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Purinergic P2Y₂ Receptors Induce Increased MCP-1/CCL2 Synthesis and Release from Rat Alveolar and Peritoneal Macrophages¹

Leanne Stokes and Annmarie Surprenant²

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 P2X₇ receptors are well known to mediate the rapid release of proinflammatory IL-18 and IL-1 β . 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 P2X₇ receptor. ATP γ S increased the release of the proinflammatory chemokine, MCP-1 (MCP-1/CCL2). Pharmacological profiling identified the receptor responsible as the P2Y₂ receptor. Brief activation (10 min) of P2Y₂ 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 P2Y₂R-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 P2Y₂ 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 targeted to specific nucleotide receptors (4–6). Two families of plasma membrane receptors mediate the actions of extracellular nucleotides, the metabotropic 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 P2Y₁ and/or P2Y₁₁ 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- α , and chemokines MCP-1/CCL2 and macrophage inflammatory protein-1 α (MIP-1 α) (10–12). Activation of these same receptors can also inhibit leukotriene D4-induced release of TNF- α , IL-8, and MIP-1 β from human mast cells (13), whereas an increase in both TNF- α and IL-8 release has been found in human eosinophils and astrocytes as a result of P2Y₆ or other undetermined P2Y receptor subtypes (14–16). The P2Y₂ 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, P2X₁, P2X₄, and P2X₇ receptors, coexist in every immune effector cell and cell line so far examined, although protein expression has not been as routinely observed and only P2X₇ receptors have been implicated in altered cytokine release (4, 6, 7, 19). There is unequivocal evidence from studies with P2X₇ receptor knockout mice (20, 21) and highly selective P2X₇ receptor antagonists (22, 23) that activation of P2X₇ receptors in endotoxin-primed monocytes and macrophages results in a rapid

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³ Abbreviations used in this paper: PLC, phospholipase C; BzATP, 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate; MIP-1 α , macrophage inflammatory protein-

1 α ; RI, relative index; PPADS, 4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonoxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid.

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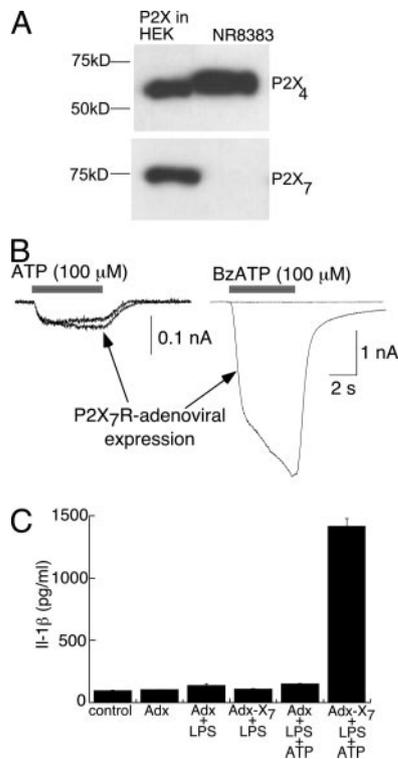


FIGURE 1. Absence of P2X₇ receptors but intact IL-1 β signaling in NR8383 alveolar macrophage. *A*, Western blot for rat P2X₄ and rat P2X₇ protein in transfected HEK cells expressing the respective receptors and in NR8383 cells as indicated. Only P2X₄R protein is detected in NR8383 cells. *B*, Membrane currents recorded from control adenoviral and P2X₇R-expressing adenoviral infected cells in response to ATP or BzATP as indicated. ATP at 100 μ M maximally activates P2X₄R but minimally activates P2X₇R while BzATP at 100 μ M maximally activates P2X₇R but minimally activates P2X₄R. *C*, IL-1 β 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-X₇+LPS (P2X₇R-adenovirus + LPS), Adx+LPS+ATP (empty adenovirus + LPS + 1 mM ATP 30 min), Adx-X₇+LPS+ATP (P2X₇R-adenovirus + LPS + ATP). Data are mean \pm SEM of three experiments.

