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The $P2Y_{11}$ Receptor Mediates the ATP-Induced Maturation of Human Monocyte-Derived Dendritic Cells[1]

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Recently, it has been shown that ATP and TNF-α synergize in the activation and maturation of human dendritic cells (DC); the effect of ATP was reproduced by hydrolysis-resistant derivatives of ATP and was blocked by suramin, suggesting the involvement of a P2 receptor, but the particular subtype involved was not identified. In this report we confirm that ATP and various derivatives synergize with TNF-α and LPS to induce the maturation of human monocyte-derived DC, as revealed by up-regulation of the CD83 marker and the secretion of IL-12. The rank order of potency of various analogs (AR-C67085 > adenosine 5′-*O*-(3-thiotriphosphate) = 2′- and 3′-*O*-(4-benzoyl-benzoyl) ATP > ATP > 2-methylthio-ATP) was close to that of the recombinant human $P2Y_{11}$ receptor. Furthermore, these compounds activated cAMP production in DC, in a xanthine-insensitive way, consistent with the involvement of the $P2Y_{11}$ receptor, which among P2Y subtypes has the unique feature of being dually coupled to phospholipase C and adenylyl cyclase activation. The involvement of the $P2Y_{11}$/cAMP/protein kinase A signaling pathway in the nucleotide-induced maturation of DC is supported by the inhibitory effect of H89, a protein kinase A inhibitor. Taken together, our results demonstrate that ATP activates DC through stimulation of the $P2Y_{11}$ receptor and subsequent increase in intracellular cAMP. *The Journal of Immunology,* **2001, 166: 7172–7177.**

Dendritic cells (DC)[3] are the most potent APC of the immune system, with a unique ability to induce primary immune responses (1). Circulating precursors home to tissues where they reside as immature DC dedicated to Ag capture via high endocytic and phagocytic activities. Following Ag uptake and processing, DC migrate to secondary lymphoid organs, where they mature and become APCs able to select and activate Ag-specific lymphocytes. This coordinated process of maturation is characterized by a series of phenotypic and functional changes; DC express a unique set of molecules, including Ag recognition/uptake receptors, accessory molecules, cytokines, chemokines, and chemokine receptors, which are differentially expressed at various stages of maturation. Especially, after maturation, DC display high stable MHC-peptide complexes and costimulatory molecules and produce cytokines such as IL-12. Mature/activated DC also express high levels of the CD83 molecule, a member of the Ig superfamily with unknown function, but which is relatively restricted to DC and therefore is often used as marker of DC maturation (2, 3).

Other inflammatory mediators such as PGE_2 participate in the maturation of DC. On its own, PGE_2 has little capacity to induce maturation of DC, but it acts synergistically with TNF-α, via an increase in the cAMP level (4). Vasoactive intestinal peptide, a well-known stimulus of cAMP accumulation, also synergizes with TNF-α in inducing DC maturation and IL-12 release (5). Extracellular nucleotides, which are released from damaged cells (6), could also be involved in DC biology (7). On the one hand, ATP triggers apoptosis of DC (8). On the other hand, it has been shown recently by different laboratories that extracellular ATP synergizes with TNF-α in the activation and maturation of monocyte-derived human DC. Indeed, ATP transiently enhanced endocytosis, which was followed by the up-regulation of DC surface markers (CD54, CD80, CD86, CD83), but also of MHC II molecules, IL-12 secretion, and an increased T cell stimulatory capacity (9, 10). ATP also induces the release of cytokines such as TNF-α by LPS-matured DC (7). From these data, it can be postulated that ATP might be a potent stimulus for the initiation of immune responses and that ATP derivatives could be considered as new agents, potentially interesting in vaccination strategies. In this context, it is crucial to identify the subtype of the receptor mediating this ATP effect, which is the aim of this work. ATP activates cells through the purinergic P2X or P2Y receptors; the former are ligand-gated ion channels, while the latter are protein G-coupled receptors (6, 11). Berchtold et al. (9) have recently shown by RT-PCR that human DC express all members of the P2Y subfamily ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$) and at least five P2X receptors ($P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, and $P2X_7$). Using single-cell Ca^{2+} imaging, Liu et al. (12) suggested that human DC express functional $P2Y_1$, $P2Y_2$, and/or $P2Y_4$ as well as a 2-methylthio-ATP (2-MeSATP)- and UTP-insensitive receptors. Recently, Schnurr et al. (10) showed that the effect of ATP on activation and maturation of DC was reproduced by hydrolysis-resistant derivatives of ATP and was blocked by suramin, suggesting the involvement of a P2 receptor.

