0026-895X/04/6604-909–920$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 66:909–920, 2004

Vol. 66, No. 4
3260/1174167
Printed in U.S.A.

Maitotoxin Induces Biphasic Interleukin-1β Secretion and Membrane Blebbing in Murine Macrophages[S]

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Received February 6, 2004; accepted June 22, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Maitotoxin (MTX) is a potent shellfish toxin widely used as an in vitro tool for increasing intracellular Ca^{2+} and studying Ca^{2+}-dependent processes. MTX also induces membrane blebbing and nonselective pores similar to those elicited by the P2X7 receptor (P2X7R), an ATP-gated cation channel expressed in inflammatory leukocytes. We therefore tested whether MTX treatment of lipopolysaccharide-primed murine macrophages would mimic the ability of activated P2X7R to induce secretion of the proinflammatory cytokine interleukin-1β (IL-1β). MTX at $\leq$0.6 nM predominantly induced processing and nonlytic release of mature IL-1β (mIL-1β), whereas >0.6 nM of MTX induced cytolytic release of unprocessed proIL-1β. MTX-dependent release of mIL-1β (but not cytolysis) was inhibited by the elimination of the *trans*-plasma membrane K^+ gradient. MTX-induced cytokine release and cytolysis were both abrogated in the absence of extracellular Ca^{2+}. On the other hand, extracellular glycine (5 mM) blocked MTX-induced cytolytic release of proIL-1β without affecting regulated secretion of mIL-1β. Because MTX has profound effects on plasma membrane permeability, we used time-lapse videography to examine the morphologic response of individual macrophages to MTX. MTX treatment led to biphasic propidium dye uptake and dilated blebbing coincident with cytolysis. Glycine completely blocked the second, lytic phase of dye uptake and prevented MTX-induced bleb dilation. These results indicate that the inflammatory macrophage can assemble the necessary signaling components to initiate both regulated and lytic release of IL-1β in response to MTX. This suggests that the hyperactivation of proinflammatory cytokine secretion may be a significant component of the in vivo response to MTX during shellfish seafood poisoning.

Maitotoxin (MTX), from the dinoflagellate *Gambierdiscus toxicus*, is among the most potent marine toxins known and plays a significant role in ciguatera seafood poisoning (Takahashi et al., 1982). MTX at doses as low as 0.2 μg/kg can kill mice, and sublethal doses induce marked abnormalities in all tissues examined (Legrand et al., 1982; Terao et al., 1988, 1989). MTX has been extensively studied for its ability to stimulate increases in intracellular Ca^{2+} via activation of Ca^{2+}-permeable, nonselective cation channels (CaNSC) (Gusovsky and Daly, 1990; Dietl and Volkl, 1994; Bielfeld-Ackermann et al., 1998). Electrophysiological analysis of patch-clamped fibroblasts has revealed that Na^+, K^+, and Ca^{2+} can all permeate the MTX-induced cation channels (Martinez-Francois et al., 2002). MTX has also been shown to mediate formation of a cytolytic/oncotic pore (COP) with properties identical to that facilitated by the activation of the P2X7 nucleotide receptor (P2X7R), as measured by vital dye uptake (Schilling et al., 1999a,b). In addition, MTX induces formation of dilated membrane blebs in endothelial cells similar to the blebs observed in macrophages upon ATP activation of the P2X7R (Estacion and Schilling, 2001; Verhoef et al., 2003). MTX-dependent pore formation and blebbing is biphasic: the initial phase is slow and allows the passage of molecules <800 Da, and the blebs are small (1–3 μm), whereas the second phase represents cytolysis as measured by rapid dye uptake, LDH release, and massive expansion of the blebs (Estacion and Schilling, 2001, 2002). This

This work was supported in part by National Institutes of Health (NIH) grant GM36387 (to G.R.D.) and HL65323 (to W.P.S.). P.A.V. was partially supported by NIH grant T32-GM07250.

[S] The online version of this article (available at http://mdpharm.aspetjournals.org) contains supplemental materials.

ABBREVIATIONS: MTX, maitotoxin; CaNSC, Ca^{2+}-permeable, nonselective cation channel(s); COP, cytolytic/oncotic pore; P2X7R, P2X7 receptor; LDH, lactate dehydrogenase; IL-1β, interleukin-1β; proIL-1β, 33-kDa precursor protein (procytokine); LPS, lipopolysaccharide/endotoxin; mIL-1β, mature interleukin-1β; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; BSS, buffered saline solution; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; AG126, (3-hydroxy-4-nitrobenzylidene)malononitrile.

An erratum has been published:
http://molpharm.aspetjournals.org/content/66/5/1359.full.pdf

cytolysis is sensitive to the cytoprotectant glycine, which acts by an unknown mechanism to block LDH release and the second phase of vital dye uptake (Estacion et al., 2003).

Given its ability to induce rapid cell death secondary to the disruption of cellular ion homeostasis, MTX is likely to trigger innate immune responses and inflammation in in vivo settings. However, few studies have evaluated the effects of MTX on specific components of the innate immune response or the actions of MTX on inflammatory effector cells such as monocytes, macrophages, and neutrophils. The secretion of proinflammatory IL-1β remains a poorly understood component of the innate immune response. In response to primary inflammatory stimuli, such as bacterial lipopolysaccharide (LPS) or tumor necrosis factor-α, monocytes and macrophages synthesize IL-1β as a 33-kDa procytokine that accumulates in the cytosol (Dinarello, 1996). For IL-1β to be secreted as a mature, biologically active mediator, appropriate stimuli are required to assemble a multiprotein complex that facilitates processing of caspase-1 from its zymogenic, 45-kDa form to its active p20/p10 tetrameric form (Martinon et al., 2002; Kahlenberg and Dubyak, 2004). Activated caspase-1 then cleaves proIL-1β to its mature 17-kDa form, and both caspase-1 and IL-1β are subsequently released into the extracellular environment by a nonclassic secretory mechanism (MacKenzie et al., 2001)

Many stimuli that induce caspase-1 processing and secretion of IL-1β share an ability to modulate ionic flux leading to the loss of K^+, gain of Na^+ and Ca^{2+}, and eventual cytolysis. These include extracellular ATP acting via the P2X7R, which is predominantly expressed in monocytes and macrophages (Di Virgilio et al., 2001; Solle et al., 2001), and the potassium ionophore nigericin (Perregaux and Gabel, 1994). Other bacterial toxins, as well as antimicrobial protegrins, have also been shown to mediate IL-1β secretion via induction of K^+ efflux and cell death (Bhakdi et al., 1990; Perregaux et al., 2002). Recent studies have evaluated possible mechanisms for coupling ATP-activated P2X7R to rapid IL-1β processing and release by examining P2X7R-dependent events such as Ca^{2+} and K^+ flux, caspase activation, microvesiculation, pore formation, membrane blebbing, apoptosis, and necrosis (Ferrari et al., 1997b; MacKenzie et al., 2001; Le Feuvre et al., 2002; Gudipaty et al., 2003; Verhoef et al., 2003; Kahlenberg and Dubyak, 2004).

Given the similar effects of MTX and most IL-1β secretagogues on the disruption of ionic homeostasis, pore formation, and membrane blebbing, we tested whether MTX might be a stimulus for IL-1β secretion. We observed that MTX can induce the release of mature IL-1β (mIL-1β) from LPS-primed macrophages by a mechanism that can be dissociated from MTX-induced cytolysis and simultaneously induce dramatic morphologic changes. These results indicate that MTX-induced IL-1β release involves signaling pathways shared by other IL-1β secretagogues and suggest that proinflammatory cytokine release may play an important role in the toxic effects of MTX.

Materials and Methods

Materials. MTX was obtained from Wako Bioproducts (Richmond, VA) and stored as a stock solution in ethanol at −20°C. *Escherichia coli* O111:B4 LPS was obtained from List Biological Laboratories Inc. (Campbell, CA). ATP and glycine were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies for ELISA analysis of murine IL-1β were obtained from Pierce Endogen (Rockford, IL). The monoclonal anti-IL-1β antibody 3ZD, which recognizes both the 33-kDa proIL-1β and mature 17-kDa IL-1β in immunoblot analysis, was provided by the Biological Resources Branch of the National Cancer Institute-Frederick Cancer Research and Development Center, whereas the p38 MAPK antibody (sc-535) and horseradish peroxidase (HRP)-coupled secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For the analysis of LDH release, the cytotoxicity detection kit from Roche Diagnostics (Indianapolis, IN) was used. The vital dye YoPro was purchased from Molecular Probes (Eugene, OR). LPS was purchased from List Biological Laboratories Inc. (Campbell, CA). All other salts and chemicals were of reagent grade.

Cell Culture. The BAC1.2F5 macrophage cell line, a clone of the simian virus 40-transformed murine macrophage cell line BAC1, was maintained using protocols described previously (Verhoef et al., 2003).

Induction of MTX-Dependent IL-1β Secretion and Cytolysis. Cells were plated to 80 to 90% confluence. LPS (1 μg/ml) in Dulbecco's modified Eagle's medium/10% calf serum/1% penicillin-streptomycin was added to the cells, followed by incubation at 37° for 4 h to induce proIL-1β synthesis. The culture medium was removed, cells were washed with PBS, and buffered saline solution (BSS) containing 25 mM HEPES, 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 0.01% bovine serum albumin, pH 7.4, was added to the cells. In experiments using high K^+ medium, 130 mM KCl replaced 130 mM NaCl. For experiments conducted in the absence of Ca^{2+}, $CaCl_2$ was omitted from the extracellular media. For experiments using inhibitors, the agents were preincubated with the cells for at least 15 min before the addition of MTX and were present in the media throughout the duration of the MTX treatment. MTX was then added directly, and after the specified time period, the extracellular media were collected, centrifuged briefly to sediment any detached cells, and analyzed by immunoblot and ELISA. The remaining adherent cells were lysed in buffer containing 25 mM HEPES, pH 7.7, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0, 0.1% Triton X-100, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM dithiothreitol, and 100 μg/ml phenylmethylsulfonyl fluoride.