Western blot assay for protein expression of P2X₇R. No P2X₇R protein could be observed in contrast to the very high protein levels of P2X₄R present in these cells (Fig. 1A). Second, whole-cell patch clamp experiments showed that the ATP analog, BzATP (30–300 μ M) evoked an inward current of <50% of maximum ATP response in control cells (Fig. 1B), which is in keeping with its action as a weak partial agonist at rat P2X₄Rs (6, 7). After adenoviral infection with rP2X₇Rs, BzATP-evoked currents (100 μ M BzATP) were 10–15-fold greater than currents evoked by 100 μ M ATP (Fig. 1B), and were reduced by >95% by 100 nM Brilliant Blue G (data not shown); these BzATP-evoked responses are typical of rP2X₇R-activated currents (6, 7, 22, 23). Third, we measured IL-1 β levels in the supernatant from LPS-primed NR8383 macrophage after ATP stimulation but found none (Fig. 1C). The more potent P2X₇R agonist, BzATP (100 μ M) or ATP γ S (100 μ M) also did not evoke any IL-1 β release from LPS-primed NR8383 macrophages ($n = 2$, data not shown). However, when we introduced the P2X₇R protein into these cells by use of adenoviral constructs, ATP now induced very large amounts of IL-1 β release from LPS-primed NR8383 macrophage (Fig. 1C). These results solidly support our initial assumption and also show that it is only the absence of P2X₇R protein, and not any defect in IL-1 β pro-

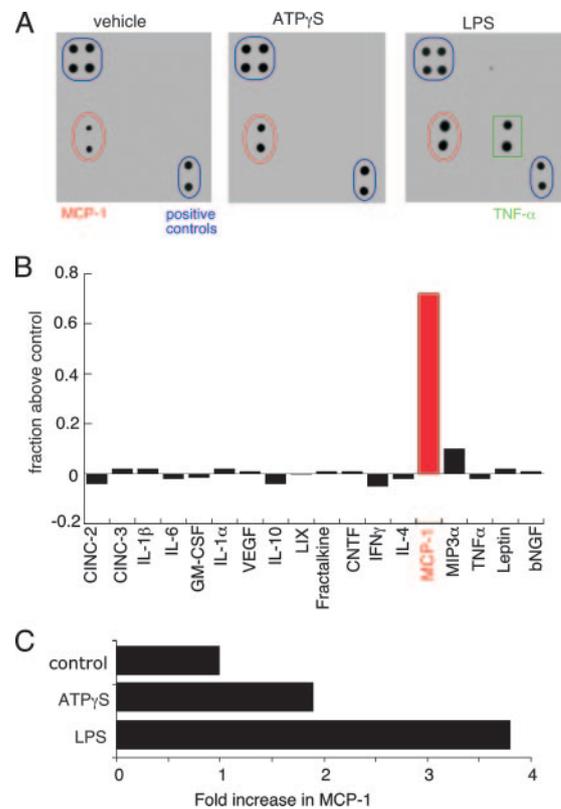


FIGURE 2. Protein array reveals MCP-1 is constitutively released in NR8383 macrophage and its release is increased by ATP γ S. *A*, Examples of raw data obtained from incubation with supernatant obtained from NR8383 cells after control (vehicle), ATP γ S (100 μ M for 2 h), or LPS (100 ng/ml for 4 h) 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- α 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 γ S and LPS plotted as fold increase over basal (control) levels.

cessing or release, that accounts for the lack of effect of ATP on IL-1 β 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 γ S (Fig. 2). We used ATP γ S in the first instance to prevent the possible breakdown of nucleotides by ectonucleotidases (8–10). In the absence of stimulation only one of these compounds, MCP-1, was observed to be released constitutively (Fig. 2A). The addition of ATP γ 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. 2B). 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- α . Consistent with this, LPS significantly and selectively increased the release of both MCP-1 and TNF- α from NR8383 macrophage (Fig. 2, A and C). Fig. 2C plots the relative amounts of constitutively released MCP-1 and the fold increase induced by either ATP γ S alone or LPS alone; ATP γ S and LPS resulted in an \sim 2-fold and 3.5-fold increase in MCP-1 release respectively.