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[3] Abbreviations used in this paper: DC, dendritic cell; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AR-C67085, 2-propylthio-β, γ-dichloromethylene-D-ATP; BzATP, 2′- and 3′-*O*-(4-benzoyl-benzoyl) ATP; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide H-89 · 2HCl; 2-MeSATP, 2-methylthio-ATP; 8-p-SPT, 8-(*p*-sulfophenyl)theophyline; db-cAMP, dibutyryl cAMP.

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In this report, we show that nucleotides induce maturation of DC, in synergy with TNF-α and LPS, through activation of the $P2Y_{11}$ receptors coupled to cAMP accumulation.

Materials and Methods

Media and materials

The culture medium used in this study was RPMI 1640 supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 25 mM HEPES buffer, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μg/ml gentamicin (all purchased from Life Technologies, Merelbeke, Belgium), and 5×10^{-5} M 2-ME (Merck, Darmstadt, Germany). Recombinant human GM-CSF or Leucomax was supplied by Novartis (Basel, Switzerland), and recombinant human IL-4 was supplied by R&D Systems (Abingdon, U.K.).

ATP, ADP, adenosine, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), 2′- and 3′-*O*-(4-benzoyl-benzoyl) ATP (BzATP), dATP, UTP, UDP, dibutyryl cAMP (db-cAMP), and LPS were obtained from Sigma (St. Louis, MO). 2-MeSATP and 8-(*p*-sulfophenyl)theophyline (8-p-SPT) were purchased from Research Biochemicals International (Natick, MA). H-89 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Rolipram was a gift from the Laboratoires Jacques Logeais (Trappes, France). AR-C67085 was a gift from Drs. Turner and Leff (Astrazeneca R&D Charnwood, Loughborough, U.K.). TNF-α was a gift from Dr. Adolf (Roche, Mannheim, Germany).

DC generation and maturation

Immature human DC were derived from adherent peripheral blood monocytes of normal donors as described by Romani et al. (13). In brief, PBMCs were isolated from leukocyte-enriched buffy coats by standard density gradient centrifugation on Lymphoprep solution from Nycomed (Oslo, Norway) and resuspended in complete medium, and 2×10^8 PBMCs were allowed to adhere for 2 h at 37°C at 5% CO_2 in air in 75-cm^2 cell culture flasks. Nonadherent cells were removed and rinsed adherent cells were cultured in 15 ml of complete medium supplemented with 800 U/ml of GM-CSF and 1000 U/ml of IL-4. GM-CSF and IL-4 were added twice a week. After 6 days of incubation, cells were replated at $3–4 \times 10^5$ cells/ml in 24-multiwell plates in complete medium with GM-CSF and IL-4. Nucleotides or other tested agents were added the next day for 48 h. The purity of each cell preparation has been evaluated by determining the expression of CD1a, HLA-DR, CD14, and CD3; 90–95% of cells were CD1a and HLA-DR positive.

Flow cytometric analysis

Cells staining was performed using FITC-conjugated anti-human CD83 (IgG1,κ, HB15e) supplied by BD PharMingen (San Diego, CA) on 2×10^5 cells in 100 μl of PBS with 0.1% sodium azide for 30 min in the dark at 4°C. After washing with 2 ml of PBS, samples were analyzed on a FACSort (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed and presented using WinMDI (version 2.6) software (J. Trotter, The Scripps Research Institute, San Diego, CA); the number of gated events were at least 5000. EC_{50} values were obtained by curve fitting (Sigma Plot, version 2.0).

