The Journal of Immunology

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*J Immunol* 2007; 179:6016-6023; ;
doi: 10.4049/jimmunol.179.9.6016
http://www.jimmunol.org/content/179/9/6016



*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

# Purinergic $P2Y_2$ Receptors Induce Increased MCP-1/CCL2 Synthesis and Release from Rat Alveolar and Peritoneal Macrophages[1]

**Leanne Stokes and Annmarie Surprenant[2]**

**Macrophages play a key role in inflammation by synthesis and release of proinflammatory cytokines and chemokines. Extracellular nucleotides released at sites of tissue damage may be an early danger signal for immune cells, and ATP-gated $P2X_7$ receptors are well known to mediate the rapid release of proinflammatory IL-18 and IL-1$\beta$. However, there is little direct evidence for the involvement of other purine receptor subtypes in the release of other cytokines or chemokines. We initially used protein arrays to address whether extracellular ATP can release cytokines and/or chemokines from rat NR8383 alveolar macrophage, which lack the $P2X_7$ receptor. ATP$\gamma$S increased the release of the proinflammatory chemokine, MCP-1 (MCP-1/CCL2). Pharmacological profiling identified the receptor responsible as the $P2Y_2$ receptor. Brief activation (10 min) of $P2Y_2$ receptors increased MCP-1 mRNA levels within 30 min and increased its release at 60 min. Similar results were obtained from rat peritoneal macrophages. We investigated likely downstream signaling cascades that may be involved, specifically the canonical $G_q$-mediated phospholipase C (PLC) and subsequent MAP kinase pathways, and $G_i/G_o$-mediated signaling. We could find no evidence for these pathways being involved in the $P2Y_2$R-induced increase in mRNA levels although inhibition of PLC blocked the UTP-induced increased release of MCP-1. Thus, the PLC-activated pathway can account for the increased release of MCP-1, but a novel signaling pathway may be involved in the increase in MCP-1 mRNA by activation of $P2Y_2$ receptors in alveolar and peritoneal macrophage.** *The Journal of Immunology,* **2007, 179: 6016–6023.**

Extracellular nucleotides (ADP, ATP and UTP) are released from secretory cells such as nerve terminals, mast cells and platelets via regulated (i.e., exocytotic) release mechanisms, as well as from nonsecretory cells such as epithelia and vascular endothelia via poorly understood constitutive release mechanisms (1–3). Nucleotide release occurs in response to both physiological stimuli (nerve activity, blood flow, digestion) and pathological stimuli (hypoxia, infection, inflammation, trauma). In particular, the ability of these released nucleotides to modulate the release of cytokines and/or chemokines from immune effector cells has generated increasing interest in regards to the development of novel anti-inflammatory therapeutics targetted to specific nucleotide receptors (4–6). Two families of plasma membrane receptors mediate the actions of extracellular nucleotides, the metabotroptic G-protein coupled P2Y purinergic receptors, and the ionotropic P2X purinergic receptor ion channels (5, 7).

There are eight mammalian metabotropic P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$), most of which ($P2Y_{1,2,4,6,11}$) couple to $G_q$-protein-mediated stimulation of the phospholipase C (PLC)[3] signaling cascade (3, 5, 8, 9) and it is this group of P2Y receptor subtypes that have been implicated in alterations in chemokine and cytokine release. In human monocyte-derived dendritic cells, activation of $P2Y_1$ and/or $P2Y_{11}$ receptors has been shown to stimulate release of the anti-inflammatory cytokine IL-10, and to inhibit the endotoxin-induced release of the proinflammatory cytokine TNF-$\alpha$, and chemokines MCP-1/CCL2 and macrophage inflammatory protein-1$\alpha$ (MIP-1$\alpha$) (10–12). Activation of these same receptors can also inhibit leukotriene D4-induced release of TNF-$\alpha$, IL-8, and MIP-1$\beta$ from human mast cells (13), whereas an increase in both TNF-$\alpha$ and IL-8 release has been found in human eosinophils and astrocytes as a result of $P2Y_6$ or other undetermined P2Y receptor subtypes (14–16). The $P2Y_2$ receptor is highly expressed in virtually all hemopoietic cells where its activation in eosinophils and neutrophils has been found to stimulate chemotaxis and superoxide production and to induce characteristics of monocytes to macrophage differentiation in a number of monocytic cells and cell lines (2, 9, 17, 18). However, it is not known whether activation of this receptor subtype directly alters cytokine or chemokine release in any of these cell types or what signaling pathways may be involved.

There are seven members of the P2X receptor family ($P2X_{1-7}$), each of which can form nucleotide-gated ion channels when expressed as homomeric or heteromeric subunit assemblies (7). mRNA for three of these, $P2X_1$, $P2X_4$, and $P2X_7$ receptors, coexist in every immune effector cell and cell line so far examined, although protein expression has not been as routinely observed and only $P2X_7$ receptors have been implicated in altered cytokine release (4, 6, 7, 19). There is unequivocal evidence from studies with $P2X_7$ receptor knockout mice (20, 21) and highly selective $P2X_7$ receptor antagonists (22, 23) that activation of $P2X_7$ receptors in endotoxin-primed monocytes and macrophages results in a rapid

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Received for publication February 13, 2007. Accepted for publication August 27, 2007.

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[3] Abbreviations used in this paper: PLC, phospholipase C; BzATP, 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate; MIP-1$\alpha$, macrophage inflammatory protein-1$\alpha$; RI, relative index; PPADS, 4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid.

release of bioactive IL-1$\beta$ and IL-18 (19, 24). However, it is the existence of this P2X receptor subtype in immune cells that complicates studies of involvement of other purine receptors in altered cytokine or chemokine function because IL-1$\beta$ itself is the "early release" cytokine classically known to evoke the subsequent release of numerous other proinflammatory molecules (25).