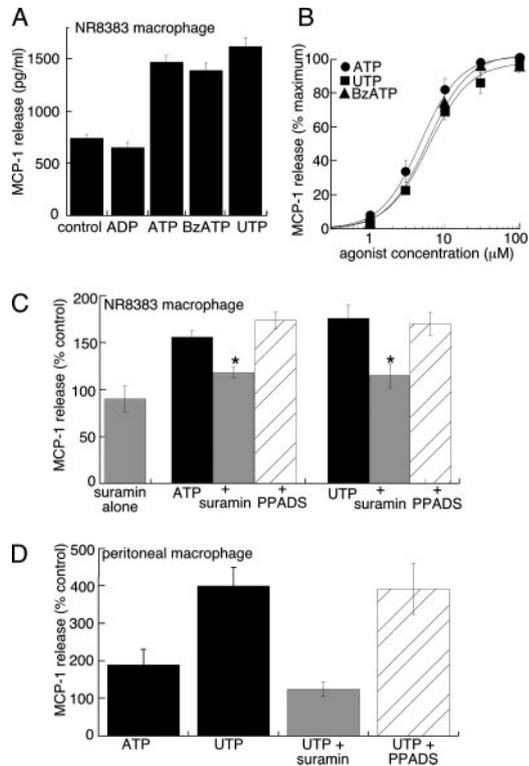


FIGURE 3. P2Y₂ receptors mediate increased MCP-1 release from NR8383 alveolar macrophage and rat peritoneal macrophage. *A*, Representative data obtained from ELISAs for MCP-1 released into the medium after 2 h incubation with each of the agonists (100 μM) as indicated; all data obtained from a single 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 μM) but not PPADS (30 μM) blocks ATP (30 μM and UTP (100 μM)-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 μM) 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.

ATP-induced increase in MCP-1 release is due to the activation of P2Y₂ receptors

We first examined whether P2X receptors are involved in the ATP-evoked increased release of MCP-1. Of the seven P2X receptors, only P2X₁R and P2X₄R are expressed in these cells (Ref. 26 and Fig. 1A). P2X₁Rs are potently activated by the ATP analog, αβmeATP (7), but this P2X₁R agonist (1–100 μM) had no effect on levels of MCP-1 (control vs αβmeATP: 1318 pg/ml vs 1350 pg/ml *n* = 3). Rat P2X₄Rs are insensitive to inhibition by the P2X/P2Y receptor antagonist, suramin (29), but suramin (10, 30 μM) completely blocked the actions of ATP to increase the release of MCP-1 (Fig. 3C). Therefore, we next examined the potential P2Y receptor candidates, P2Y₁, P2Y₂, P2Y₄, and P2Y₁₂ receptors because mRNA for these receptors are present in NR8383 cells (26). P2Y₁ and P2Y₁₂ receptors are preferentially activated by ADP with P2Y₁ showing potent inhibition by PPADS (8, 9), but neither ADP (100 μM) nor PPADS (10, 30 μM) had any effect on MCP-1 release (Fig. 3A,C). P2Y₂ and P2Y₄ 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 P2Y₂ receptors but inactive at P2Y₄ receptors (8, 30). We found that ATP, BzATP, and

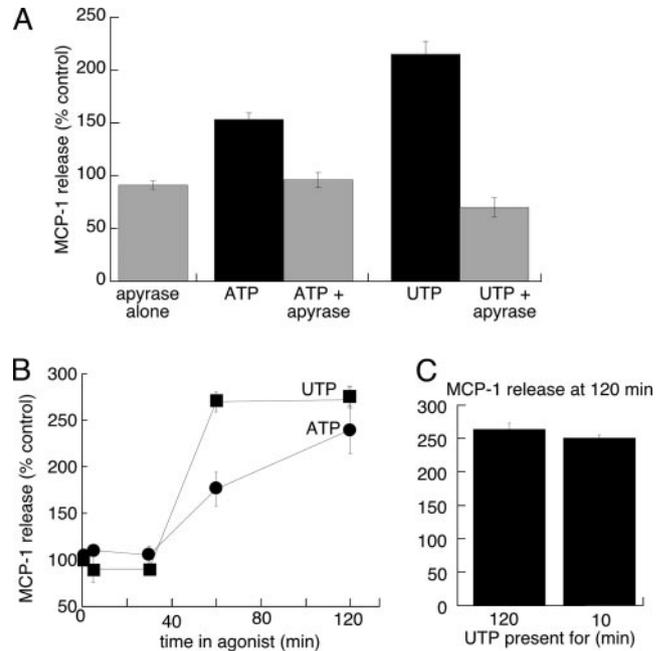


FIGURE 4. Kinetic properties of constitutive and P2Y₂R-stimulated release of MCP-1. *A*, MCP-1 released, expressed as percent vehicle-control, under conditions indicated; in these experiments, MCP-1 release over 2 h was measured in each case, *n* = 3. Apyrase did not alter basal MCP-1 release (1483 ± 38 pg/ml in this set of experiments) but was effective to block the actions of exogenously applied ATP or UTP (100 μM). *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 μM) 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).