Quantitation of IL-12

Human IL-12 was quantified by ELISA using a commercially available kit (BioSource International, Camarillo, CA) that recognizes both bioactive p70 heterodimer and free p40 subunit.

cAMP measurements

Cells were preincubated for 20 min in RPMI 1640 supplemented with 25 mM HEPES buffer and 25 μM rolipram and incubated in the same medium for 10 min in the presence of the agonists. The incubation was stopped by the addition of 0.1 M HCl. The incubation medium was dried up, resuspended in water, and diluted as required. cAMP was quantified by RIA after acetylation as previously described (14).

Quantitative RT-PCR assay of $P2Y_{11}$ mRNA

Total RNA was isolated using the Tripur isolation reagent from Roche Diagnostics (Basel, Switzerland). To avoid DNA contamination, 1 μg of total RNA was treated with DNA-free from Ambion (Austin, TX). RT was performed with 500 ng of RNA using the Superscript II preamplification system with random hexamers (Life Technologies). The primers and TaqMan fluorogenic probe for $P2Y_{11}$ (GenBank accession no. AF030335) were obtained from Eurogentec (Seraing, Belgium): forward (nt 19–39), 5′-CTG CCC TGC CAA CTT CTT G-3′; reverse $P2Y_{11}$ (nt 76–96), 5′-CAG TAT GGG CCA CAG GAA GTC-3′; and probe (nt 45–69), 5′-(Fam)-TGC CGA CGA CAA ACT CAG TGG GTT-(Tamra)-3′. TaqMan PCR assays were performed in duplicate on cDNA samples or RNA in 96-well optical plates on an Applied Biosystems Prism 7700 Sequence Detection system (PE Applied Biosystems, Foster City, CA). For each 25-μl reaction, 1/20 RT product was mixed with 12.5 μl of 2× TaqMan Universal PCR Master Mix (PE Applied Biosystems), primers (300 nM each), and fluorogenic probe (200 nM). PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Results for $P2Y_{11}$ were normalized to GAPDH (Eurogentec). Data were analyzed with Sequence Detector software (SDS version 1.6; PE Applied Biosystems).

Results

Maturation of DC was studied in this work by measuring the expression of the CD83 marker and the production of IL-12. As previously reported, DC incubated 48 h with TNF-α (1 ng/ml) modestly expressed the CD83 protein at their surfaces (Fig. 1). The TNF-α effect involved only a portion of the population (~20%) due to the low concentration of the cytokine. When DC were stimulated with ATP alone (1 mM), we also detected a small portion of the cell population (~30%) that was weakly CD83 positive (Fig. 1). TNF-α and ATP acted synergistically, so that 90% of the cell population was CD83 positive, and the synergy was also apparent on the level of expression, as judged by the increase in the mean