Immunoblot Analysis of Cell-Associated and Extracellular Proteins. For the assessment of cell-associated proteins, cell lysates were separated by SDS-polyacrylamide gel electrophoresis (15%) and electrophoretically transferred to PVDF membranes under rapid transfer conditions (in 1× Towbins buffer containing 3.03 g of Tris + 14.4 g of glycine + 200 ml of methanol per liter) for 45 min at 1 mA. PVDF membranes were rinsed in immunoblot buffer (10 mM Tris, pH 7.4, 0.9% NaCl, 0.05% Tween 20, and 1 mM EDTA) and blocked for 1 h in immunoblot buffer containing 4% nonfat dried milk. PVDF membranes were then incubated in primary antibody in immunoblot buffer containing 4% milk at room temperature for 1 h. Primary antibody concentrations were 1:1000 for IL-1β and 1:1000 for p38 MAPK. Membranes were then washed (1 × 15 min and 2 × 5 min) and incubated with 1:5000 dilutions of horseradish peroxidase-conjugated secondary antibody in 4% milk-immunoblot buffer for 1 h at room temperature. Membranes were washed and developed using chemiluminescent reagents (SuperSignal; Pierce Endogen) for 0.5 to 5.0 min and exposed to Eastman Kodak X-ray film (Eastman Kodak, Rochester, NY). For the assessment of proteins in the extracellular media, soluble proteins were acid-precipitated using 72 μl of 100% trichloroacetic acid and 15 μl of 10% cholic acid per 1 ml of extracellular media. The precipitated proteins were rinsed in acetone, followed by dissolution in Laemmli buffer.

ELISA Analysis of IL-1β Release. Aliquots (1–25 μl) of extracellular media samples were assayed for IL-1β content by sandwich ELISA. In brief, a 96-well plate was coated with 1 μg/ml primary anti-murine IL-1β overnight and then blocked with 4% BSA in PBS for 1 h. Plates were washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, and 0.2% Tween 20). Aliquots of media samples or

murine IL-1β standards, diluted to 50 μl with PBS, were added to the blocked wells together with 50 μl of the second, biotinylated anti-murine IL-1β antibody (0.2 μg/ml). The plates were incubated at room temperature for 2 h and then washed three times. The captured immune complexes were colorimetrically detected by subsequent incubations with streptavidin-HRP conjugate (0.1 μg/ml), washing, development with tetramethyl benzidine substrate for HRP, and absorbance measurement using a SoftMax Pro plate reader (Molecular Devices, Sunnyvale, CA).

LDH Release Assays for Cytolysis. Methods were followed according to the manufacturer (Roche Diagnostics). In brief, cells were cultured in 24-well dishes in 500 μl of media per well. Upon completion of the experiment, the extracellular media were removed, and the cells were lysed in the aforementioned lysis buffer (50 μl). Either 10 μl of cell lysate or 100 μl of extracellular media was analyzed for LDH activity. The results are expressed as a percentage of total LDH released, which was obtained by dividing the amount of LDH detected extracellularly by the sum total of LDH detected within the cell and the amount detected extracellularly, times 100.

Measurement of K^+ Release in Response to MTX. BAC1 macrophages on 12-well plates were incubated at 37°C in 1 ml of NaCl BSS or in modified BSS containing 0 mM $CaCl_2$ or 5 mM glycine. Cells were treated with the indicated MTX concentrations for 30 min or for the indicated times. Reactions were terminated by rapid aspiration of the extracellular medium in each well. The adherent cells in each well were then extracted overnight with 1 ml of 10% HNO_3. K^+ content in these nitric acid extracts was assayed by atomic absorbance spectrophotometry. Duplicate or triplicate wells were run for all test conditions in each separate experiment, and the measured K^+ contents were averaged.

Microscopy. BAC1 macrophages were sparsely seeded on circular glass coverslips prepared by ethanol sterilization followed by incubation in complete Dulbecco's modified Eagle's medium before the addition of cells. Cells were used within 1 to 3 days of seeding. The coverslips were mounted in temperature-controlled perfusion chambers and placed on the stage of a Leica inverted microscope (Leica, Wetzlar, Germany). All experiments were conducted in the BSS used for the IL-1β release experiments. The cells were illuminated with light from a 175-W Sutter Lambda LS lamp (Sutter Instrument Company, Novato, CA) using a Leica L5 cube. Epifluorescence was recorded using a SPOT camera (Diagnostic Instruments, Sterling Heights, MI), and images were acquired and analyzed using SimplePCI imaging software (Compix Inc., Cranberry Township, PA). During each experiment, phase and fluorescence image pairs were collected at 30-s intervals with shutter controllers switching between light and fluorescent illumination. Phase images were contrast-enhanced and digitally merged with the corresponding fluorescent images, and time-lapse videos were created using the SimplePCI software. YoPro fluorescence per cell (in arbitrary fluorescence units) was assessed using the SimplePCI software to highlight the regions of interest around the cell, whereas blebbing was assessed during visual inspection of the time-lapse movies. In certain instances, time-lapse images and movies were generated without merging fluorescence information for better resolution of phase-contrast images. All time-lapse images were analyzed as movies in which images were collected at 30-s intervals and sequenced together at seven images per second. All figures are representative data from at least three separate experiments.

Results

MTX Induces IL-1β Release from Macrophages in a Concentration-Dependent Manner. MTX has much in common with ATP acting at the P2X7R. Both activate nonselective cation channels, both trigger the formation of pores permeable to molecules <800 Da, both can facilitate cytolysis, and both induce dilated blebbing of the plasma membrane (Ferrari et al., 1997a; Schilling et al., 1999a; Estacion and Schilling, 2001; Verhoef et al., 2003). We therefore tested whether MTX could induce processing and/or release of IL-1β from macrophages similar to that elicited by the activation of the P2X7R. For all experiments, BAC1 macrophages were primed with 1 μg/ml LPS for 4 h to induce proIL-1β synthesis and initiate other proinflammatory transcriptional changes. Cells were then washed and equilibrated in minimal HEPES-BSS before the addition of MTX. Figure 1A shows immunoblot analysis of the acid-precipitated extracellular media from cells treated 30 min with increasing concentrations of MTX. The release of processed 17-kDa mIL-1β was observed with MTX concentrations as low as 0.06 nM, with increased p17 detected in response to 0.3 nM and a diminution in p17 secretion at higher concentrations; 3 nM MTX was incapable of eliciting p17 mIL-1β secretion. The release of 33-kDa unprocessed proIL-1β was detected beginning at 0.3 nM MTX and increased in a concentration-dependent manner.

Given that MTX has been shown to induce cytolysis and bleb dilation in endothelial cells, we considered the possibility that elevated concentrations of MTX compromised the capacity of macrophages to process and release mIL-1β in a regulated manner, instead leading to cytolysis and release of proIL-1β (Estacion and Schilling, 2001). To test this hypothesis, we probed the immunoblots for p38 MAPK as an intracellular marker of similar molecular mass to p33 IL-1β that might be released in the context of cytolysis. p38 MAPK was released in a MTX concentration-dependent manner, similar to that characterizing the release of 33-kDa proIL-1β (Fig. 1A). This suggested that lower MTX concentrations facilitate a regulated, nonlytic release of mIL-1β, whereas higher MTX doses induce a cytolytic release of unprocessed IL-1β. Hereafter, we refer to the release of caspase-1-processed mIL-1β as "regulated" and the release of unprocessed, immature proIL-1β as "lytic".

Figure 1B indicates the results of ELISA of samples from similar experiments in which the MTX concentration-IL-1β-release relationship was evaluated at the 30-min time point. The IL-1β ELISA antibodies have higher affinity for mIL-1β over proIL-1β and are more sensitive at detecting mIL-1β than immunoblot analysis. This ELISA therefore represents both a confirmatory and highly quantitative technique for evaluating MTX-induced IL-1β secretion. The observed concentration-response relationship was sharp and biphasic, with the small increase in MTX from 0.06 to 0.6 nM leading to a dramatic increase in mIL-1β release, which was followed by a reduced signal at progressively higher MTX concentrations. The ELISA-detectable IL-1β observed at higher concentrations of MTX represents a combination of a small amount of mIL-1β and significant amounts of proIL-1β, as indicated in Fig. 1A. Figure 1C illustrates immunoblot analysis of the cell-associated protein fraction of the experiment described in Fig. 1A. Regardless of MTX concentration, no 17-kDa mIL-1β was detected intracellularly, consistent with previous analyses of stimulus-induced IL-1β secretion (Gudipaty et al., 2003). Increasing concentrations of MTX led to a progressive reduction in the cell-associated proIL-1β and p38 MAPK, thus correlating with the increase in extracellular proIL-1β and p38 MAPK noted in Fig. 1A.

Figure 1D shows the results of LDH release assays for cytolysis induced by MTX. This assay depends on LDH as a large (>100 kDa) cytosolic marker protein that is found out-

side the cell only in the context of a loss of membrane integrity. As with p38 MAPK and p33 proIL-1β, a significant release of LDH (indicative of cytolysis) was observed at MTX concentrations >0.3 nM, with more than 75% cytolysis at 3 nM.

To initiate caspase-dependent IL-1β processing and secretion, the ATP-activated P2X7R must allow efflux of K^+ and influx of Ca^{2+} across the cell membrane (Perregaux and Gabel, 1994; Gudipaty et al., 2003; Kahlenberg and Dubyak, 2004). The ability of MTX to induce nonselective, noninactivating cation channels has been extensively described, and we confirmed the ability of MTX to trigger rapid and sustained K^+ efflux from the macrophages (Fig. 1E). At concentrations >30 pM, MTX induced 2-fold reductions in intracellular K^+ content (Fig. 1A).