UTP were all equally effective at increasing MCP-1 release, with half-maximal concentrations (EC₅₀ values) of 4.5 ± 0.6 μM, 2.5 ± 0.6 μM, and 3.6 ± 0.3 μM respectively (Fig. 3B). Similar EC₅₀ values for these agonists have been reported for heterologously expressed P2Y₂ 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 μM) and UTP (10, 100 μM) 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. 3D). This concentration of agonist (10 μM) evoked maximum MCP-1 release as higher concentrations of ATP and UTP (100 μM) did not evoke significantly greater MCP-1 release, and suramin (30 μM) 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. 3D).

Constitutive release of MCP-1 does not involve P2Y₂ receptors

We examined the possibility that the constitutive release of MCP-1 may be due to low-level activation of P2Y₂ receptors by constitutively released ATP by preincubating cells in apyrase to hydrolyze extracellular nucleotides, if present. Apyrase alone did not alter the constitutive release of MCP-1, whereas it abolished the increased release of MCP-1 induced by either ATP or UTP (Fig. 4A). We also examined the effects of preincubation with suramin and

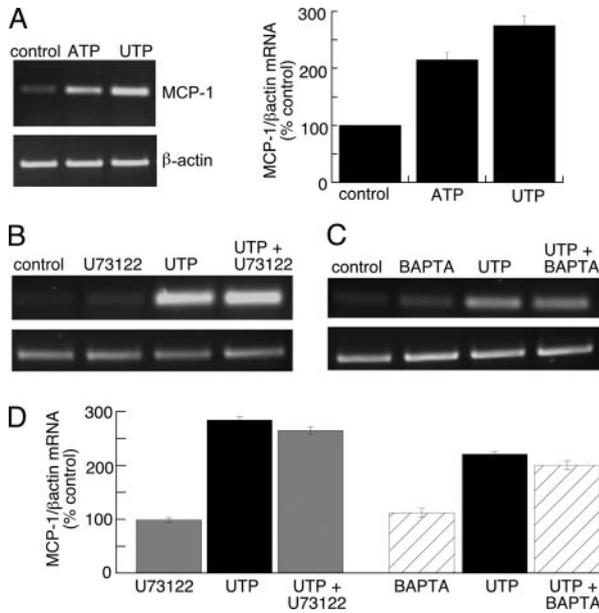


FIGURE 5. ATP and UTP increase MCP-1 mRNA levels in alveolar macrophage via PLC-independent signaling. *A*, Representative semiquantitative RT-PCR data for MCP-1 (and β -actin) mRNA before and 30 min after agonist application (100 μ M); PCR products were quantitated using densitometry and RI calculated.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

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

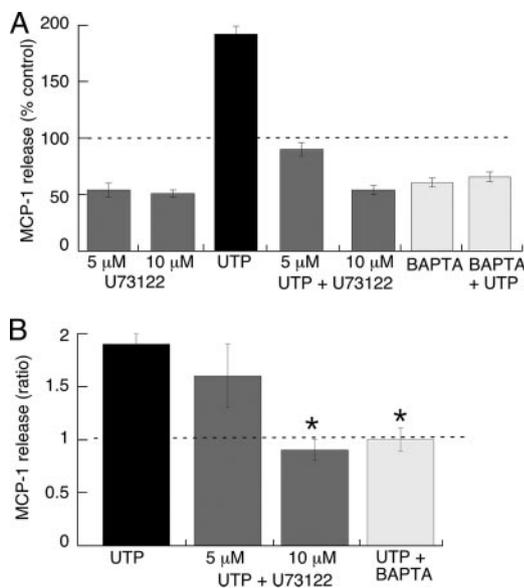


FIGURE 6. PLC signaling cascade mediates P2Y₂R-induced increased MCP-1 release. *A*, MCP-1 release under conditions as indicated, $n = 3-6$ in each case. *B*, Data from experiments shown in *A* plotted as a ratio of MCP-1 release (UTP/control; UTP in U73122/U73122 alone; UPT in BAPTA-AM/BAPTA-AM alone); 10 μ M U73122 (but not 5 μ M) as well as BAPTA-AM (10 μ M) abolished UTP (10 μ M)-induced facilitation of MCP-1 release. Control MCP-1 release was 1337 ± 62 pg/ml and 1420 ± 36 pg/ml for these two sets of experiments.