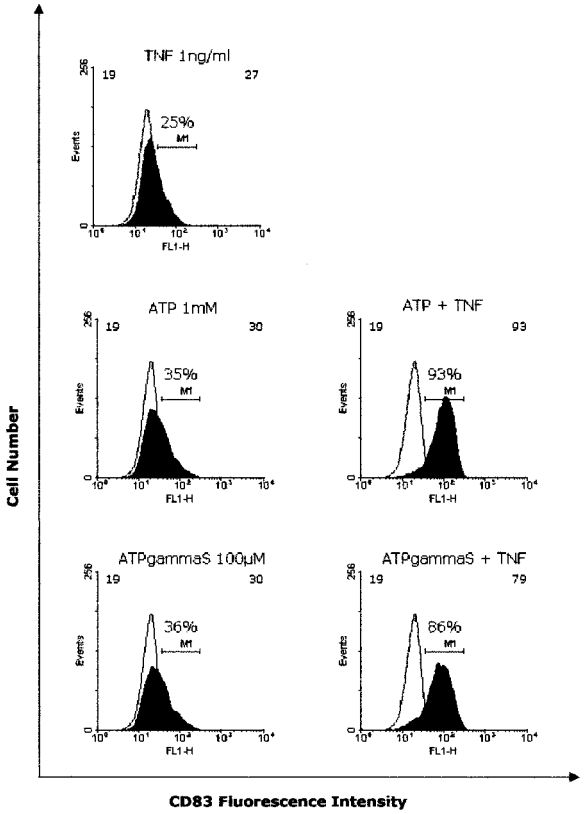

FIGURE 1. Effects of the combination of TNF-α with ATP or ATPγS on CD83 expression in human DC. Day 7 DC were incubated with ATP (1 mM), ATPγS (100 μM), TNF-α (1 ng/ml), or the combination of ATP or ATPγS plus TNF-α. After 48 h, cells were harvested, and CD83 expression was measured by flow cytometry. Control cells (3% of $CD83^+$ cells) are represented by an open histogram and stimulated cells are shown by a filled histogram. These data are representative of at least four independent experiments. The number in the *left corner* corresponds to the $CD83^+$ mean of control cells and that in the *right corner* corresponds to the $CD83^+$ mean of stimulated cells.

fluorescence intensity (Fig. 1). Because DC exhibit a strong ectonucleotidase activity (9), the ATP effect could be due to its degradation into ADP, AMP, and adenosine. Indeed, in some preparations of DC (five of seven), adenosine and ADP synergized with TNF-α to differentiate DC. This effect was lower than that of ATP at the same concentration and was inhibited by 8-p-SPT, an A_2 receptor antagonist (data not shown). Moreover, we tested the effect of ATPγS, a hydrolysis-resistant analog of ATP. At 100 μM, ATPγS reproduced the effect of ATP 1 mM (Fig. 1), and its effect was insensitive to 8-p-SPT (data not shown), suggesting the involvement of a P2 receptor.

We thereafter compared the concentration-action curves characterizing the effects of nucleotides on CD83 expression in DC treated with TNF-α (Fig. 2*A*). The rank order of agonist potency was AR-C67085 > ATPγS = BzATP > ATP. In the presence of TNF-α, the EC_{50} values were 4.0 ± 1.3 μM for AR-C67085 and 27 ± 4.0 μM for ATPγS (mean of three independent experiments). The EC_{50} for AR-C67085 alone was 36.2 ± 4.6 μM (data