The NR8383 rat alveolar macrophage cell line does not appear to express functional $P2X_7$ receptors, but does express functional $P2Y_1$, $P2Y_2$, and $P2X_4$ receptors as assayed by electrophysiological and calcium flux measurements (26) and therefore may provide a valuable model in which to examine potential roles of these purinergic receptors in cytokine and chemokine release. We used these cells initially in a cytokine/chemokine array assay to ask whether extracellular ATP acting on purinergic receptors alters release profiles. We found that constitutive release of MCP-1 from these cells, and from rat peritoneal macrophage, was greatly increased by ATP or UTP acting through $P2Y_2$ receptors. The increased release was associated with increased levels of MCP-1 mRNA. The UTP-induced increased MCP-1 release, but not the increased mRNA levels, was blocked by inhibition of PLC. The $P2Y_2$ receptor-induced increased MCP-1 mRNA levels also could not be attributed to involvement of MAPK, PI3K, elevated intracellular calcium levels, or $G_{i/o}$ signaling, which may suggest a novel signaling pathway activated by $P2Y_2$ receptors in alveolar and peritoneal macrophages.

## Materials and Methods

### *Cell culture*

The NR8383 rat alveolar macrophage cell line (American Type Culture Collection) was maintained in F12-K medium (Invitrogen Life Technologies) supplemented with 15% bovine FCS (Invitrogen Life Technologies) at 37°C in a humidified 5% $CO_2$ incubator. Rat peritoneal macrophages were isolated by lavage of the peritoneal cavity with 5 ml of PBS. The collected cells were centrifuged and resuspended in DMEM containing 10% FCS and 2 mM L-glutamine plus 100U/ml penicillin and 100 $\mu$g/ml streptomycin (Invitrogen Life Technologies). Adenoviral constructs were generated for rat $P2X_7$-EE using the BD Clontech Adenovirus Expression System-1. All protocols involving rats were approved and in accord with University of Sheffield institutional committee on animal welfare.

### *Reagents*

ATP, UTP, 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate (BzATP), ADP, ATP$\gamma$S, suramin, 4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid (PPADS), cycloheximide, LPS (0155:B55 strain), wortmannin, LY294002, and apyrase Grade VI were purchased from Sigma. BAPTA-AM, U73122, PD98059, and SB203580 were from Calbiochem.

### *Protein array*

The RayBiotech rat cytokine Ab array I was purchased from Tebu-Bio and performed as per the manufacturer's instructions. Twenty-four hours before stimulation, $5 \times 10^6$ cells were plated in 60 mm petri dishes in F12-K medium containing 5% FCS.

Medium was removed, cells were washed twice, and then cells were incubated with vehicle, 100 ng/ml LPS as a positive control, or 100 $\mu$M ATP$\gamma$S for 4 h in a total volume of 1 ml. Cell-free supernatants were collected and added to preblocked protein array membranes overnight at 4°C. Array membranes were washed three times with wash buffer and incubated with biotin-conjugated anti-cytokine Abs for 2 h at room temperature. After washing, streptavidin HRP was added for 2 h. Protein array membranes were exposed to detection buffer for 1 min and then exposed to Kodak Bio-Max MS film (Sigma-Aldrich). The resulting membrane films were analyzed using GeneSnap/GeneTools software (Syngene) to quantify spot density.

### *Western blotting*

Western blotting was performed as detailed previously (27) according to standard protocols and proteins were visualized using anti-Glu-Glu primary Ab and HRP-conjugated secondary Ab (both at 1/2000 dilution), followed by detection using the ECL-plus kit and Kodak Bio-Max MS film.

### *ELISAs and secretion experiments*

A duo-set ELISA development kit for rat IL-1$\beta$ was purchased from R&D Systems. Ninety-six-well ELISA plates were coated with anti-IL-1$\beta$ capture Ab at 0.8 $\mu$g/ml in PBS and the detection Ab, biotinylated goat anti-rat IL-1$\beta$ was used at 350 ng/ml. Recombinant rat IL-1$\beta$ was used as standard over the range of 62–4000 pg/ml. Cells were plated at $5 \times 10^5$ cells per well in F-12K medium containing 15% FCS. Adenoviral constructs ($rP2X_7$ or control adenovirus) were added 48 h before release studies. Culture medium was removed and replaced with 200 $\mu$l 5% FCS medium containing LPS at 100 ng/ml or 1 $\mu$g/ml. A paired Ab set to detect MCP-1 by ELISA was purchased from BD Pharmingen; it used mouse anti-rat MCP-1 (C4) at 2 $\mu$g/ml and biotinylated mouse anti-rat MCP-1 (B4) at 1 $\mu$g/ml. Recombinant rat MCP-1 (Peprotech) was used as standard over the range of 62–4000pg/ml. Twenty-four hours before stimulation, NR8383 cells were plated at a density of $2 \times 10^5$ cells/well in 24-well plates (Nunc) in F12-K medium containing 1% FCS. Medium was then removed and replaced with serum-free medium containing nucleotides with or without test compounds to a final volume of 250 $\mu$l/well. At indicated times supernatants were pipetted from each well, briefly centrifuged to pellet any loose cells, and 100 $\mu$l supernatant was added to the ELISA plate. Experiments were performed in triplicate in each case.

Because control constitutive release of MCP-1 varied widely from experiment to experiment (500 pg/ml to 3000 pg/ml over a 2-h time period), but not within a single experiment, all agonists and test compounds were normalized to the control value from the same experiment. For data shown in the figures, the absolute values (mean ± SEM) of control release are given in the corresponding figure legends.

### *Semiquantitative RT-PCR*

Four $\times 10^6$ NR8383 cells were stimulated with vehicle, nucleotides (100 $\mu$M ATP or UTP) in presence or absence of test inhibitors for 30 min in F-12K medium containing 5% FCS. Cells were removed from petri dishes by scraping and centrifuged, and the cell pellet was used to extract total RNA using the RNAeasy kit (Qiagen). Five micrograms total RNA was reverse transcribed using Superscript III Rnase H- reverse transcriptase (Invitrogen Life Technologies) and 2.5 $\mu$M oligo dT. Primers for rat MCP-1 were designed using accession number NM_031530 and sequences were rMCP-1 sense: 5′ GCT GCT ACT CAT TCA CTG GCA AG-3′ and rMCP-1 antisense: 5′-GGT CAA GTT CAC ATT CAA AGG TGC-3′. Primers for $\beta$-actin were sense: GGC TCT CTT CCA GCC TTC CTT CTT G and antisense: CAC AGA GTA CTT GCG CTC AGG AGG. PCR was performed with Bioline *Taq* polymerase, 2.5 mM $MgCl_2$, 125 $\mu$M each dNTP, and 1 $\mu$M each primer. Thermal cycling was performed in a Hybaid PCR machine and cycling conditions were 94°C for 2 min followed by 20 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min, and a final elongation at 72°C for 5 min. The expected PCR product sizes were 260 bp (MCP-1) and 1.1 kb ($\beta$-actin). Preliminary experiments were performed to ensure that the number of PCR cycles performed (20) were nonsaturating. MCP-1 and $\beta$-actin PCR products were run on the same 1% agarose gel containing ethidium bromide and quantitated by densitometry using GeneSnap/GeneTools software (Syngene). For each sample, the relative index (RI) was calculated as RI = densitometry of MCP-1 PCR/densitometry of $\beta$-actin PCR.