Kinetic Analysis of MTX-Induced IL-1β Release and Cytolysis. Figure 2A illustrates immunoblot analysis of extracellular media from macrophages treated with either 0.06, 0.6, or 6 nM MTX for increasing times from 5 to 30 min. At 0.06 nM, MTX elicited the release of mainly mIL-1β after a >20-min lag period, whereas 0.6 nM MTX induced detectable secretion of both mIL-1β and proIL-1β within 15 min, followed by increased release of mainly proIL-1β thereafter. At 6.0 nM, MTX only induced secretion of proIL-1β, beginning at 15 min, suggesting that the cells were lysing before they were able to initiate the signal cascades that would lead to effective processing and release of mIL-1β. This was supported by the immunoblot for p38 MAPK as a marker of cytolysis, which revealed that little or no extracellular p38 MAPK was detectable at early time points of MTX treatment but that robust release of p38 MAPK was observed, coincident with the release of p33 proIL-1β. ELISA of media from cells exposed to different MTX concentrations revealed that MTX was an efficacious IL-1β secretagogue even at picomolar concentrations but that longer exposure periods were necessary to observe this effect (Fig. 2B). Furthermore, the lower concentration was more efficient in facilitating mature IL-1β release rather than proIL-1β release given that the ratio of p17 to p33 at 30 min was higher with the 0.06 nM stimulus than with the 0.6 nM stimulus. This suggests that nanomolar MTX eventually favors the release of proIL-1β rather than mIL-1β, consistent with an effect on cytolysis

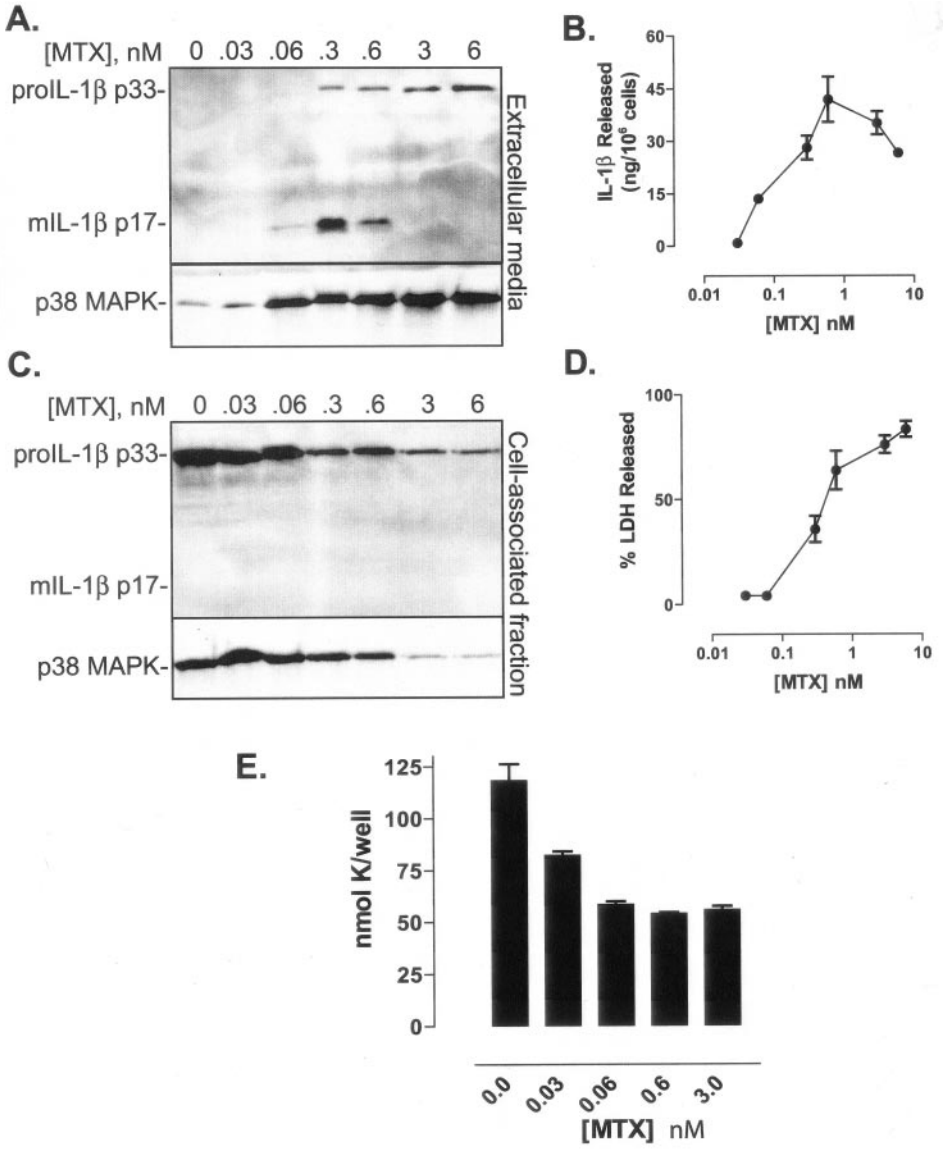

Fig. 1. MTX induces IL-1β release and K^+ efflux from LPS-primed macrophages in a concentration-dependent manner. BAC1 macrophages were plated at 1×10^6/ml, LPS-primed (1 μg/ml) for 4 h, and then transferred to standard NaCl BSS containing 130 mM NaCl, 25 mM HEPES, pH 7.5, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 0.01% BSA. In the case of immunoblot analysis, the results represent experiments performed at least three times, whereas ELISA/LDH data were performed at least twice, in triplicate. A, macrophages stimulated with the indicated concentrations of MTX for 30 min. The extracellular media were collected and analyzed by immunoblot using an antibody that detects both unprocessed 33-kDa proIL-1β and the 17-kDa mIL-1β or p38 MAPK as a marker of cytolysis. B, extracellular media from experiments similar to those described in A quantitatively analyzed for IL-β content using an ELISA with higher avidity for mIL-1β over proIL-1β. C, cell-associated protein fractions from A analyzed for proIL-1β, mIL-1β, or p38 MAPK content by immunoblot. D, samples from the experiment described in B assayed for LDH release as a separate marker of cytolysis. E, cells treated with the indicated concentrations of MTX for 30 min and then analyzed for the loss of intracellular K^+ using atomic absorbance spectrophotometry.

rather than regulated secretion of IL-1β. To further support this possibility, we analyzed the extracellular media for released LDH and found that 0.06 nM MTX did not elicit LDH release at any time point examined. At 0.6 nM, MTX induced significant LDH release by 15 min and cytolysis of 25 to 50% of cells by 30 min (Figs. 2C and 1D). At 6.0 nM, MTX triggered LDH loss even at 5 min and cytolysis of >80% of the cell population by 30 min (Figs. 2C and 1D). These time courses paralleled the release of proIL-1β illustrated in the Fig. 2A immunoblots.

MTX-Induced Release of IL-1β but Not Cytolysis Is Blocked by Elevated Extracellular K^+. Perregaux and Gabel (1994) have demonstrated that the incubation of LPS-primed macrophages in saline containing KCl rather than NaCl as the predominant extracellular salt potently inhibits ATP-induced or nigericin-induced IL-1β secretion. We have previously demonstrated that ATP stimulation of BAC1 macrophages in KCl-BSS does not induce proteolytic activation of caspase-1 and that K^+ efflux through the P2X7R specifically activates signal transduction pathways leading to stable caspase-1 activity (Gudipaty et al., 2003; Kahlenberg and Dubyak, 2004). To test whether a similar pathway was activated by MTX, we compared MTX concentration-response relationships using LPS-primed macrophages bathed in standard BSS or in KCl-BSS, in which the 130 mM NaCl was replaced by equimolar KCl. Figure 3A shows immunoblot analysis of the acid-precipitated extracellular media from such an experiment in which LPS-treated macrophages were subjected to increasing concentrations of MTX for 30 min in KCl-BSS. No processing of proIL-1β to mIL-1β was initiated at the lower MTX concentrations; therefore, no release of mature 17-kDa IL-1β was evident. In contrast, the MTX-induced release of unprocessed proIL-1β was intact and comparable to that observed in NaCl-BSS (Fig. 1A). These results imply that MTX is capable of facilitating the K^+ efflux necessary for stable activation of caspase-1 and initiation of the downstream IL-1β secretion machinery (i.e., regulated secretion as opposed to lytic release) and that K^+ efflux is not required for cytolysis. To further quantify the effect of elevated extracellular K^+ on MTX-induced IL-1β release, we compared the ELISA-detectable IL-1β from MTX concentration-response experiments conducted in KCl-BSS with those conducted in standard BSS (same data as Fig. 1B). Figure 3B shows that the amount of ELISA-detectable IL-1β released was markedly reduced and the concentration-response relationship was not biphasic, because the IL-1β detected is only proIL-1β rather than a mix of proIL-1β and mIL-1β. That MTX-dependent cytolysis was responsible for proIL-1β release observed in KCl-BSS was supported by the analysis of LDH release in Fig. 3C. MTX induced release of LDH at concentrations as low as 0.3 nM; >1 nM MTX elicited lysis of 75 to 80% of the macrophage population within 30 min (Fig. 3C). These results support the idea that MTX-dependent IL-1β release is characterized by two distinct components, regulated and lytic, as previously suggested. Moreover, the regulated secretion of processed mIL-1β, but not the lytic release of proIL-1β, can be blocked with elevated extracellular K^+.