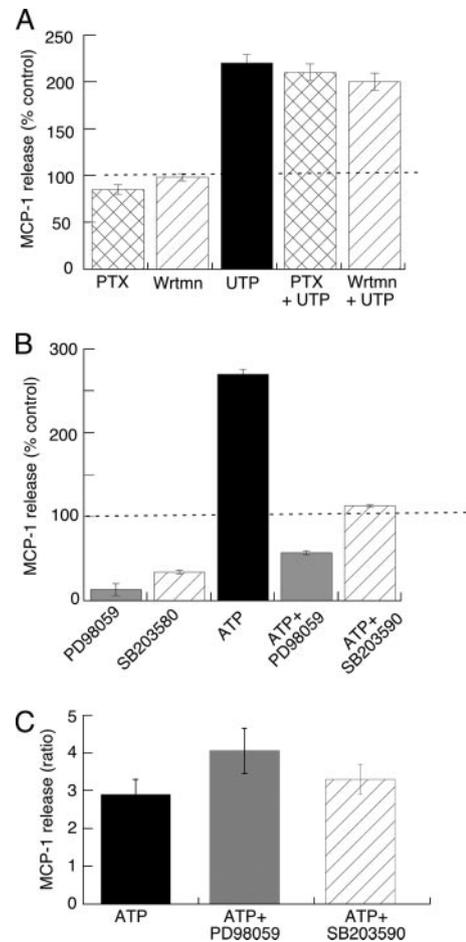


FIGURE 7. Different signaling cascades are involved in basal vs P2Y₂R-induced MCP-1 release. *A*, Pertussis toxin (1 μ g/ml overnight) or wortmannin (10 μ M) did not alter basal or UTP (10 μ M) induced MCP-1 release, $n = 3$ for each condition. *B*, PD98059 and SB203580 (10 μ M) markedly inhibited basal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

(100–300 μ 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 P2Y₂R-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*).

P2Y₂R activation increases MCP-1 mRNA levels via PLC-independent pathway

The delayed increased release of MCP-1 in response to P2Y₂R 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 β -actin and found significantly increased levels within 30 min after stimulation with ATP or UTP (Fig. 5A). P2Y₂R 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- κ B via G β γ protein coupling to PI3K (8, 9, 31–33). However, the PLC inhibitor, U73122 (5 or 10 μ 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).

P2Y₂R 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 μ M (but not at 5 μ M) completely prevented the increased release of MCP-1 by UTP (Fig. 6). Both concentrations of U73122 decreased basal release of MCP-1 by \sim 50% and the absolute amounts of MCP-1 induced by UTP were concomitantly reduced in the presence of either concentration of U73122 (Fig. 6A). 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. 6B). Similarly, preincubation of the cells with BAPTA-AM inhibited basal MCP-1 release by \sim 55% (Fig. 6A) and also prevented the UTP-induced increase in release, as evidenced by the calculated MCP-1 release ratio (Fig. 6B). We examined the actions of a number of other inhibitors of pathways linked to P2Y₂ 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. 7A). 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 P2Y₂ 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 P2Y₂ 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 P2X₇ receptors because P2X₇Rs unequivocally couple to the rapid release of IL-1 β from activated macrophages (usually via LPS priming) (4, 19–21); IL-1 β 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 P2X₇ receptors and so do not release IL-1 β in response to extracellular ATP. Because these cells possess all other aspects of the IL-1 β cascade, as evidenced by their capacity to release fully processed IL-1 β in response to ATP after ectopic expression of P2X₇R, 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 P2Y₁, P2Y₂, P2Y₁₂, and P2X₄ receptors, each of which can be observed to be functional in electrophysiological assays (26). We used a supramaximal concentration of the nonhydrolyzable ATP analog, ATP γ 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 P2Y₁R actions because P2Y₁Rs are ADP-sensitive and, thus, unlikely to be activated by ATP γ S (8); however, use of ADP β S (an nonhydrolyzable ADP analog) in this type of cytokine/chemokine array may well identify a P2Y₁R-specific pathway.

