion but, rather, that it is unlikely to be the transporter. Clearly secretion of IL-1β is a complicated event that is influenced by cell type, and by the mode of stimulus.

Materials and Methods

Materials

RPMI 1640 and DMEM culture media, fetal bovine serum (FBS), glutamine and a streptomycin-penicillin antibiotic solution were all purchased from Invitrogen (UK).

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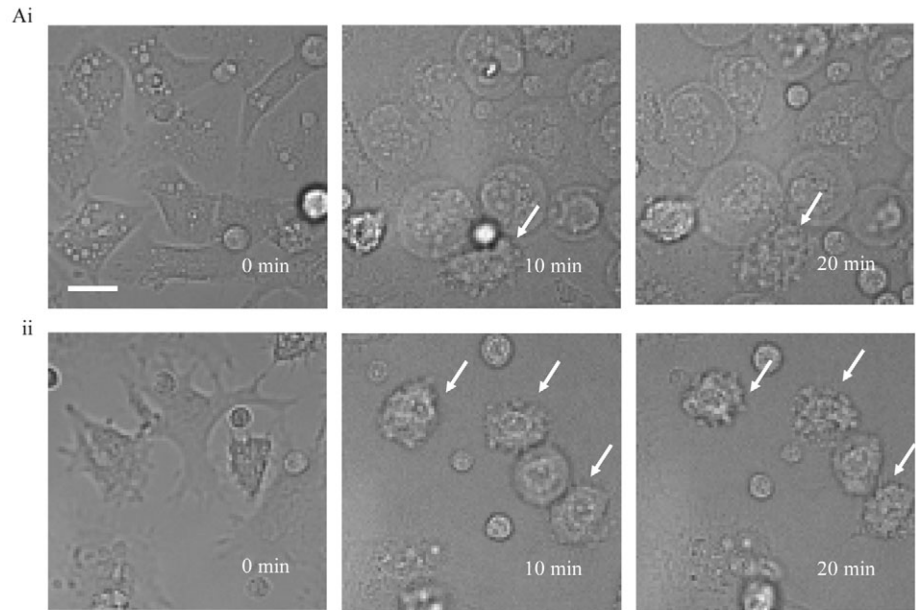