We further compared the effects of elevated extracellular K^+ on MTX-induced externalization of IL-1β versus LDH by conducting kinetic experiments using increasing concentrations of MTX (0.06, 0.6, and 6.0). Compared with the findings with control cells (i.e., in NaCl-BSS) in Fig. 2B, LPS-primed macrophages bathed in KCl-BSS released much less ELISA-detectable IL-1β at all timepoints in response to these concentrations of MTX (Fig. 3D). The modest IL-1β release noted with 6 nM MTX was caused by the release of proIL-1β as indicated in the immunoblot analyses in Fig. 3A. In contrast to its ability to markedly attenuate the rate of ELISA-detectable IL-1β release, the high extracellular K^+ concentration did not diminish the rates of MTX-induced cytolysis, as indicated by the accumulation of extracellular LDH (Fig. 3E compared with Fig. 2C). The release of LDH nearly parallels

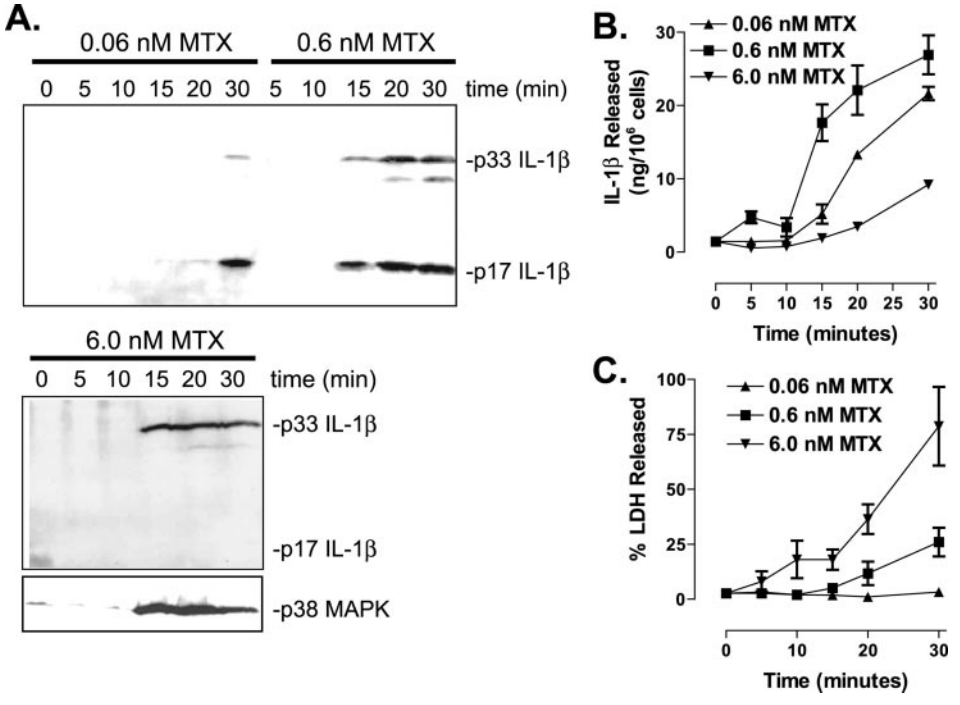

Fig. 2. Kinetics of MTX-induced IL-1β secretion from murine macrophages. BAC1 macrophages were plated at 1×10^6/ml, LPS-primed (1 μg/ml) for 4 h, and then transferred to standard NaCl BSS containing 130 mM NaCl, 25 mM HEPES, pH 7.5, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 0.01% BSA. In the case of immunoblot analysis, the results represent experiments performed at least three times, whereas ELISA/LDH data were performed at least twice, in triplicate. All comparison experiments were paired such that they were conducted using different wells in the same plate of cells treated under different conditions on the same day. A, extracellular media from macrophages exposed to either 0.06, 0.6, or 6.0 nM MTX for the indicated times analyzed by immunoblot for proIL-1β and mIL-1β release. B, samples from the experiment described in A analyzed for IL-1β release evoked by 0.06 (▲), 0.6 (■), or 6.0 nM (▼) MTX by ELISA, as described in Fig. 1. C, samples from the experiment described in A analyzed for LDH release evoked by 0.06 (▲), 0.6 (■), or 6.0 nM (▼) MTX, as described in Fig. 1.

the release of IL-1β indicated in Fig. 3D, again suggesting that the cytokine is being released by cytolysis.

MTX Effects on Regulated and Lytic Release of IL-1β Require Extracellular Ca^{2+}. We and others have shown that the rapid release of processed IL-1β is Ca^{2+}-dependent (Gudipaty et al., 2003). MTX-regulated cation channels are both Ca^{2+}-permeable and Ca^{2+}-regulated given previous reports that state that the ability of MTX to trigger its target nonselective cation channels requires extracellular Ca^{2+} (Martinez-Francois et al., 2002; Wisnoskey et al., 2004). This presumably reflects an allosteric effect of extracellular Ca^{2+} on the binding of MTX to these channels. We therefore tested how the removal of extracellular Ca^{2+} from the media would affect the MTX-dependent release of IL-1β by either the regulated or lytic mechanisms. As shown in Fig. 4A, concentrations of MTX as high as 3 nM were unable to elicit IL-1β release from the cells in the absence of extracellular Ca^{2+}, whereas at 6 nM, MTX induced modest release (compare with control data in Fig. 1B). Likewise, Fig. 4B indicates that MTX only induced modest cytolysis (as indicated by LDH release) at the highest concentration tested, 6.0 nM. We also tested the ability of MTX to trigger K^+ release by incubating the cells with either 0.3 or 3.0 nM MTX in Ca^{2+}-free NaCl-BSS (Fig. 4C). Under these conditions, MTX induced little K^+ efflux compared with control data in Fig. 1E, indicating that MTX was unable to effectively perturb ionic homeostasis in the absence of extracellular Ca^{2+}.

Glycine Blocks MTX-Induced Cytolysis but Not the Regulated Release of mIL-1β. Glycine has been extensively studied as a cytoprotectant acting by an unknown mechanism to attenuate cytolysis induced by a wide range of pronecrotic stimuli (Zhong et al., 2003). We have recently observed that glycine protects against both MTX-induced cytolysis of endothelial cells and P2X7R-dependent cytolysis of macrophages (P. A. Verhoef and G. R. Dubyak, unpublished observations; Estacion et al., 2003). We therefore tested whether glycine might block the lytic release of IL-1β induced by MTX and whether this blockade would be coincident with cytoprotection as measured by the attenuation of LDH release. We tested the concentration dependence of MTX-induced IL-1β secretion and cytolysis in BAC1 macrophages incubated in the presence of 5 mM glycine. Figure 5A shows the anti-IL-1β immunoblot of the extracellular media from cells treated with increasing concentrations of MTX for 30 min in the presence of 5 mM glycine. In the presence of glycine, macrophages were still able to process and release mIL-1, but no extracellular proIL-1β was detected at any concentration of MTX tested (Fig. 1A). The detection of p38 MAPK released into the extracellular media was also markedly reduced but not eliminated in the presence of glycine.