### *Electrophysiology*

Conventional whole-cell patch clamp recordings were obtained using HEKA EPC9 patch clamp amplifier as detailed previously (22, 26). Recordings were made using the following intracellular to extracellular solutions (mM): 145 NaCl, 10 HEPES, 10 EGTA: 145 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 2 KCl, 10 HEPES, 10 glucose (pH 7.3). Agonists were applied via fast perfusion system (RSC delivery system, Biologic). Recordings were performed 24–48 h post adenoviral infection.

## Results

### *NR8383 alveolar macrophages lack $P2X_7$ receptors but not IL-1$\beta$ signaling cascade*

An important assumption in the present study was that NR8383 macrophages lack $P2X_7$Rs and therefore any nucleotide action on other cytokines or chemokines that may occur could not be due to $P2X_7$R-induced release of IL-1$\beta$. We made this assumption because we had previously found no evidence for their functional coupling to either ionic currents or calcium influx (26). However, low levels of $P2X_7$R mRNA can be detected in these cells (26) and therefore we addressed the validity of this assumption, first by

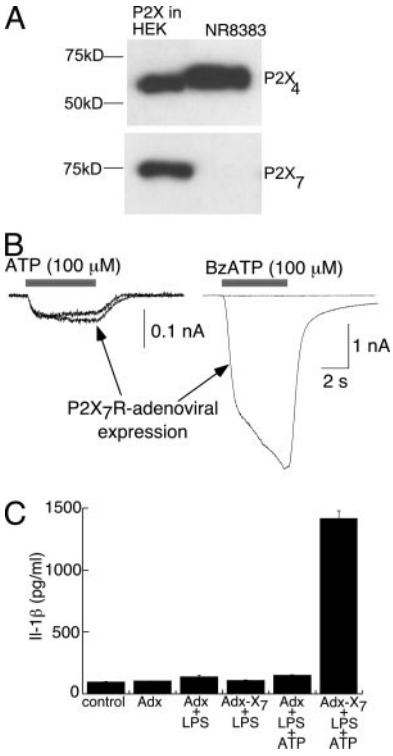


**FIGURE 1.** Absence of $P2X_7$ receptors but intact IL-1$\beta$ signaling in NR8383 alveolar macrophage. *A,* Western blot for rat $P2X_4$ and rat $P2X_7$ protein in transfected HEK cells expressing the respective receptors and in NR8383 cells as indicated. Only $P2X_4R$ protein is detected in NR8383 cells. *B,* Membrane currents recorded from control adenoviral and $P2X_7R$-expressing adenoviral infected cells in response to ATP or BzATP as indicated. ATP at 100 $\mu$M maximally activates $P2X_4R$ but minimally activates $P2X_7R$ while BzATP at 100 $\mu$M maximally activates $P2X_7R$ but minimally activates $P2X_4R$. *C,* IL-1$\beta$ released into the medium from NR8383 macrophage under the following conditions: control (vehicle only), Adx (empty adenovirus alone), Adx+LPS (empty adenovirus + 4 h LPS 100 ng/ml), Adx-$X_7$+LPS ($P2X_7R$-adenovirus + LPS), Adx+LPS+ATP (empty adenovus + LPS + 1 mM ATP 30 min), Adx-$X_7$+LPS+ATP ($P2X_7R$-adenovirus + LPS + ATP). Data are mean ± SEM of three experiments.

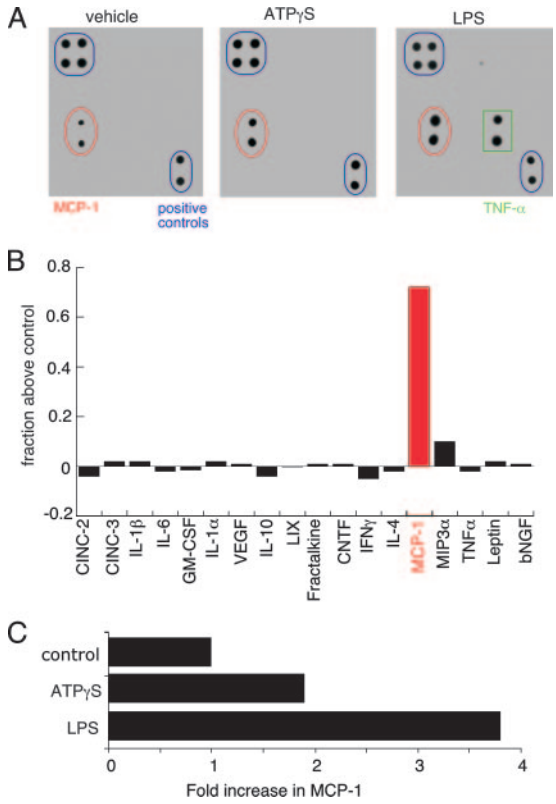


**FIGURE 2.** Protein array reveals MCP-1 is constitutively released in NR8383 macrophage and its release is increased by ATP$\gamma$S. *A,* Examples of raw data obtained from incubation with supernatant obtained from NR8383 cells after control (vehicle), ATP$\gamma$S (100 $\mu$M for 2 h), or LPS (100 ng/ml for 4 h) as indicated. Positive controls are circled in blue and were used to provide quantitative analysis of spots. MCP-1 is circled in red and was the only compound released under basal conditions. TNF-$\alpha$ is indicated by green squares; its increase by LPS was used as our positive control. *B,* Summary of all data obtained from experiments illustrated in *A*, where data are plotted as fraction above control. *C,* Comparison of changes in MCP-1 induced by ATP$\gamma$S and LPS plotted as fold increase over basal (control) levels.