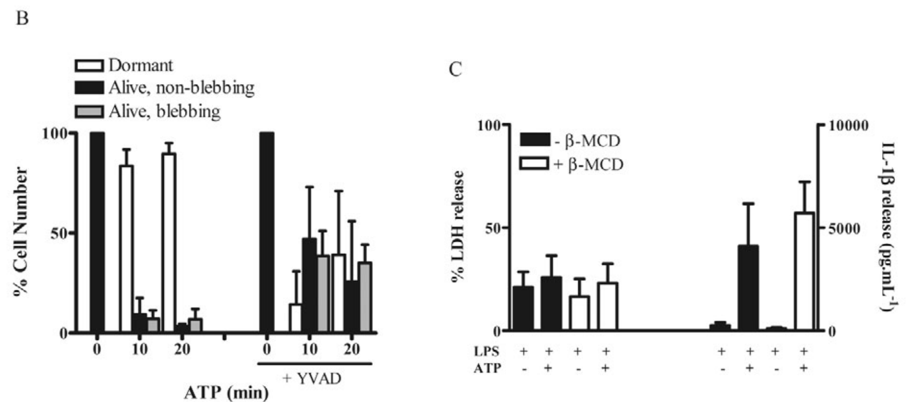

Fig. 7. Physical properties of IL-1β secretion. (A) Stills from a bright-field movie sequence of LPS-treated (1 μg/ml, 2 hours) murine macrophages stimulated with ATP (5 mM) in the absence (i) and presence (ii) of the caspase-1 inhibitor Ac-YVAD-fmk (50 μM). Time in minutes (min) after the addition of ATP is indicated. Arrows indicate blebbing cells. Bar, 10 μm. (B) The effects of ATP and Ac-YVAD-fmk in the above movies were quantified, with cells classified as dormant (white bars), alive but not blebbing (black bars), and alive and blebbing (gray bars). Data are the mean ± s.d. of cells counted from three independent experiments. (C) The effects of cholesterol depletion using β-MCD (7.5 mM, 30 minutes) on cell death (LDH release, left *y*-axis) and IL-1β release (right *y*-axis) from LPS-treated (1 μg/ml, 2 hours) murine macrophages in the absence or presence of ATP (5 mM, 10 minutes). β-MCD-treated cells, white bars; control cells, black bars. Data are the mean ± s.d. of duplicate wells from three independent cultures.

Bacterial lipopolysacharide (LPS, *Escherichia coli* 026:B6), ATP, the caspase inhibitor Ac-YVAD-fmk, poly-D-lysine (PDL), methyl-β-cyclodextrin (β-MCD) and muramyl dipeptide (MDP, N-acetylmuramyl-L-alanyl-D-isoglutamine) were purchased from Sigma (UK). Mouse anti-IL-1β antibody used for immunocytochemistry was purchased from R&D Systems (UK) and mouse anti-IL-1β antibody used for immunoblot (S329) was a kind gift from the National Institute of Biological Standards and Controls (NIBSC, UK). The antibodies against human early endosomal antigen-1 (EEA1) and human cathepsin D (CD) were purchased from abcam (UK) and Upstate (UK), respectively. Alexa Fluor-488 anti-goat and Alexa Fluor-594 anti-rabbit IgGs were purchased from Invitrogen. ProLong Gold mounting medium containing DAPI and the transfection reagent Lipofectamine 2000 were also purchased from Invitrogen. The pCMV-Tag 4 expression vector was purchased from Stratagene and the mouse IL-1β cDNA was supplied as an IMAGE clone (3989461) from the MRC Geneservice (UK). C57BL/6J mice were supplied by Harlan (UK) and *beige* mice (C57BL/6J-*Lyst*$^{bg\text{-}J}$/J) were supplied by The Jackson Laboratory (USA).

Molecular biology

Three forms of mouse IL-1β (pro-IL-1β, mature IL-1β and an intermediate form of IL-1β) were sub-cloned into a C-terminal FLAG expression vector as follows. The nucleotide sequences corresponding to the two caspase-1 cleavage sites on the mouse pro-IL-1β molecule (Thornberry et al., 1992) were identified and primers were designed to incorporate a tetracysteine motif (Griffin et al., 1998) at the N-terminus of a full-length 31-kDa protein, an intermediate 27-kDa protein and the mature 17-kDa protein. The corresponding nucleotide sequences were amplified to generate the DNA sequence with *Eco*RI and *Xho*I overhangs (restriction enzymes purchased from Roche). These sequences were then cloned into the pCMV-Tag 4 expression vector. All expressed proteins therefore contained a C-terminal FLAG epitope.

Cell culture and transfection

Macrophages were prepared from adult male mice as described previously (Le Feuvre et al., 2002). Briefly, following sacrifice, the peritoneal cavity was lavaged with 8 ml RPMI 1640 containing 5% FBS and 100 μg/ml streptomycin and 100 IU penicillin. The medium was recovered, the cells collected by centrifugation (80 ***g***, 5 minutes) and seeded onto a 24-well plate (some with 13-mm glass coverslips) at a density of 1×10^6 cells/ml. The macrophages were incubated overnight (37°C, 5% CO_2). To induce the synthesis of pro-IL-1β, macrophages were incubated with 1 μg/ml LPS for 2 hours. Where indicated, cells were stimulated with 5 mM ATP. HEK 293 cells were passaged and maintained in DMEM containing 5 mM glutamine, 10% FBS and 100 μg/ml streptomycin and 100 IU penicillin. One day prior to experimentation HEK 293 cells were seeded at a density of 5×10^5 cells/ml onto PDL-coated glass coverslips. The following day HEK 293 cells were transfected using Lipofectamine 2000 according to manufacturer's instructions with a DNA:Lipofectamine reagent ratio of 1:3. At 24 hours following transfection the cells were either fixed or lysates harvested as discussed below.