Agonist concentration curves and antagonist profiles clearly identified P2Y₂R as the sole receptor underlying nucleotide-mediated MCP-1 release. ATP, UTP, and BzATP all showed similar maximum responses and EC₅₀ values of 4–6 μ M, values which are very similar to those obtained in heterologous expression assays of rat P2Y₂R (30). These agonist profiles rule out P2Y₁R (ATP, UTP, and BzATP-insensitive), P2Y₁₂R (UTP and BzATP-insensitive), and P2X₄R (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 P2Y₂Rs (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 μ 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 μ M had no effect on either constitutive nor ATP/UTP-evoked MCP-1 release. These results provide further support for selective activation of P2Y₂R (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 μ M will inhibit virtually all P2X and P2Y receptors so far examined (7, 8). For example, in a recent study, ATP γ S (10 and 100 μ 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 μ 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 μ M) but not PPADS (30 μ M). In these macrophage the EC₅₀ values for ATP and UTP were \sim 10 μ M. These results are consistent with P2Y₂R 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 P2Y₂R-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 P2Y₂ receptors although we found no evidence for the involvement of either of the other two signaling cascades that are less prominently associated with P2Y₂R 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 P2Y₂R-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 P2Y₂R 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 P2Y₂R-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 P2Y₂R 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), P2Y₂Rs 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 ρ -GTPase-dependent signaling. The present study provides the first demonstration that P2Y₂R activation and its canonical PLC signaling cascade may also link to chemotaxis by inducing the release of MCP-1 from macrophage.

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Disclosures

The authors have no financial conflict of interest.

References

- Hasko, G., and C. Szabo. 1998. Regulation of cytokine and chemokine production by transmitters and co-transmitters of the autonomic nervous system. *Biochem. Pharmacol.* 56: 1079–1087.
- Lazarowski, E. R., and R. C. Boucher. 2001. UTP as an extracellular signalling molecule. *News Physiol. Sci.* 16: 1–5.
- Fields, R. D., and G. Burnstock. 2006. Purinergic signalling in neuron-glia interactions. *Nat. Rev. Neurosci.* 7: 423–436.
- Di Virgilio, F., P. Chiozzi, D. Ferrari, S. Falzoni, J. M. Sanz, A. Morelli, M. Torboli, G. Bolognesi, and O. R. Baricordi. 2001. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* 97: 587–600.
- Abbracchio, M. P., G. Burnstock, J. M. Boeynaems, E. A. Barnard, J. L. Boyer, C. Kennedy, G. E. Knight, M. Fumagalli, C. Gachet, K. A. Jacobson, and G. A. Weisman. 2006. Update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol. Rev.* 58: 281–341.
- Baraldi, P. G., F. Di Virgilio, and R. Romagnoli. 2004. Agonists and antagonists acting at P2X₇ receptor. *Curr. Top. Med. Chem.* 4: 1707–1717.
- North, R. A. Molecular physiology of P2X receptors. *Physiol. Rev.* 82: 1013–1067.
- von Kugelgen, I. 2006. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. *Pharmacol. Ther.* 110: 415–432.
- Boeynaems, J.-M., D. Communi, N. Suarez-Gonzalez, and B. Robaye. 2005. Overview of P2 receptors. *Semin. Thromb. Hemostasis* 31: 139–149.
- Horkmans, M., B. Marcet, F. Marteau, F. Bulte, A. Maho, M. Parmentier, J.-M. Boeynaems, and D. Communi. 2006. Extracellular adenosine nucleotides inhibit the release of major monocyte recruiters by human monocyte-derived dendritic cells. *FEBS Lett.* 580: 747–754.
- Marteau, F., D. Communi, J.-M. Boeynaems, and N. Suarez-Gonzalez. 2004. Involvement of multiple P2Y receptors and signalling pathways in the action of adenosine nucleotides diphosphates on human monocyte-derived dendritic cells. *J. Leukocyte Biol.* 76: 796–803.
- La Sala, A., S. Sebastiani, D. Ferrari, F. Di Virgilio, M. Idzko, J. Norgauer, and G. Girolomoni. 2002. Dendritic cells exposed to extracellular adenosine triphosphate acquire the migratory properties of mature cells and show a reduced capacity to attract type 1 T lymphocytes. *Blood* 99: 1715–1722.
- Feng, C., A. G. Mery, E. M. Beller, C. Favot, and J. A. Boyce. 2004. Adenosine nucleotides inhibit cytokine generation by human mast cells through a G_s-coupled receptor. *J. Immunol.* 173: 7539–7547.
- Cox, M. A., B. Gomes, K. Palmer, K. Du, M. Wiekowski, B. Wilburn, M. Petro, C. C. Chou, C. Desquitado, M. Schwarz, et al. 2005. The pyrimidineric P2Y₆ receptor mediates a novel release of proinflammatory cytokines and chemokines in monocytic cells stimulated with UDP. *Biochem. Biophys. Res. Commun.* 330: 467–473.
- Warny, M., S. Aboudola, S. C. Robson, J. Sevigny, D. Communi, S. P. Soltoff, and C. P. Kelly. 2001. P2Y₆ nucleotide receptor mediates monocyte interleukin-8 production in response to UDP or lipopolysaccharide. *J. Biol. Chem.* 276: 26051–26056.