Western blot assay for protein expression of $P2X_7R$. No $P2X_7R$ protein could be observed in contrast to the very high protein levels of $P2X_4R$ present in these cells (Fig. 1*A*). Second, whole-cell patch clamp experiments showed that the ATP analog, BzATP (30–300 $\mu$M) evoked an inward current of <50% of maximum ATP response in control cells (Fig. 1*B*), which is in keeping with its action as a weak partial agonist at rat $P2X_4Rs$ (6, 7). After adenoviral infection with $rP2X_7Rs$, BzATP-evoked currents (100 $\mu$M BzATP) were 10–15-fold greater than currents evoked by 100 $\mu$M ATP (Fig. 1*B*), and were reduced by >95% by 100 nM Brilliant Blue G (data not shown); these BzATP-evoked responses are typical of $rP2X_7R$-activated currents (6, 7, 22, 23). Third, we measured IL-1$\beta$ levels in the supernatant from LPS-primed NR8383 macrophage after ATP stimulation but found none (Fig. 1*C*). The more potent $P2X_7R$ agonist, BzATP (100 $\mu$M) or ATP$\gamma$S (100 $\mu$M) also did not evoke any IL-1$\beta$ release from LPS-primed NR8383 macrophages ($n = 2$, data not shown). However, when we introduced the $P2X_7R$ protein into these cells by use of adenoviral constructs, ATP now induced very large amounts of IL-1$\beta$ release from LPS-primed NR8383 macrophage (Fig. 1*C*). These results solidly support our initial assumption and also show that it is only the absence of $P2X_7R$ protein, and not any defect in IL-1$\beta$ processing or release, that accounts for the lack of effect of ATP on IL-1$\beta$ release from these cells.

### *ATP increases the release of MCP-1 from macrophage*

We used a protein microarray to examine the constitutive release of a panel of 18 cytokines and chemokines from NR8383 cells and in response to ATP or the nonhydrolysable ATP analog, ATP$\gamma$S (Fig. 2). We used ATP$\gamma$S in the first instance to prevent the possible breakdown of nucleotides by ectonucleotidasdes (8–10). In the absence of stimulation only one of these compounds, MCP-1, was observed to be released constitutively (Fig. 2*A*). The addition of ATP$\gamma$S resulted in a significant increase in the release of this chemokine without any significant increase in levels of the other cytokines or chemokines examined (Fig. 2*B*). We used LPS as a positive control in these experiments because LPS treatment is well known to increase the synthesis and release of MCP-1 (28) as well as TNF-$\alpha$. Consistent with this, LPS significantly and selectively increased the release of both MCP-1 and TNF-$\alpha$ from NR8383 macrophage (Fig. 2, *A* and *C*). Fig. 2*C* plots the relative amounts of constitutively released MCP-1 and the fold increase induced by either ATP$\gamma$S alone or LPS alone; ATP$\gamma$S and LPS resulted in an ~2-fold and 3.5-fold increase in MCP-1 release respectively.

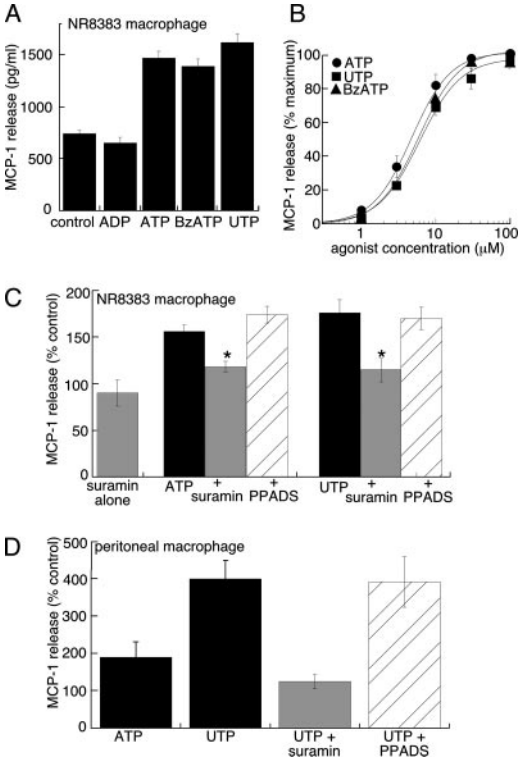


**FIGURE 3.** $P2Y_2$ receptors mediate increased MCP-1 release from NR8383 alveolar macrophage and rat peritoneal macrophage. *A,* Representative data obtained from ELISAs for MCP-1 released into the medium after 2 h incubation with each of the agonists (100 $\mu$M) as indicated; all data obtained from a single experiment and is representative of four to six similar experiments. *B,* Agonist concentration-response curve for ATP (○), UTP (□), and BzATP (△) obtained from all experiments as indicated in *A* where MCP-1 release is expressed as percent maximum from individual experiments; $n = 2$–5 for each point. *C,* Suramin (30 $\mu$M) but not PPADS (30 $\mu$M) blocks ATP (30 $\mu$M and UTP (100 $\mu$M)-induced increase in MCP-1; control constitutive release from this set of experiments was 2212 ± 73 pg/ml, $n = 3$. *D,* ATP and UTP (100 $\mu$M) also increased MCP-1 released into the supernatant from primary cultures of peritoneal macrophage in a suramin-sensitive manner; $n = 3$–6 experiments in each case.