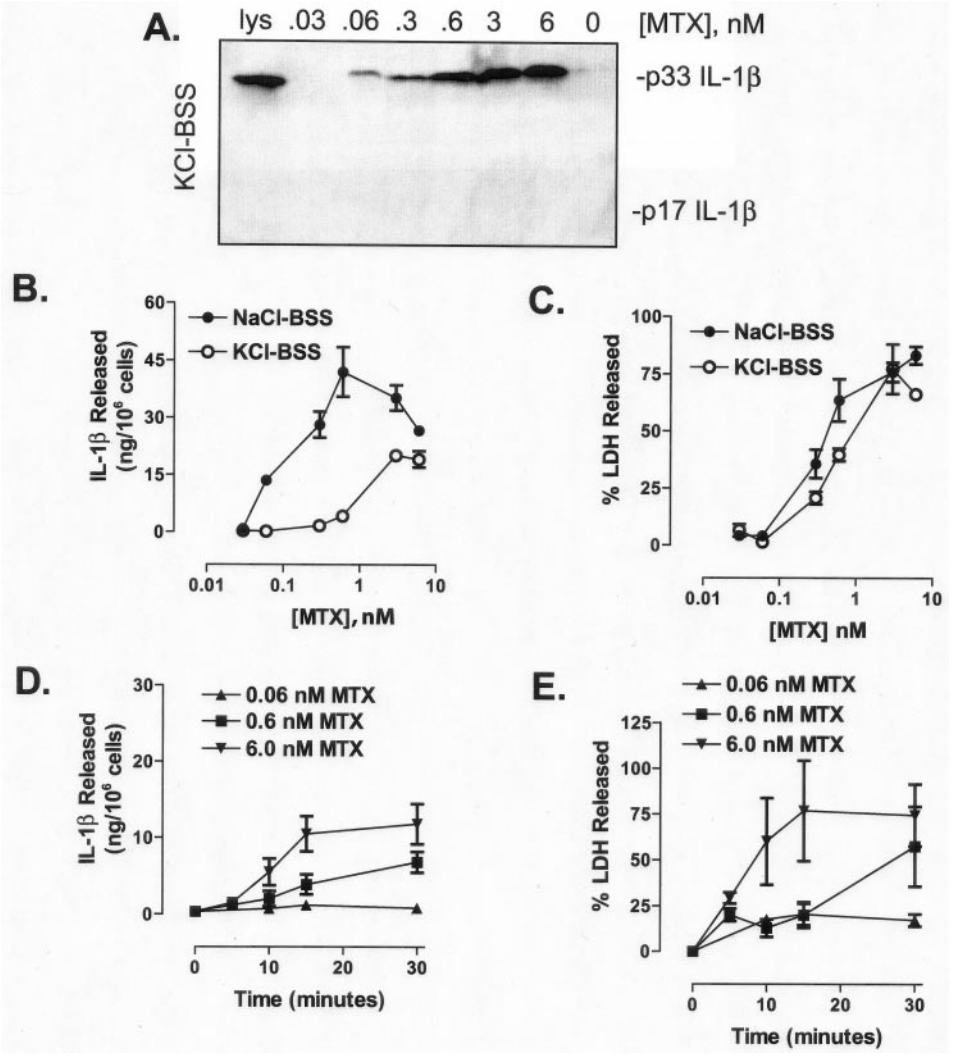

Fig. 3. MTX-induced release of mIL-1β, but not cytolysis, is blocked by elevated extracellular K^+. BAC1 macrophages were plated at 1×10^6/ml, LPS-primed (1 μg/ml) for 4 h, and then transferred to the standard NaCl-BSS or KCl-BSS with 130 mM KCl substituted for 130 mM NaCl. In the case of immunoblot analysis, the results represent experiments performed at least three times, whereas ELISA/LDH data were repeated at least twice, in triplicate. All comparison experiments were paired such that they were conducted using different wells in the same plate of cells treated under different conditions on the same day. A, macrophages stimulated with the indicated concentrations of MTX for 30 min in KCl-BSS that lacks extracellular Na^+. The extracellular media were collected and analyzed by immunoblot using an antibody that detects both unprocessed 33-kDa proIL-1β and the 17-kDa mIL-1β. The initial lane labeled as "lys" shows the total immunoreactive IL-1β content of corresponding whole-cell lysates. B, extracellular media samples from cells treated with the indicated MTX concentration for 30 min in KCl-BSS (○) analyzed for mIL-1β release by ELISA to determine whether the substitution of KCl for NaCl affected MTX-induced IL-1β secretion. For comparison, data from the equivalent experiment shown in Fig. 1B conducted with NaCl-BSS (●) are replotted here. C, extracellular media samples [KCl-BSS (○) versus NaCl-BSS (●)] from the IL-1β release experiments illustrated in B additionally analyzed for LDH release. D, extracellular media from macrophages exposed either to 0.06 (▲), 0.6 (■), or 6.0 nM (▼) MTX in KCl-BSS for the indicated times analyzed by ELISA for IL-1β release. E, samples (from the experiment described in D) analyzed for LDH release evoked by 0.06 (▲), 0.6 (■), or 6.0 nM (▼) MTX.

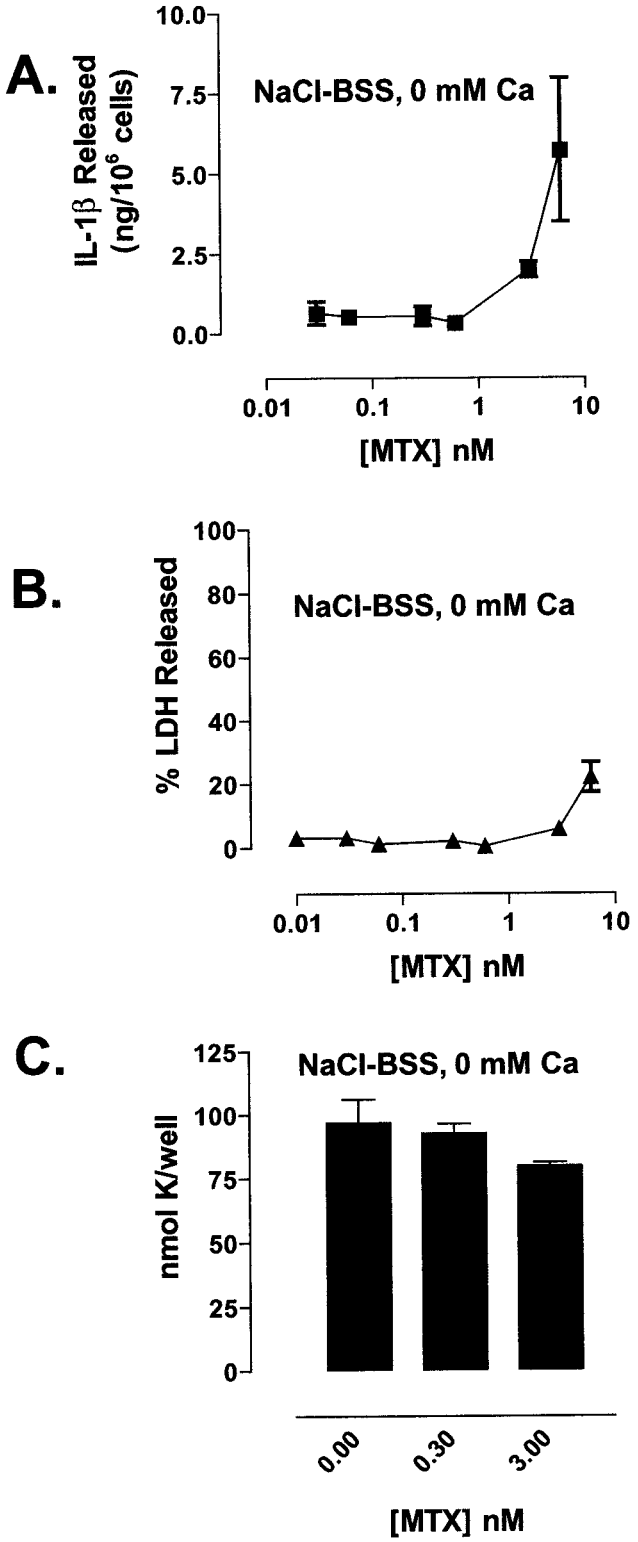

Fig. 4. The removal of extracellular Ca^{2+} blocks MTX-induced K^+ release, IL-1β secretion, and cytolysis. BAC1 macrophages were plated at 1×10^6/ml, LPS-primed (1 μg/ml) for 4 h, and then transferred to Ca^{2+}-free NaCl-BSS media (with 0 mM $CaCl_2$ and 1 mM $MgCl_2$). The experiments were performed twice in triplicate. A, macrophages stimulated with the indicated concentrations of MTX for 30 min in Ca^{2+}-free NaCl-BSS. The extracellular media were collected and analyzed by ELISA. B, samples from the experiment described in A analyzed for LDH release. C, cells bathed in Ca^{2+}-free-NaCl-BSS, treated with the indicated concentrations of MTX for 30 min, and then analyzed for the loss of intracellular K^+ using atomic absorbance spectrophotometry.

ELISA analysis of the extracellular media from a similar experiment (Fig. 5B) indicated that the initial phase of the [MTX]IL-1β release relationship (<0.6 nM) was similar to that observed in the absence of glycine (Fig. 1B); in contrast, release was significantly reduced at concentrations of >0.6 nM MTX (Fig. 5B). These results strongly suggest that the IL-1β normally detected by ELISA at higher MTX concentrations in the absence of glycine is due largely to proIL-1β released by a glycine-sensitive cytolytic pathway. Figure 5C illustrates the anti-IL-1β immunoblot analysis of the cell-associated protein fraction corresponding to the extracellular samples in Fig. 5A. No mIL-1β was detected in these glycine-treated cells, and most of the intracellular proIL-1β was retained even during stimulation with 6 nM MTX, in contrast to the control experiments shown in Fig. 1C conducted in the absence of glycine. The amount of p38 MAPK within the cells also remained largely constant, indicating that glycine protected macrophages against the lytic release of both proIL-1β and p38 MAPK. This was further supported by the assay of extracellular LDH levels that revealed that glycine blocked nearly all MTX-induced LDH release over the entire range of tested MTX concentrations (Fig. 5D). We also tested the ability of MTX to induce K^+ release in the presence or absence of glycine and found that 5 mM extracellular glycine did not affect the MTX-induced cation fluxes (Fig. 5E).

Kinetic Analysis of MTX-Induced IL-1β Release and Cytolysis in the Presence of Extracellular Glycine. Immunoblot analysis of extracellular media from a kinetic study of IL-1β release in the presence of 5 mM glycine indicated that glycine markedly reduced the release of 33-kDa proIL-1β induced by 0.06, 0.6, or 6.0 nM MTX at all time points examined (compare Figs. 6A and 2A). In contrast, the time courses of MTX-induced release of mature IL-1β were not significantly reduced at any concentration, compared with the glycine-free conditions. The results of ELISA analysis of an experiment similar to that conducted for Fig. 6A are shown in Fig. 6B. As indicated, 0.06 nM MTX elicited the release of some mIL-1β (consistent with Fig. 6A) over time, whereas 0.6 nM MTX induced robust mIL-1β secretion, notable at the 20-min timepoint. At 6.0 nM, MTX induced a smaller amount of IL-1β release relative to that elicited with 0.6 nM; this IL-1β signal mainly reflected mIL-1β rather than proIL-1β, given the immunoblot analysis in Fig. 6A. Analysis of the extracellular media from the experiment shown in Fig. 6B revealed no release of LDH at any time during exposure to 0.06 or 0.6 nM MTX and only minor release at 30 min for 6.0 nM MTX in the presence of glycine (Fig. 6C). These results demonstrate that the lytic phase of MTX-induced IL-1β release can be effectively blocked by glycine without affecting the regulated secretion of proteolytically processed mIL-1β.