16. Khine, A. A., L. Del Sorbo, R. Vaschetto, S. Voglis, E. Tullis, A. S. Slutsky, G. P. Downey, and H. Zhang. 2006. Human neutrophil peptides induce interleukin-8 production through the P2Y₆ signaling pathway. *Blood*. 107: 2936–2942.
17. Bagchi, S., Z. Liao, F. A. Gonzalez, N. E. Chorna, C. I. Seye, G. A. Weisman, and L. Erb. 2005. The P2Y₂ nucleotide receptor interacts with α_v integrins to activate G_o and induce cell migration. *J. Biol. Chem.* 280: 39050–39057.
18. Chen, Y., R. Corriden, Y. Inoue, L. Yip, N. Hashiguchi, A. Zinkernagel, V. Nizet, P. P. Insel, and W. G. Junger. 2006. ATP release guides neutrophil chemotaxis via P2Y₂ and A3 receptors. *Science* 314: 1792–1795.
19. Ferrari, D., C. Pizzirani, E. Adinolfi, R. M. Lemoli, A. Curti, M. Idzko, E. Panther, and F. Di Virgilio. 2006. The P2X₇ receptor: a key player in IL-1 processing and release. *J. Immunol.* 176: 3877–3883.
20. Labasi, J. M., N. Petrushova, C. Donovan, S. McCurdy, P. Lira, M. M. Payette, W. Brissette, J. R. Wicks, L. Audoly, and C. A. Gabel. 2002. Absence of the P2X₇ receptor alters leukocyte function and attenuates an inflammatory response. *J. Immunol.* 168: 6436–6445.
21. Chessell, I. P., J. P. Hatcher, C. Bountra, A. D. Michel, J. P. Hughes, P. Green, J. Egerton, M. Murfin, J. Richardson, and W. L. Peck. 2005. Disruption of the P2X₇ purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* 114: 386–396.
22. Stokes, L., L.-H. Jiang, L. Alcaraz, J. Bent, K. Bowers, M. Fagura, M. Furber, M. Mortimore, M. Lawson, J. Theaker, et al. 2006. Characterization of a selective and potent antagonist of human P2X₇ receptors, AZ11645373. *Br. J. Pharmacol.* 149: 880–887.
23. Honore, P., D. Donnelly-Roberts, M. T. Namovic, G. Hsieh, C. Z. Zhu, J. P. Mikusa, G. Hernandez, C. Zhong, D. M. Gauvinn, P. Chandran, et al. 2006. A-740003, a novel and selective P2X₇ receptor antagonist, dose-dependently reduces neuropathic pain in rat. *J. Pharmacol. Exp. Ther.* 319: 1376–1385.
24. Sluyter, R., J. G. Dalitz, and J. S. Wiley. 2004. P2X₇ receptor polymorphism impairs extracellular adenosin 5'-triphosphate-induced interleukin-18 release from human monocytes. *Genes Immun.* 5: 588–591.
25. Dinarello, C. Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist. 1998. *Int. Rev. Immunol.* 16: 457–499.
26. Bowler, J. W., R. J. Bailey, R. A. North, and A. Surprenant. 2003. P2X₄, P2Y₁, and P2Y₂ receptors on rat alveolar macrophages. *Br. J. Pharmacol.* 140: 567–575.
27. Young, M. T., P. Pelegrin, and A. Surprenant. 2006. Identification of Thr²⁸³ as a key determinant of P2X₇ receptor function. *Br. J. Pharmacol.* 149: 261–268.
28. Leonard, E. J., A. Skeel, T. Yoshimura, and J. Rankin. 1993. Secretion of monocyte chemoattractant protein-1 (MCP-1) by human mononuclear phagocytes. *Adv. Exp. Med. Biol.* 351: 55–64.
29. Buell, G., C. Lewis, G. Collo, R. A. North, and A. Surprenant. 1996. An antagonist-insensitive P2X receptor expressed in epithelia and brain. *EMBO J.* 15: 55–62.