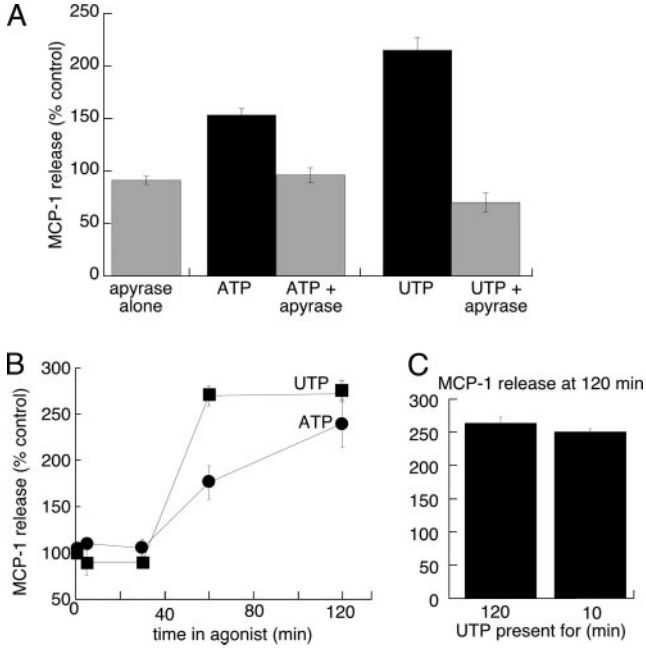


**FIGURE 4.** Kinetic properties of constitutive and $P2Y_2R$-stimulated release of MCP-1. *A,* MCP-1 released, expressed as percent vehicle-control, under conditions indicated; in these experiments, MCP-1 release over 2 h was measured in each case, $n = 3$. Apyrase did not alter basal MCP-1 release (1483 ± 38 pg/ml in this set of experiments) but was effective to block the actions of exogenously applied ATP or UTP (100 $\mu$M). *B,* Kinetics of MCP-1 release into the medium during continuous incubation with ATP or UTP as indicated; significantly increased release occurred only 60 min after agonist application. *C,* MCP-1 released during 2 h with UTP (100 $\mu$M) present for the entire 2 h or present for the initial 10 min only; $n = 4$ (basal constitutive release at 2 h time point was 1540 ± 30 pg/ml in this set of experiments).

### *ATP-induced increase in MCP-1 release is due to the activation of $P2Y_2$ receptors*

We first examined whether P2X receptors are involved in the ATP-evoked increased release of MCP-1. Of the seven P2X receptors, only $P2X_1R$ and $P2X_4R$ are expressed in these cells (Ref. 26 and Fig. 1*A*). $P2X_1Rs$ are potently activated by the ATP analog, $\alpha\beta$meATP (7), but this $P2X_1R$ agonist (1–100 $\mu$M) had no effect on levels of MCP-1 (control vs $\alpha\beta$meATP: 1318 pg/ml vs 1350 pg/ml $n = 3$). Rat $P2X_4Rs$ are insensitive to inhibition by the P2X/P2Y receptor antagonist, suramin (29), but suramin (10, 30 $\mu$M) completely blocked the actions of ATP to increase the release of MCP-1 (Fig. 3*C*). Therefore, we next examined the potential P2Y receptor candidates, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}$ receptors because mRNA for these receptors are present in NR8383 cells (26). $P2Y_1$ and $P2Y_{12}$ receptors are preferentially activated by ADP with $P2Y_1$ showing potent inhibition by PPADS (8, 9), but neither ADP (100 $\mu$M) nor PPADS (10, 30 $\mu$M) had any effect on MCP-1 release (Fig. 3*A,C*). $P2Y_2$ and $P2Y_4$ receptors are equally activated by both ATP and UTP and inhibited by suramin, but these receptors can be pharmacologically distinguished because the ATP analog, BzATP, is an agonist at $P2Y_2$ receptors but inactive at $P2Y_4$ receptors (8, 30). We found that ATP, BzATP, and UTP were all equally effective at increasing MCP-1 release, with half-maximal concentrations ($EC_{50}$ values) of 4.5 ± 0.6 $\mu$M, 2.5 ± 0.6 $\mu$M, and 3.6 ± 0.3 $\mu$M respectively (Fig. 3*B*). Similar $EC_{50}$ values for these agonists have been reported for heterologously expressed $P2Y_2$ receptors (30).

We obtained similar results from acutely isolated rat peritoneal macrophage. We found that MCP-1 was constitutively released from these cells, that ATP (10, 100 $\mu$M) and UTP (10, 100 $\mu$M) both resulted in an increased release of MCP-1, and that suramin but not PPADS abolished the UTP-induced increase in MCP-1 release (Fig. 3*D*). This concentration of agonist (10 $\mu$M) evoked maximum MCP-1 release as higher concentrations of ATP and UTP (100 $\mu$M) did not evoke significantly greater MCP-1 release, and suramin (30 $\mu$M) also blocked MCP-1 release evoked by these higher concentrations ($n = 3$). Whereas maximum responses to ATP and UTP were the same in NR8383 alveolar macrophage (Fig. 3, *A* and *B*), the maximum response to UTP was approximately twice that of ATP in primary cultures of rat peritoneal macrophages (Fig. 3*D*).

### *Constitutive release of MCP-1 does not involve $P2Y_2$ receptors*

We examined the possibility that the constitutive release of MCP-1 may be due to low-level activation of $P2Y_2$ receptors by consitutively released ATP by preincubating cells in apyrase to hydrolyze extracellular nucleotides, if present. Apyrase alone did not alter the consitutive release of MCP-1, whereas it abolished the increased release of MCP-1 incuded by either ATP or UTP (Fig. 4*A*). We also examined the effects of preincubation with suramin and

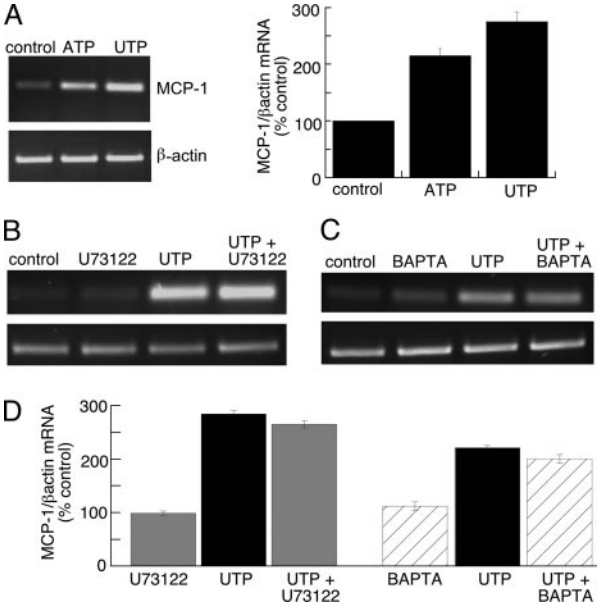


**FIGURE 5.** ATP and UTP increase MCP-1 mRNA levels in alveolar macrophage via PLC-independent signaling. *A,* Representative semiquantitative RT-PCR data for MCP-1 (and $\beta$-actin) mRNA before and 30 min after agonist application (100 $\mu$M); PCR products were quantitated using densitometry and RI calculated. Results are normalized (MCP-1/$\beta$-actin) from all similar experiments, $n = 4$. *B* and *C,* Representative gels from RT-PCR for MCP-1 and $\beta$-actin from cells treated as indicated; neither U73122 (10 $\mu$M, *B*) nor BAPTA-AM (10 $\mu$M, *C*) altered UTP-induced increase in MCP-1 mRNA, as summarized in *D* for all similar experiments, $n = 4$.