Digitonin permeabilization

Macrophages were plated onto glass coverslips as described. Following the 2-hour LPS incubation (where indicated, a 5-minute pulse of 5 mM ATP was applied) macrophages were incubated with digitonin (Calbiochem, UK) in culture medium for progressively increasing times. At the end of each time point supernatants were removed and cells were fixed in 4% PFA. Using the described immunocytochemistry protocol, the cells were then stained for IL-1β and CD.

Detection of IL-1β by ELISA

Measurement of IL-1β released into the culture supernatant of LPS-treated and ATP-treated macrophages was as described elsewhere (Brough et al., 2002). Briefly,

IL-1β release was measured using a specific sandwich ELISA generously provided by Steve Poole of the NIBSC (UK).

Immunoblotting and immunocytochemistry

Following the experiment, supernatants and lysates were harvested and prepared in sample buffer containing 1% β-mercaptoethanol. Samples were boiled and then separated by electrophoresis on 12% SDS-acrylamide gels. Proteins were transferred to a nitrocellulose membrane and blotted with a sheep anti-mouse IL-1β polyclonal serum followed by a horseradish peroxidase (HRP)-conjugated goat anti-sheep antibody; subsequent exposure was with enhanced chemi-luminesence reagents (ECL, Amersham, UK). For the cathepsin D immunoblot, membranes were blotted with a rabbit anti-human cathepsin D (CD) antibody (4 μg/ml) followed by a HRP-conjugated anti-rabbit antibody. The positive antigen control for the cathepsin D immunoblot was supplied and used according to the manufacturer's instructions (Upstate, UK).

Cells grown on glass coverslips were used for immunocytochemistry. Following the experiment, cells were fixed with 4% paraformaldehyde (PFA) in PBS. Cells were permeabilized with 0.1% Triton X-100 and then quenched with 0.25% ammonium chloride. A blocking step of 1 hour using 5% BSA was used prior to the simultaneous incubation of the sample with an affinity purified goat anti-mouse IL-1β antibody (1 μg/ml) and either a rabbit anti-human EEA1 antibody (2.5 μg/ml) or a rabbit anti-human CD antibody (10 μg/ml), in 5% BSA for 1 hour. The polyclonal IL-1β antibody was purified from a goat immunized with recombinant, mature mouse IL-1β. It recognizes both pro- IL-1β and mature IL-1β. Alexa Fluor-488 donkey anti-goat IgG (1 μg/ml) and Alexa Fluor-594 donkey anti-rabbit IgG (1 μg/ml) were used in 5% BSA for secondary detection. The coverslips were washed with PBS and mounted onto a glass slide using ProLong Gold mounting medium containing DAPI.

Microscopy

All the wide-field, high-resolution Deltavision and live cell microscopy was carried out at the Core Bioimaging Facility at the Faculty of Life Sciences, University of Manchester. All offline analysis of images and movies used ImageJ software (http://rsb.info.gov/ij/).

Lactate dehydrogenase assay

Release of the enzyme lactate dehydrogenase (LDH) from the cells was performed using the CytoTox-96 assay (Promega, UK) according to manufacturer's instructions.

Data analysis

Data were presented as the mean ± s.d. of at least three separate cultures. Differences between two groups were identified using the Student's *t*-test. In all immunoblot and immunocytochemistry experiments, the figures shown are representative of three independent experiments from three separate cultures.

The authors are grateful to members of the Rothwell laboratory for useful discussions and for reviewing the manuscript. The work was funded by the Medical Research Council (MRC) UK. We are also grateful to Peter March of the Bioimaging Facility at the University of Manchester.

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