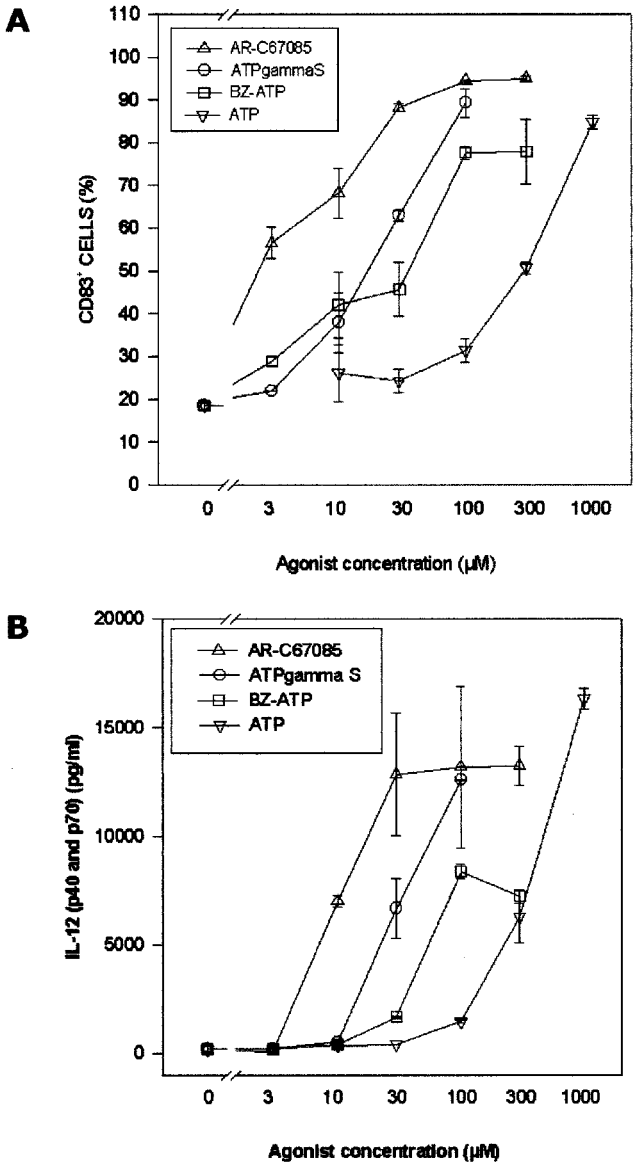

FIGURE 2. Concentration-action curves for AR-C67085, ATPγS, BzATP, and ATP on CD83 expression (*A*) and on IL-12 secretion (*B*) in human DC. The cells were incubated with AR-C67085, ATPγS, BzATP, and ATP at various concentrations for 48 h in the presence of TNF-α (1 ng/ml); CD83 expression was measured by flow cytometry (*A*); and supernatants were harvested for IL-12 ELISA (*B*). Data are given as the mean ± range of duplicate points and are representative of three independent experiments.