PPADS on consitutive MCP-1 release and found that suramin or PPADS at concentrations $\leq$30 $\mu$M had no significant effect on constitutive release (Fig. 3*B*) but that higher concentrations

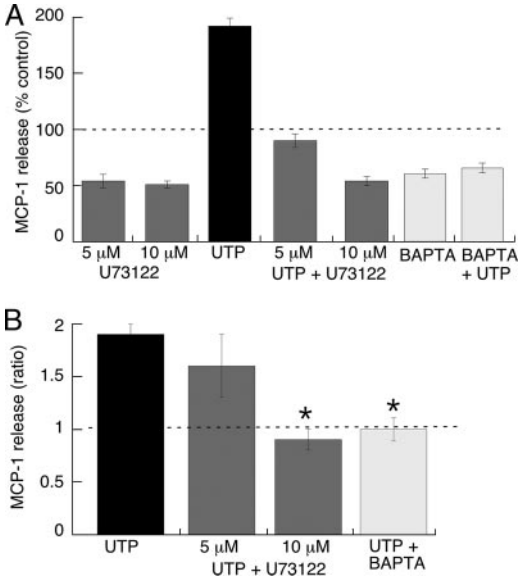


**FIGURE 6.** PLC signaling cascade mediates $P2Y_2R$-induced increased MCP-1 release. *A,* MCP-1 release under conditions as indicated, $n = 3$–6 in each case. *B,* Data from experiments shown in *A* plotted as a ratio of MCP-1 release (UTP/control; UTP in U73122/U73122 alone; UPT in BAPTA-AM/BAPTA-AM alone); 10 $\mu$M U73122 (but not 5 $\mu$M) as well as BAPTA-AM (10 $\mu$M) abolished UTP (10 $\mu$M)-induced facilitation of MCP-1 release. Control MCP-1 release was 1337 $\pm$ 62 pg/ml and 1420 $\pm$ 36 pg/ml for these two sets of experiments.

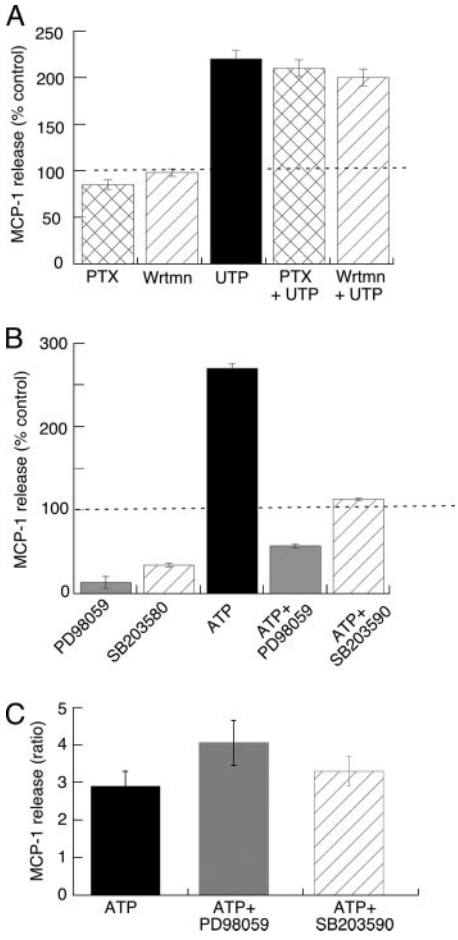


**FIGURE 7.** Different signaling cascades are involved in basal vs P2Y2R-induced MCP-1 release. *A,* Pertussis toxin (1 $\mu$g/ml overnight) or wortmannin (10 $\mu$M) did not alter basal or UTP (10 $\mu$M) induced MCP-1 release, $n = 3$ for each condition. *B*, PD98059 and SB203580 (10 $\mu$M) markedly inhibited basal MCP-1 release but did not alter the relative increase induced by ATP (30 $\mu$M), as indicated by the MCP-1 release ratio data summarized in *C*, $n = 4$ for each; control MCP-1 release was 502 $\pm$ 34 pg/ml for this set of experiments.

(100–300 $\mu$M) virtually abolished all MCP-1 release (data not shown). These actions are most likely due to several well-documented nonspecific actions of these compounds (7, 8).

### *Kinetics of $P2Y_2R$-induced increase in MCP-1 release*

We examined the kinetics of MCP-1 release in response to the continued application of ATP or UTP; the earliest time point at which MCP-1 was significantly increased over control was 60 min after addition of either agonist and levels reached maximum at 120 min after agonist addition (Fig. 4*B*). We then asked whether a brief pulse of agonist would also lead to subsequent increased release of MCP-1 by applying UTP for 10 min and assaying for MCP-1 release 120 min later. We found a 10 min pulse of UTP was as effective to increase levels of MCP-1 release as was the continued presence of agonist for the entire duration (Fig. 4*C*).

### *$P2Y_2R$ activation increases MCP-1 mRNA levels via PLC-independent pathway*

The delayed increased release of MCP-1 in response to $P2Y_2R$ activation suggested a signaling cascade involving increased synthesis. Therefore, we used a semiquantitative RT-PCR method to

examine levels of MCP-1 mRNA relative to $\beta$-actin and found signficantly increased levels within 30 min after stimulation with ATP or UTP (Fig. 5*A*). $P2Y_2R$ coupling through $G_q$ protein activation can lead to increase transcription through at least three pathways: activation of PLC leading to Ras/Raf/MAP Kinase/ERK1/2 signaling, activation of PLC leading to phosphorylation of CREB, or activation of NF-$\kappa$B via G$\beta\gamma$ protein coupling to PI3K (8, 9, 31–33). However, the PLC inhibitor, U73122 (5 or 10 $\mu$M), did not alter the UTP-induced increase in MCP-1 mRNA nor did chelation of intracellular calcium by incubating cells with BAPTA-AM (Fig. 5, *B–D*).