MTX Treatment of Macrophages Induces Dilated Blebbing Followed by Abrupt Influx of Propidium Dye. MTX has recently been shown to induce the formation of large dilated blebs in endothelial cells, but the effect of MTX on macrophage membrane dynamics has not been evaluated. Given our previous studies that examined rapid changes in macrophage morphology in the context of ATP-stimulated IL-1β release, we used similar time-lapse videography to compare the time-dependent effects of MTX on membrane dynamics and permeability in individual macrophages (MacKenzie et al., 2001; Verhoef et al., 2003). BAC1

macrophages were plated on coverslips for microscopy, equilibrated in standard NaCl-BSS, and then stimulated with 0.3 nM MTX at the 5-min time-point in the video recordings. These experiments (and all subsequent microscopy experiments) were conducted at 37°C using BSS supplemented with 200 nM YoPro, a propidium dye that enters cells in response to MTX-dependent pore formation and produces intense fluorescence upon binding intracellular RNA and DNA. MTX was used at a concentration of 0.3 nM because this concentration induced both regulated and cytolytic release of IL-1β (Figs. 1 and 3). The initial response of macrophages to MTX was decreased motility that was followed, after a 5-min lag, by a gradual and near-linear accumulation of YoPro over the next 6 to 7 min (Fig. 7 and the movie in supplemental data S1). At ~13 min after the addition of MTX, some cells began to extrude large, dilated blebs and initiate a second and much more rapid phase of YoPro fluorescence indicative of an abrupt and dramatic increase in dye uptake and labeling of nuclear DNA (see supplemental data S1). By 30 min after MTX exposure, all macrophages within the visual field had formed dilated blebs and progressed to the abrupt phase of YoPro influx. The second, rapid phase of YoPro uptake was temporally correlated with LDH release and presumably reflects lysis at the single cell level. Quantification of four separate experiments revealed that 281/281 cells demonstrated membrane blebbing and biphasic dye uptake. The increase in YoPro fluorescence of individual cells was monitored and is indicated in the final panel of Fig. 7 as a function of time. Although all cells eventually demonstrated membrane blebbing and biphasic dye uptake, there was broad heterogeneity on an individual cell basis with regard to the timing and initiation of bleb formation and the second, abrupt phase of YoPro influx.

Glycine Blocks MTX-Induced Dilated Blebbing and the Abrupt Phase of YoPro Influx. We next tested the effect of glycine on MTX-induced dilated blebbing and YoPro dye uptake in macrophages. BAC1 macrophages were equilibrated in BSS containing 5 mM glycine before stimulation

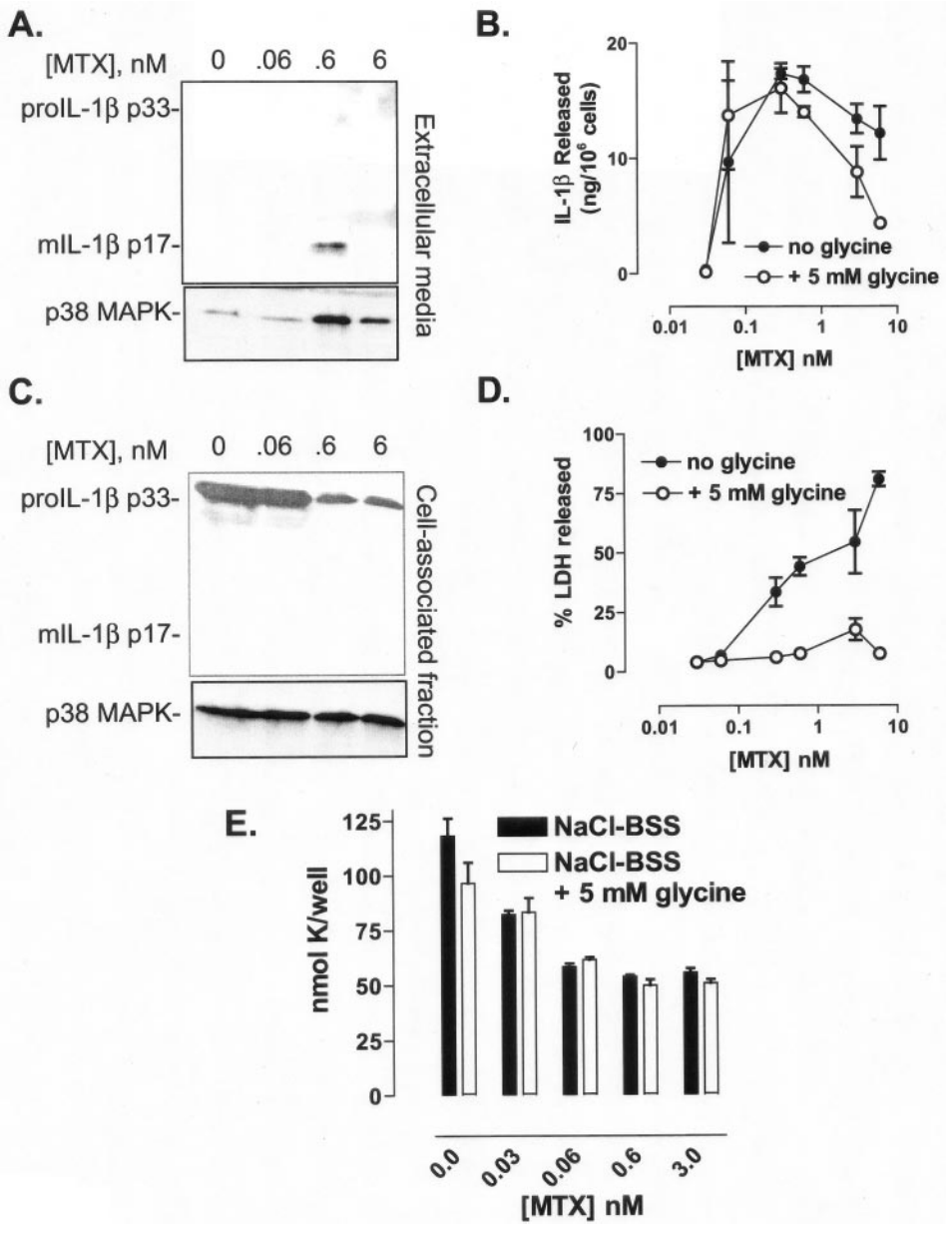

Fig. 5. Glycine does not affect MTX-induced IL-1β-regulated secretion or MTX-induced K^+ release but blocks MTX-induced cytolysis. BAC1 macrophages were plated at 1×10^6/ml, LPS-primed (1 μg/ml) for 4 h, and then transferred to the standard NaCl-BSS supplemented with or without 5 mM glycine. In the case of immunoblot analysis, the results represent experiments performed at least three times, whereas ELISA/LDH studies were performed at least twice, in triplicate. All comparison experiments were paired such that they were conducted using different wells in the same plate of cells treated under different conditions on the same day. A, macrophages stimulated with the indicated concentrations of MTX in the presence of 5 mM glycine for 30 min. The extracellular media were collected and analyzed by immunoblot using an antibody that detects both unprocessed 33-kDa proIL-1β and the 17-kDa mIL-1β. The extracellular media were also analyzed for p38 MAPK. B, macrophages stimulated with the indicated concentrations of MTX in the presence (○) or absence (●) of 5 mM glycine for 30 min. Extracellular media samples were collected and quantitatively analyzed for IL-β content using an ELISA with higher avidity for mIL-1β over proIL-1β. C, cell-associated protein fractions from A analyzed for proIL-1β, mIL-1β, and p38 MAPK content by immunoblot. D, samples from the experiments described in B additionally assayed for LDH release as a separate marker of MTX-induced cytolysis in the presence (○) or absence (●) of 5 mM glycine. E, cells treated with the indicated concentrations of MTX in the presence or absence of glycine for 30 min and then analyzed for the loss of intracellular K^+ using atomic absorbance spectrophotometry.

with 0.3 nM MTX. Glycine blocked both the bleb dilation and the second, cytolytic phase of YoPro uptake (Fig. 8 and supplemental data S2). Quantification of four separate experiments revealed that 0/251 cells exhibited bleb dilation and the abrupt phase of dye uptake even when the MTX exposure was extended to more than an hour. In contrast, the slow YoPro influx (indicative of macropore formation) elicited by MTX was not inhibited by glycine. Control experiments of YoPro uptake by macrophages incubated for >60 min in the absence of MTX, but with glycine, showed that this slow dye influx was dependent on MTX rather than steady-state uptake because of basal permeability or pinocytosis (data not shown).

Discussion

Maitotoxin is an important component of ciguatera (shellfish) seafood poisoning with a remarkable potency, inducing effects at subnanomolar concentrations (Takahashi et al., 1982). Although it has been largely used as a tool to study Ca^{2+}-dependent cellular functions (Gusovsky and Daly, 1990), it has also recently been shown to facilitate the recruitment/activation of a nonselective pore, membrane blebbing, and cytolysis (Estacion and Schilling, 2001). Because these are all functions induced by many of the known stimuli for IL-1β secretion (which itself remains poorly understood), we tested the effects of MTX on IL-1β processing and release from murine macrophages. We found that MTX stimulated the release of IL-1β from macrophages with two distinct phases. At picomolar concentrations (~0.06 nM), MTX induced a regulated release of processed, mature 17-kDa IL-1β

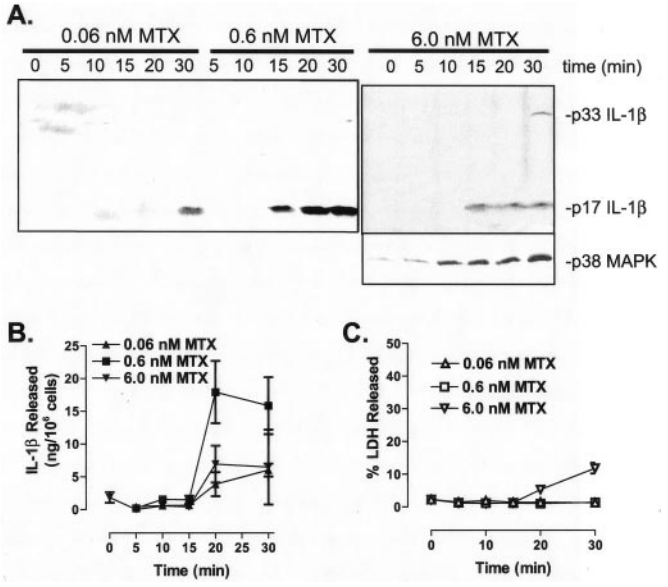

Fig. 6. Effects of glycine on time courses of MTX-induced IL-1β release and cytolysis. BAC1 macrophages were plated at 1×10^6/ml, LPS-primed (1 μg/ml) for 4 h, and then transferred to the standard NaCl-BSS supplemented with or without 5 mM glycine. In the case of immunoblot analysis, the results represent experiments performed at least three times, whereas ELISA/LDH studies were performed at least twice, in triplicate. All comparison experiments were paired such that they were conducted using different wells in the same plate of cells treated under different conditions on the same day. A, extracellular media from macrophages exposed to either 0.06, 0.6 nM, or 6.0 nM MTX in the presence of 5 mM glycine for the indicated times analyzed by immunoblot for proIL-1β, mIL-1β release, or p38 MAPK (for 6.0 nM MTX). B, samples from the experiment described in A analyzed for IL-1β release evoked by 0.06 (▲), 0.6 (■), or 6.0 nM (▼) MTX by ELISA, as described in Fig. 1. C, extracellular media samples and cell lysates from the experiment described in E analyzed for LDH release evoked by 0.06 (△), 0.6 (□), or 6.0 nM (▽) MTX in the presence of 5 mM glycine.