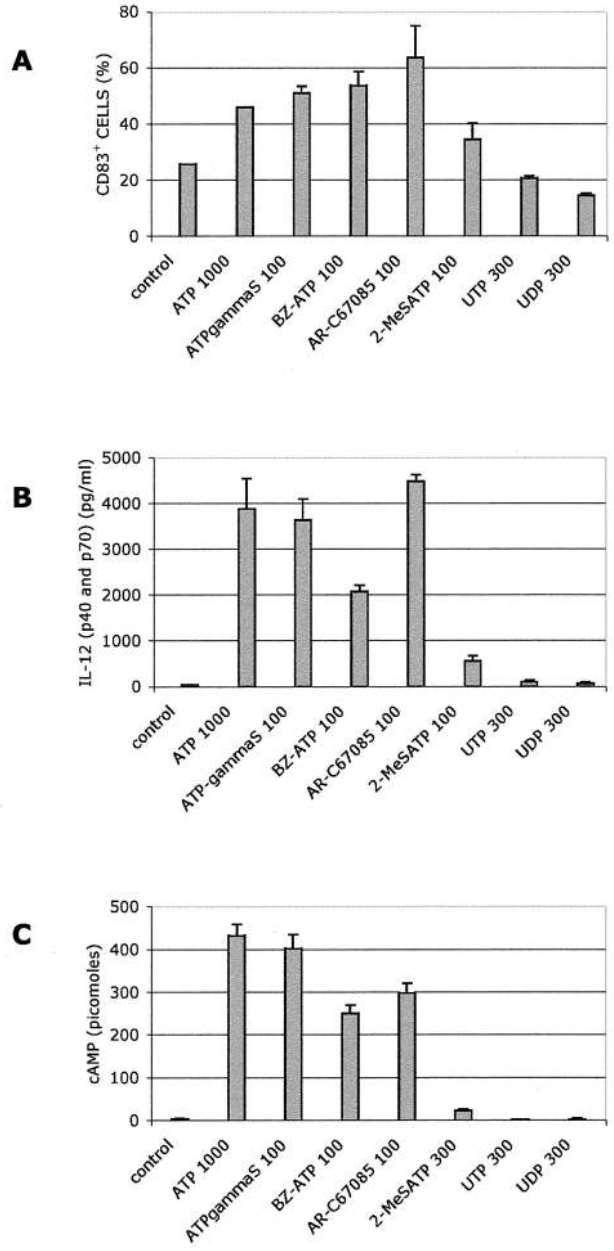

FIGURE 3. Effects of the different nucleotides, alone or in combination with TNF-α, on CD83 expression (*A*), IL-12 production (*B*), and cAMP accumulation (*C*) in human DC. *A*, DC were incubated with nucleotides at maximal concentration in combination with TNF-α (1 ng/ml). After 48 h, cells were harvested and CD83 expression was measured by flow cytometry. Data are given as the mean ± range of duplicate points and are representative of at least four independent experiments. *B*, DC were incubated with nucleotides at maximal concentration in combination with TNF-α (1 ng/ml). After 48 h, supernatants were harvested for IL-12 ELISA. Data are given as the mean ± range of duplicate points and are representative of at least three independent experiments. *C*, DC were preincubated for 20 min in RPMI 1640/HEPES buffer with rolipram and incubated in the same medium for 10 min in the presence of nucleotides at maximal concentration. cAMP was quantified by RIA after acetylation. Data are given as the mean ± SD of triplicate points and are representative of three independent experiments. The values close to the name of the agonist represent the micromolar concentrations.

not shown). Similar concentration-action curves were observed for IL-12 release (Fig. 2*B*).

As shown on Fig. 3, *A* and *B*, UTP and UDP did not enhance the expression of CD83 or IL-12 production, while 2-MeSATP had a very small effect. In the same experiment, we measured the production of cAMP in DC (Fig. 3*C*). ATP, ATPγS, BzATP, and AR-C67085 stimulated the production of cAMP, while UTP and UDP were inactive, and 2-MeSATP had a very modest effect. This increase in cAMP was insensitive to 8-p-SPT, an A_2 receptor antagonist (data not shown). We have confirmed the observation by Schnurr et al. (10) that α,β-methylene-ATP and β,γ-methylene-ATP are active on DC, as evaluated by CD83 expression and IL-12 release, and found that this activity was associated with an increase in cAMP as it was in CHO-K1 cells expressing the human recombinant $P2Y_{11}$ (data not shown).

Both the rank order of potency characterizing the DC-differentiating effect and the xanthine-insensitive stimulation of cAMP by these nucleotides are consistent with the involvement of $P2Y_{11}$ receptors (15, 16). Therefore, the $P2Y_{11}$ mRNA was quantified by RT-PCR using TaqMan fluorogenic detection. These experiments revealed a significant expression of $P2Y_{11}$ messengers, which was similar in monocytes, immature DC, and DC activated by TNF-α (Table I).

To ascertain that the stimulation of the cAMP/protein kinase A signaling pathway mediates the maturation effect of the nucleotides observed here, we tested the effects of activators or inhibitors of the cAMP signaling pathways such as db-cAMP (a cell-permeable activator of the protein kinase A), forskolin (an activator of the adenylyl cyclase), and H-89 (an inhibitor of the protein kinase A) (17) on the expression of CD83 and the IL-12 production. The cAMP analog by itself up-regulated the expression of CD83 and synergized with TNF-α to mature DC. The production of IL-12 induced by db-cAMP (100 μM) was comparable to that obtained in response to ATPγS (data not shown). In the same manner, CD83 expression was induced by forskolin (10 μM; data not shown). Due to the high toxicity of H-89 after 24 and 48 h of treatment, we modified the protocol of stimulation to test the effect of this compound. The DC were pretreated with H-89 for 90 min and then stimulated by ATPγS alone or with TNF-α for 2 h (in the presence of H-89); thereafter, they were cultured overnight with or without TNF-α. Treatment of the cells with H-89 inhibited the up-regulation of CD83 expression by ATPγS (100% inhibition with ATPγS alone and 70% inhibition with ATPγS in the presence of TNF-α), while the effect of TNF-α alone was unchanged (Fig. 4*A*). H-89 also inhibited the release of IL-12 induced by ATPγS alone or in combination with TNF-α (Fig. 4*B*).

ATP (1 mM) or ATPγS (100 μM) was also able to act in synergy with LPS (1 ng/ml), another inflammatory mediator, on CD83 expression (Fig. 5, *A* and *C*). When the production of IL-12 was studied, we also observed a synergy between ATP or ATPγS and LPS (Fig. 5, *B* and *D*).