### *$P2Y_2R$ activation increases MCP-1 release via the PLC-dependent pathway*

In contrast to the lack of effect of PLC blockade on MCP-1 mRNA levels, U73122 at a concentration of 10 $\mu$M (but not at 5 $\mu$M) completely prevented the increased release of MCP-1 by UTP (Fig. 6). Both concentrations of U73122 decreased basal release of MCP-1 by ~50% and the absolute amounts of MCP-1 induced by UTP were comcomitantly reduced in the presence of either concentration of U73122 (Fig. 6*A*). By calculating the ratio of MCP-1 release (i.e., release in UTP/basal release vs release in UTP + U73122/release in U73122 alone) it can be seen that only the higher concentration of U73122 produced a significant inhibition of the UTP-evoked increased release in MCP-1 (Fig. 6*B*). Similarly, preincubation of the cells with BAPTA-AM inhibited basal MCP-1 release by ~55% (Fig. 6*A*) and also prevented the UTP-induced increase in release, as evidenced by the calculated MCP-1 release ratio (Fig. 6*B*). We examined the actions of a number of other inhibitors of pathways linked to $P2Y_2$ receptor activation on basal and UTP-evoked MCP-1 release. Blockade of $G_i/G_o$ coupling by preincubation with pertussis toxin, or blockade of PI3K (and ERK1/2) with wortmannin or LY294002, did not alter basal or UTP-evoked MCP-1 release (Fig. 7*A*). PD98059 and SB203580, which block MEK1 and p38 MAPK respectively, markedly reduced the basal release of MCP-1 (by >85%) but did not alter the increase in release by UTP as evidenced by the MCP-1 release ratio (Fig. 7, *A* and *B*).

## Discussion

This study has shown that brief activation of metabotropic $P2Y_2$ purinergic receptors induced increased levels of MCP-1 mRNA and a delayed release of this chemokine from rat NR8383 alveolar macrophage cell lines and from primary cultures of rat peritoneal macrophage. MCP-1 (CCL2) is the prototypic, and most studied, proinflammatory chemokine that targets monocytes and T-lymphocytes expressing the CC chemokine receptor CCR2 to recruit these cells from the bloodstream to damaged tissue (34). In addition to providing chemotactic signals, MCP-1 signals to monocyte activation and induction of the respiratory burst (35, 36). Indeed, MCP-1 has been linked to numerous roles in both adaptive and native immunity as well as in inflammatory diseases, and its synthesis and/or release is well documented to be increased in vascular endothelial and smooth muscle cells in atherosclerotic plaques, in macrophages and in glandular epithelial cells at sites of acute and chronic inflammation (34–36). Much less information exists as to the initial physiological and pathophysiological stimuli that induce MCP-1 synthesis and/or release. Results from the present study implicate extracellular nucleotides acting at the $P2Y_2$ receptor as a potentially important initial stimulus for MCP-1 release.

The multiplicity of purine receptors expressed on macrophage, together with a paucity of subtype-selective receptor antagonists, presents considerable obstacles to identifying which receptor subtype may be directly responsible for modulation of chemokine or cytokine release. This is particularly true for immune cells expressing $P2X_7$ receptors because $P2X_7Rs$ unequivocally couple to the rapid release of IL-1$\beta$ from activated macrophages (usually via LPS priming) (4, 19–21); IL-1$\beta$ can then lead to the release of numerous other proinflammatory molecules including MCP-1 (25). For this reason, we made use of the rat NR8383 alveolar macrophage cell line which the present (Fig. 1) and previous (26) study reveal do not express functional $P2X_7$ receptors and so do not release IL-1$\beta$ in response to extracellular ATP. Because these cells possess all other aspects of the IL-1$\beta$ cascade, as evidenced by their capacity to release fully processed IL-1$\beta$ in response to ATP after ectopic expression of $P2X_7R$, they should prove highly useful in further studies of chemokine/cytokine modulation by other stimuli which may directly or indirectly lead to release of ATP. Moreover, use of rat NR8383 macrophage additionally simplifies investigations of purine receptor actions because they express a more limited repertoire of receptors than other immune cells and immune cell lines, expressing mRNA for only $P2Y_1$, $P2Y_2$, $P2Y_{12}$, and $P2X_4$ receptors, each of which can be observed to be functional in electrophysiological assays (26). We used a supramaximal concentration of the nonhydrolysable ATP analog, ATP$\gamma$S, to initially screen a 17-member cytokine/chemokine protein array and found only one, MCP-1, to be significantly increased by this agonist. It should be noted that this protocol would be expected to bias against $P2Y_1R$ actions because $P2Y_1Rs$ are ADP-sensitive and, thus, unlikely to be activated by ATP$\gamma$S (8); however, use of ADP$\beta$S (an nonhydrolysable ADP analog) in this type of cytokine/chemokine array may well identify a $P2Y_1R$-specific pathway.