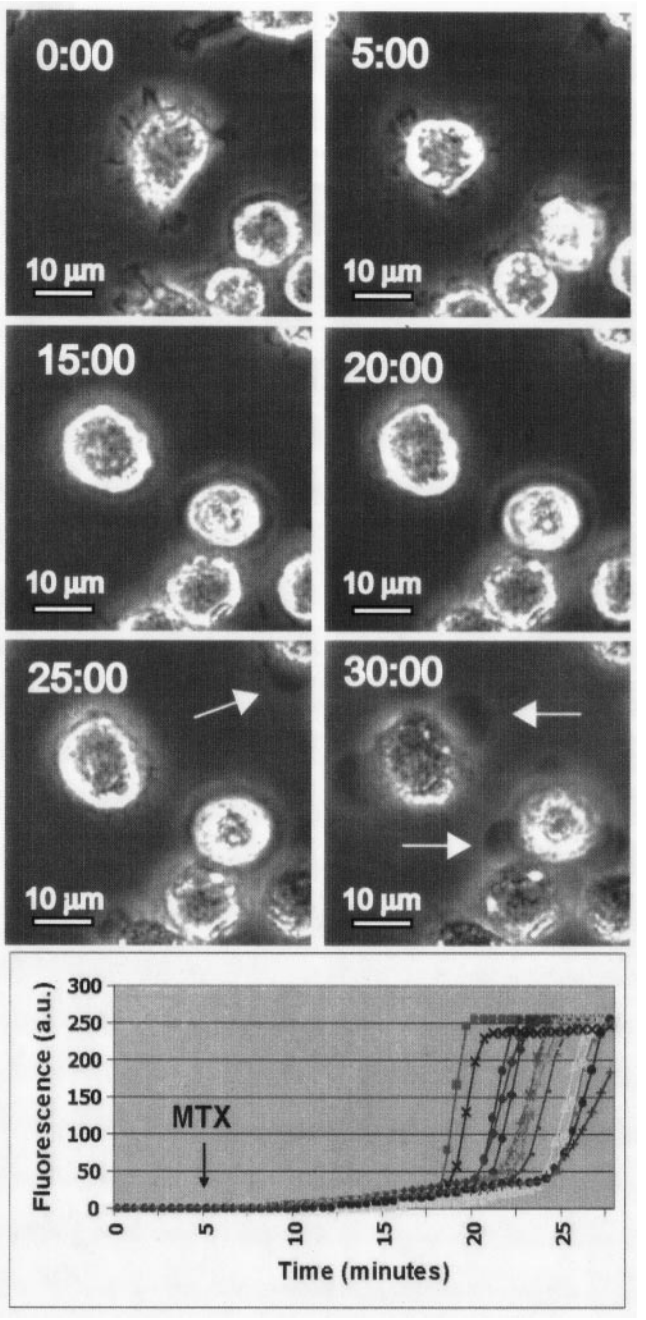

Fig. 7. MTX induces large dilated blebs and biphasic YoPro dye uptake in macrophages. BAC1 macrophages were cultured on glass coverslips as described under *Materials and Methods*, bathed in NaCl-BSS containing 200 nM YoPro at 37°C, and exposed to 0.3 nM MTX at the 5-min time point of video recording. Phase-contrast and fluorescence images were continuously recorded during the 30-min incubation with MTX (see movie in supplemental data S1); selected phase-contrast images at the indicated times are shown. Large dilated blebs (indicated by arrows) can be observed in some cells at the 25-min time point of video recording, and these blebs show massive YoPro fluorescence in the merged images (supplemental data S1). The bottom illustrates the time-dependent changes in the magnitude of YoPro fluorescence at the single cell level using 12 randomly selected macrophages in the visual field. Note the biphasic time course of dye uptake characterized by slow increases in fluorescence during the initial 12 to 20 min after exposure to MTX followed by an abrupt and very rapid increase that correlates with rapid bleb dilation (supplemental data S1).

by a mechanism sensitive to blockade by elevated extracellular K^+. MTX required extracellular Ca^{2+} to elicit its effects, because the removal of extracellular Ca^{2+} abrogated the ability of MTX to induce either regulated or lytic IL-1β release. At nanomolar (>0.6 nM) concentrations, MTX initiated the lytic release of predominantly unprocessed 33-kDa proIL-1β by a mechanism sensitive to inhibition by the cytoprotectant glycine. We also observed that MTX induced dramatic membrane blebbing and biphasic uptake of YoPro dye in these macrophages, initially via the nonselective pore and later by a lytic collapse of membrane integrity. This blebbing and the second, cytolytic phase of dye uptake were completely blocked by glycine. In contrast, glycine did not block the ability of subnanomlar MTX to induce the caspase-1-dependent processing and secretion of mature IL-1β.

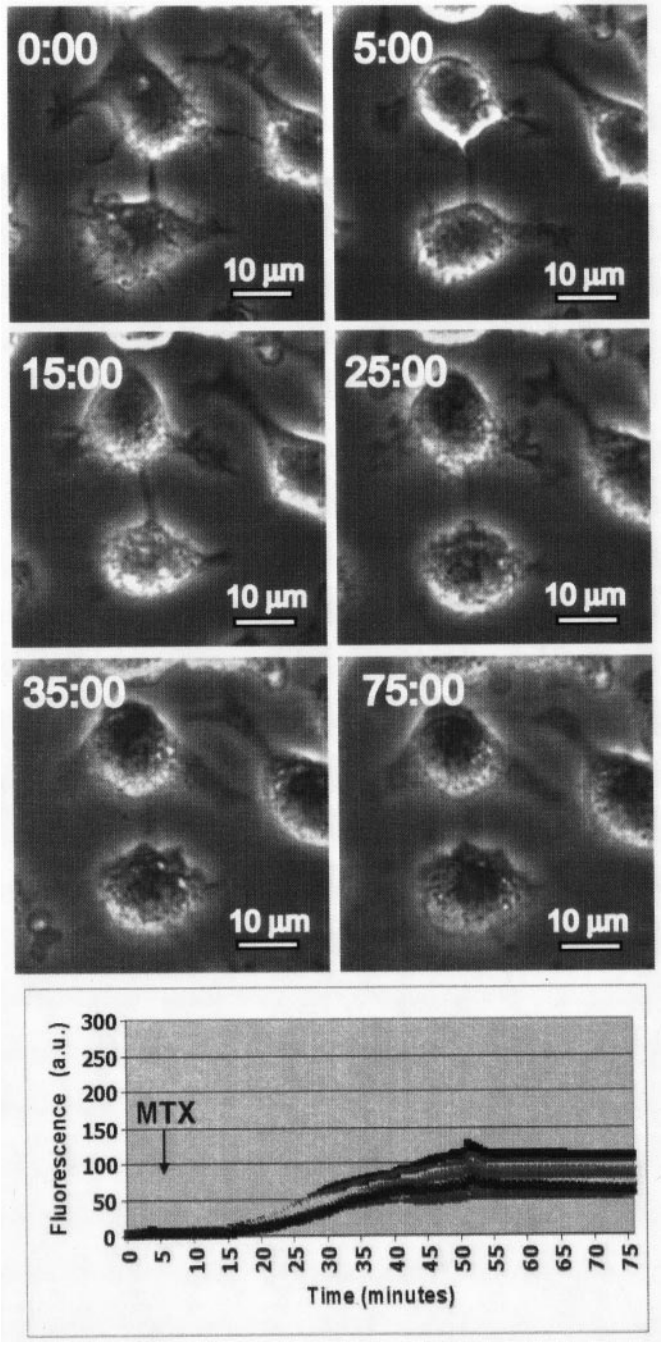

Fig. 8. Glycine blocks MTX-induced dilated blebbing and the abrupt phase of YoPro influx in macrophages. BAC1 macrophages were cultured on glass coverslips as described under *Materials and Methods*, bathed in NaCl-BSS containing 5 mM glycine and 200 nM YoPro at 37°C, and exposed to 0.3 nM MTX at the 5-min time point of video recording. Phase-contrast and fluorescence images were continuously recorded during the 80-min incubation with MTX (see movie in supplemental data S2); selected phase-contrast images at the indicated times are shown. Inspection of individual macrophages revealed no dilated blebbing even at the longest MTX exposure times. The bottom illustrates the time-dependent changes in the magnitude of YoPro fluorescence at the single cell level using 12 randomly selected macrophages in the visual field. Note that the increase in YoPro fluorescence is monophasic, rather than biphasic, indicating that glycine blocked the second phase of dye uptake that temporally correlates with dilated blebbing and cytolysis.