30. Wildman, S. S., R. J. Unwin, and B. F. King. 2003. Extended pharmacological profiles of rat P2Y₂ and rat P2Y₄ receptors and their sensitivity to extracellular H⁺ and Zn²⁺ ions. *Br. J. Pharmacol.* 140: 1177–1186.
31. Gonzalez, F. A., G. A. Weisman, L. Erb, C. I. Seye, G. Y. Sun, B. Velazquez, M. Hernandez-Perez, and N. E. Chorna. 2005. Mechanisms for inhibition of P2 receptors signalling in neural cells. *Mol. Neurobiol.* 31: 65–79.
32. Flores, R. V., M. G. Hernandez-Perez, E. Aquino, R. C. Garrad, G. A. Weisman, and F. A. Gonzalez. 2005. Agonist-induced phosphorylation and desensitization of the P2Y₂ nucleotide receptor. *Mol. Cell. Biochem.* 280: 35–45.
33. Abbott, K. L., A. M. Robida, M. E. Davis, G. K. Paviath, J. M. Camden, J. T. Turner, and T. J. Murphy. 2000. Differential regulation of vascular smooth muscle nuclear factor κ -B by G α q-coupled and cytokine receptors. *J. Mol. Cell. Cardiol.* 32: 391–403.
34. Dawson, J., W. Miltz, A. K. Mir, and C. Wiessner. 2003. Targeting monocyte chemoattractant protein-1 signalling in disease. *Exp. Opin. Ther. Targets* 7: 35–48.
35. Daly, C., and B. J. Rollins. 2003. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. *Microcirculation* 10: 247–257.
36. Charo, I. F., and M. B. Taubman. 2004. Chemokines in the pathogenesis of vascular disease. *Circ. Res.* 95: 858–866.
37. Seiffert, K., W. Ding, J. A. Wagner, and R. D. Granstein. 2006. ATP γ S enhances the production of inflammatory mediators by a human dermal endothelial cell line via purinergic receptor signalling. *J. Invest. Dermatol.* 126: 1017–1027.
38. J. M. Cavillon. 1994. Cytokines and macrophages. *Biomed. Pharmacother.* 48: 445–453.
39. Bruun, J. M., A. S. Lihn, S. B. Pedersen, and B. Richelsen. 2005. Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous adipose tissue: implication of macrophages resident in the adipose tissue. *J. Clin. Endocrinol. Metab.* 90: 2282–2289.
40. Liu, B., M. Poon, and M. B. Taubman. 2006. PDGF-BB enhances monocyte chemoattractant protein-1 mRNA stability in smooth muscle cells by downregulating ribonuclease activity. *J. Mol. Cell. Cardiol.* 41: 160–169.
41. Fan, J., N. M. Heller, M. Gorospe, U. Atasoy, and C. Stellato. 2005. The role of post-transcriptional regulation in chemokine gene expression in inflammation and allergy. *Eur. Respir. J.* 26: 933–947.
42. Rossi, L., R. Manfredini, F. Bertolini, D. Ferrari, M. Fogli, R. Zini, S. Salati, V. Salvestrini, S. Gulinelli, E. Adinolfi, S. Ferrari, F. Di Virgilio, M. Baccarani, and R. M. Lemoli. 2007. The extracellular nucleotide UTP is a potent inducer of hematopoietic stem cell migration. *Blood* 109: 533–542.
43. Weisman, G. A., M. Wang, Q. Kong, N. E. Chorna, J. T. Neary, G. Y. Sun, F. A. Gonzalez, C. I. Seye, and L. Erb. 2005. Molecular determinants of P2Y₂ nucleotide receptor function: implications for proliferative and inflammatory pathways in astrocytes. *Mol. Neurobiol.* 31: 169–183.