Agonist concentration curves and antagonist profiles clearly identified $P2Y_2R$ as the sole receptor underlying nucleotide-mediated MCP-1 release. ATP, UTP, and BzATP all showed similar maximum responses and $EC_{50}$ values of 4–6 $\mu$M, values which are very similar to those obtained in heterologous expression assays of rat $P2Y_2R$ (30). These agonist profiles rule out $P2Y_1R$ (ATP, UTP, and BzATP-insensitive), $P2Y_{12}R$ (UTP and BzATP-insensitive), and $P2X_4R$ (UTP-insensitive) (8, 30). Additionally, we found that brief stimulation ($\leq$10 min) with UTP was as effective to increase MCP-1 mRNA levels and secretion as was prolonged receptor stimulation (2 h). These kinetics are in keeping with the well known rapid and pronounced desensitization of $P2Y_2Rs$ (31). Results we obtained with the nonselective antagonists, suramin and PPADS, also provide limited, but supportive, data. We found that suramin at concentrations $\leq$30 $\mu$M abolished the ATP or UTP-evoked increases in MCP-1 without exerting any effect on MCP-1 release when applied in the absence of ATP or UTP, whereas PPADS at $\leq$30 $\mu$M had no effect on either constitutive nor ATP/UTP-evoked MCP-1 release. These results provide further support for selective activation of $P2Y_2R$ (suramin-sensitive/PPADS-insensitive) by ATP and UTP (8). However, higher concentrations of either antagonist inhibited both constitutive and agonist-induced release by >85%, thus emphasizing the nonspecific actions of these compounds and the need for caution in interpretations made based on their actions, especially in view of much data showing that concentrations >100 $\mu$M will inhibit virtually all P2X and P2Y receptors so far examined (7, 8). For example, in a recent study, ATP$\gamma$S (10 and 100 $\mu$M) was found to increase the release of a number of inflammatory mediators, including MCP-1, from the human HMEC1 epithelial cell line. In that study, suramin and PPADS were used at concentrations of 100 and 1000 $\mu$M and found to block both constitutive and agonist-induced release of all compounds measured (37); thus, providing no information as to the receptors involved.

We also found that primary cultures of rat peritoneal macrophage exhibited constitutive release of MCP-1 as well as ATP and UTP-evoked increases in MCP-1 release and the agonist-evoked increased release was prevented by suramin (10 $\mu$M) but not PPADS (30 $\mu$M). In these macrophage the $EC_{50}$ values for ATP and UTP were ~10 $\mu$M. These results are consistent with $P2Y_2R$ activation, but the maximum UTP-evoked response was 2-fold greater than the ATP-evoked response, a result that is most likely due to activation of more than a single receptor type by ATP.

In common with several studies of MCP-1 release in other cells (10, 37–39), we found substantial basal release of MCP-1 from both NR8383 alveolar macrophage and peritoneal macrophage. This was not due to presence of extracellular nucleotides because neither apyrase nor suramin altered the basal MCP-1 release. High levels of basal MCP-1 release generally have been ascribed to known or unknown conditions resulting in an activated (inflammatory) phenotype (35, 37, 39). However, we did not observe significant constitutive release of any of the other proinflammatory chemokines or cytokines assayed in our protein array, thus suggesting the high basal levels of MCP-1 in these apparently unactivated macrophages may have a functional role unrelated to inflammatory conditions.

Because MCP-1 follows the classical endoplasmic reticulum-Golgi pathway of constitutive secretion, it is reasonable to expect that the $P2Y_2R$-induced increase in MCP-1 release is a straightforward consequence of increased protein synthesis. In keeping with this expectation, we observed increased levels of MCP-1 mRNA within 15–30 min of agonist application, and a delayed release detected only 60 min after receptor stimulation. However, inhibition of PLC by U73122, or prevention of PLC-induced increases in intracellular calcium concentrations by chelation with BAPTA-AM, effectively abolished UTP-induced increased release of MCP-1 without significantly altering the increased mRNA levels. It is difficult to explain these results based on activation of a single transduction pathway (i.e., the PLC cascade) by $P2Y_2$ receptors although we found no evidence for the involvement of either of the other two signaling cascades that are less prominently associated with $P2Y_2R$ activation: $G_q$-coupled PI3K pathways and $G_i/G_o$-coupled signaling (8, 9, 31–33). An interesting possibility is suggested by a series of studies using vascular smooth muscle cells where platelet-derived growth factor and/or various serum constituents were found to increase MCP-1 mRNA levels by two distinct mechanisms: a rapid, transient enhancement of transcription and a prolonged increase in MCP-1 mRNA stability due to decreased RNase activity with the latter mechanism being the most prominent (40, 41). If $P2Y_2R$-mediated actions on MCP-1 mRNA in macrophages also involve PLC-dependent and PLC-independent mechanisms, it is likely that the identification based on blockade of either alone will be masked by the other. Further studies detailing the kinetics of MCP-1 mRNA changes and time course of its degradation in response to UTP will be required to clarify whether, and to what extent, PLC signaling is involved in this action of $P2Y_2R$ activation in macrophages. In any event, UTP and ATP-evoked MCP-1 release into the medium appears to be mediated primarily, or solely, by activation of PLC signaling. In this regard, it is important to emphasize the obvious dissociation in signaling processes underlying basal MCP-1 release and $P2Y_2R$-induced increased MCP-1 release in macrophages examined in this study. That is, blockade of MEK or p38 MAPK with PD98059 and SB203580 respectively, reduced basal MCP-1 release by 80–90%, but did not alter the relative increase induced by $P2Y_2R$ activation while PLC inhibition with U73122 or BAPTA-AM decreased basal MCP-1 release by 50% but completely prevented UTP- or ATP-induced increases. Inhibition of Ras/Raf/ERK1/2 signaling with wortmannin or LY294002 altered neither basal nor UTP-induced MCP-1 release. These results suggest that specific, constitutively active, MAPK pathways (MEK and/or p38 MAPK) are primarily responsible for basal (but not stimulated) MCP-1 release and highlight the importance of measuring release ratios, rather than absolute levels, when assaying changes in constitutively released proteins.

ATP, ADP, and/or UTP have been known for the past decade to act indirectly as chemotactic signals in several immune and hemopoietic cells, including neutrophils, dendritic cells, monocytes, and recently, hemopoietic stem cells (2–4, 17, 42, 43), although the purinergic receptor subtypes involved and their underlying signaling mechanism(s) have been investigated only quite recently (17, 42). In human endothelial cells, glia (17, 43) and hemopoietic stem cells (42), $P2Y_2Rs$ signal through pertussis toxin-sensitive $G_i/G_o$ paths to engage other chemotactic or adhesion molecules, such as integrins (17) and CXCL12 (42), via $\rho$-GTPase-dependent signaling. The present study provides the first demonstration that $P2Y_2R$ activation and its canonical PLC signaling cascade may also link to chemotaxis by inducing the release of MCP-1 from macrophage.

## Acknowledgments

We thank Elizabeth Martin and Weihong Ma for valuable technical assistance.

## Disclosures

The authors have no financial conflict of interest.

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