The observation that MTX can induce significant secretion of mIL-1β within 30 min has important implications for understanding the pathogenesis of ciguatera food poisoning. Although it is believed that the initial effects of MTX destroy target tissues because of Ca^{2+} overload, the progression to death via septic shock may additionally involve a hypersecretion of proinflammatory cytokines that, in turn, affects vascular endothelial and immune cell function (Legrand et al., 1982; Terao et al., 1988, 1989). In other experiments (data not shown), we found that MTX-induced IL-1β release was blocked by the tyrphostin inhibitor AG126 in addition to being blocked by elevated extracellular K^+. That MTX-induced processing of IL-1β is sensitive to previously identified inhibitors of IL-1β secretion indicates that MTX can initiate highly regulated signal transduction cascades leading to caspase-1 activation and subsequent proteolytic maturation of IL-1β (Martinon et al., 2002; Kahlenberg and Dubyak, 2004). These results also suggest that MTX may be capable of stimulating the Ca^{2+}-dependent microvesiculation that has been proposed as the mechanism by which IL-1β is rapidly secreted, because the removal of Ca^{2+} dramatically attenuates MTX-induced IL-1β secretion (MacKenzie et al., 2001). By joining the growing list of toxins, receptor agonists, and stress conditions capable of inducing IL-1β release from monocyte/macrophages, MTX represents both an in vitro tool for investigating IL-1β release mechanisms and a possible in vivo stimulus of clinical relevance. It remains to be determined whether MTX injection or ciguatera food poisoning might lead to an increase in either circulating or tissue-limited IL-1β and whether this could be pharmacologically modulated with anti-IL-1β therapy.

The role for glycine as an anti-inflammatory and cytoprotective therapy has generated significant interest. Exposure of diverse tissues to glycine at millimolar concentrations has been shown to elicit a wide range of protective responses, including increased secretion of anti-inflammatory cytokines, blunted secretion of proinflammatory cytokines, and attenuation of ischemia/reperfusion damage and/or cold-storage injury to organs intended for transplant (Moran and Schnellmann, 1997; Wheeler et al., 2000; Zhong et al., 2003). Our results indicate that glycine may reduce the proinflammatory cytolysis of MTX-exposed target tissues and additionally attenuate the release of proinflammatory IL-1β by macrophages into extracellular compartments. Dietary supplementation to achieve therapeutic levels of glycine for cytoprotection is inexpensive and simple and therefore represents a possible therapy for diseases involving proinflammatory cytolysis leading to the hypersecretion of IL-1β (Wheeler et al., 2000).

Regardless of its possible clinical utility, glycine may prove to be a valuable tool for investigating nonclassic mechanisms

of protein export from cells. Several important cytokines and growth factors, including IL-1β, IL-18, and basic fibroblast growth factor, lack conventional signal sequences for accumulation and processing within the endoplasmic reticulum and therefore are not secreted by classic exocytotic pathways (Dinarello, 1996). For example, mature IL-1β is generated by caspase-1-mediated processing reactions within the cytosolic compartment. Three nonclassic mechanisms have been proposed to explain the export of this cytosolic pool of mature IL-1β to the extracellular compartments of inflamed tissues (Bhakdi et al., 1990; MacKenzie et al., 2001; Le Feuvre et al., 2002; Verhoef et al., 2003): 1) facilitated efflux across the plasma membrane through peptide-recognizing transport proteins, 2) inclusion of mature IL-1β within microvesicles that evaginate from the plasma membrane before release by regulated scission, and 3) cytolysis of the inflammatory macrophage after completion of the cytosolic IL-1β processing reactions. Our experiments with MTX plus glycine unequivocally demonstrate that the nonclassic secretion of mature IL-1β can be mechanistically dissociated from cytolysis.

The use of p38 MAPK as an indicator of cytolysis also raised some interesting issues regarding the mechanism by which IL-1β might be released from the MTX-treated macrophages. In general, p38 MAPK release paralleled LDH release in the context of cytolysis (Figs. 1 and 2). However, even in the presence of glycine, which prevented LDH release, p38 MAPK could be detected in parallel with mIL-1β in the extracellular media from MTX-treated macrophages (Fig. 5A). This suggests two possibilities. First, p38 MAPK may play a role in regulated IL-1β processing signals and thus be spatially colocalized with IL-1β and packaged with the cytokine into a microvesiculation-based export machinery (MacKenzie et al., 2001). We have observed that other cytosolic proteins, including caspase-1 (Kahlenberg and Dubyak, 2004) and caspase-11 (data not shown) are coordinately released with mature IL-1β from activated macrophages. On the other hand, p38 MAPK may be packaged serendipitously into the IL-1β export machinery. In either case, the contents of secreted microvesicles may reflect a variety of intracellular cytosolic proteins.

The signal transduction events initiated by MTX treatment of target cells are thought to proceed as follows. First, MTX interacts with its as yet undefined receptor on the membrane in a Ca^{2+}-dependent fashion, leading to the activation of a CaNSC that may involve members of the transient receptor potential (TRP) channel family (Chen and Barritt, 2003). The activation of such CaNSC will not only trigger increases in cytosolic Ca^{2+} and Na^{+}, it will also trigger decreased intracellular K^{+} (Martinez-Francois et al., 2002). These rapid changes in ionic flux are followed closely by the opening of the COP pathway, which can be monitored using dyes such as YoPro. During the induction of COP, MTX-induced membrane blebs can be detected, measuring 3 to 5 μm in diameter (Estacion and Schilling, 2001). Last, cells progress to cytolysis, characterized by the dramatic expansion of the membrane blebs, LDH release, and a second, abrupt phase of dye uptake. Glycine blocks this second phase of dye uptake, LDH release, and cytolysis, although the specific target of glycine inhibition remains unknown (Estacion et al., 2003). The present study provides the first documentation of the response of macrophages to MTX. Upon treatment with MTX, macrophages initiate the characteristic increase in Ca^{2+} (P. A. Verhoef and G. R. Dubyak, unpublished observations) and the induction of COP, as monitored by YoPro uptake; however, MTX-induced membrane blebs were not detected during this phase. Rather, the formation and expansion of dilated blebs was observed immediately before the second, rapid phase of dye uptake. These results suggest that the inhibitory effect of glycine on MTX-dependent cytolysis is conserved across cell types but that MTX-dependent blebbing is not necessary for regulated release of mIL-1β, because mIL-1β secretion was intact in the presence of glycine concentrations sufficient to prevent blebbing and cytolysis.

MTX-dependent blebbing per se represents an intriguing phenomenon. All cell types examined thus far undergo massive blebbing in response to MTX, even though the time course of bleb formation and its sensitivity to inhibition by glycine may vary. In contrast to our previous findings with endothelial cells, we were unable to detect the formation of smaller, MTX-dependent blebs in macrophages; however, we clearly observed bleb dilation similar to that which is correlated with MTX-induced cytolysis of endothelial cells. This suggests that the formation of smaller, visible blebs in response to MTX in macrophages may occur immediately before bleb dilation rather than over the much longer time course characterized in endothelial cells, whereby small blebs were evident for 5 to 10 min before cytolytic dilation. Our observation that MTX induced blebs in macrophages bathed in high K^{+} medium (data not shown) suggests that bleb expansion may not simply reflect passive influx of water (secondary to Na^{+} influx) and subsequent osmotic lysis; however, the possibility remains that Cl^{-} influx generates the necessary osmotic solute drive. The functional role of membrane blebbing remains unclear, but it is evident that multiple types of blebbing can be induced and that these modes of blebbing are differentially regulated (Hagmann et al., 1999; Sebbagh et al., 2001; Inbal et al., 2002; Verhoef et al., 2003).

Our analysis of membrane blebbing and dye uptake by individual macrophages during MTX exposure revealed a striking heterogeneity in temporal response that will be "averaged out" by our biochemical assays (e.g., immunoblots, ELISA, and LDH release) of MTX-induced responses in macrophage populations. This temporal heterogeneity of response at the single cell level (Figs. 7 and 8) can explain the biochemical data showing that MTX induces both regulated mIL-1β release and lytic proIL-1β release in macrophage cultures (Figs. 1–6). The experiment shown in Fig. 7 revealed that, at the 20-min time point, individual macrophages differed in their response to 0.3 nM MTX, with some cells lysing (and therefore releasing proIL-1β) and other cells remaining intact and therefore capable of releasing mIL-1β. By the 30-min time point, the response shifted strongly in favor of cell lysis over cell integrity. Thus, lower concentrations of MTX will favor activation of caspase-1-dependent processing and secretion of mIL-1β secretion from the majority of macrophages without progression to cytolysis. Higher MTX concentrations will stimulate an increasingly larger fraction of the macrophage population to lyse more rapidly and thus be unable to assemble the caspase-1 signaling complexes required for processing and secretion of mIL-1β.

In summary, we have shown that, depending on the concentration, MTX-treated macrophages can release the proin-

flammatory cytokine IL-1β by either the regulated secretion of mIL-1β or the cytolytic loss of proIL-1β. Furthermore, MTX induces membrane blebbing with a unique morphological and biochemical profile in macrophages. These results warrant future investigations regarding the role of MTX-dependent IL-1β release in the pathophysiology of ciguatera food poisoning, the distinctive signaling cascades initiated by MTX in macrophages versus endothelial cells, and the use of MTX as a tool for defining mechanisms that underlie different forms of membrane blebbing.

Acknowledgments

We are grateful to J. Michelle Kahlenberg and Dr. Alexander Garber for helpful discussions and critical reading of this manuscript.

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Correction to "Maitotoxin induces biphasic interleukin-1β secretion and membrane blebbing in murine macrophages"

In the above article [Verhoef PA, Kertesy SB, Estacion M, Schilling WP, and Dubyak GR (2004) *Mol Pharmacol* **66:**909–920], the footnote indicating that supplemental material is available for the article contains the wrong URL. The correct URL is http://molpharm.aspetjournals.org. The online version has been corrected in departure from print.

We regret this error and apologize for any confusion or inconvenience it may have caused.