Table I. *Expression of $P2Y_{11}$ and GAPDH in monocytes and DC in the presence or absence of TNF-α (5 ng/ml) during 48 h*[a]

	$P2Y_{11}$ Copy No. ± SD	GAPDH Copy No. ± SD	Ratio of $P2Y_{11}$:GAPDH ($\times 10^3$) ± SD
Monocytes	3,630 ± 171	213,605 ± 3,789	17.0 ± 0.3
DC	22,001 ± 1,594	1,086,818 ± 6,276	20.2 ± 1.5
DC TNF	15,062 ± 1,024	682,044 ± 101	22.1 ± 1.5

[a] The copy number of $P2Y_{11}$ and GAPDH is calculated using the standard curve method.

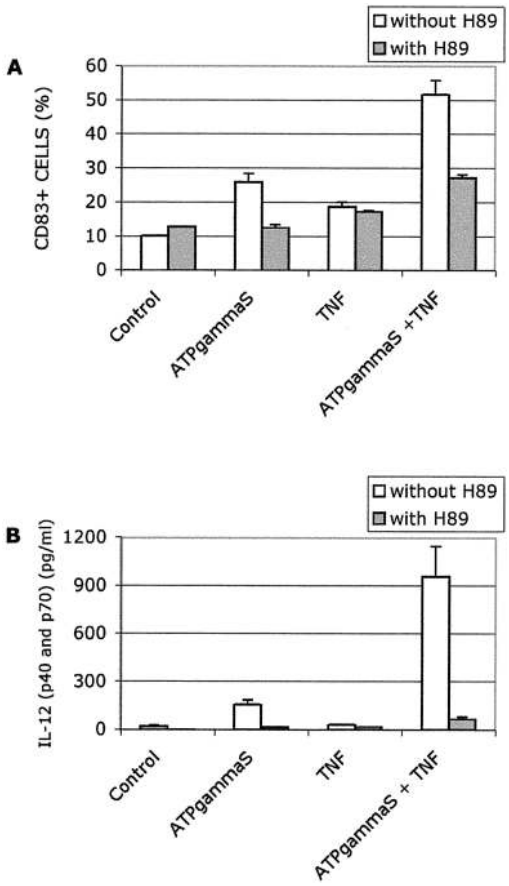

FIGURE 4. Effects of H-89 on CD83 expression (*A*) and on IL-12 secretion (*B*) in human DC. DC were pretreated with H-89 (30 μM) for 90 min, followed by stimulation in the presence of H-89 with ATPγS, either alone or in the presence of TNF-α for 2 h, then rinsed twice and cultured for 16 h in the presence or the absence of TNF-α. After 16 h, cells were harvested, CD83 expression was measured by flow cytometry (*A*), and supernatants were harvested for IL-12 ELISA (*B*). Data are given as the mean ± range of duplicate points and are representative of three independent experiments.

Discussion

Recent data argue for a crucial role of extracellular ATP on the biology of human DC. By RT-PCR, Berchtold et al. (9) have shown that human monocyte-derived DC express mRNA for all P2Y receptors known to date and at least for four P2X receptors. Using single-cell Ca^{2+} imaging, it has been suggested that human TNF-α-treated DC express functional $P2Y_1$, $P2Y_2$, and/or $P2Y_4$ and a 2-MeSATP- and UTP-insensitive P2Y receptor (12). Some biological functions mediated by these receptors have been suggested. Coutinho-Silva et al. (8) showed that ATP at millimolar concentrations induces rapid apoptotic cell death through activation of the $P2X_7$ receptor in both human and murine DC. Recently, it has been suggested that the $P2X_7$ receptor is involved in the increase in intracellular Ca^{2+}, the membrane depolarization, and the secretion of inflammatory cytokines by human DC in response to ATP (7). The same $P2X_7$ could be involved in Ag presentation by murine DC (18). In the same cells UTP and UDP, but not ATP, mobilized intracellular calcium and stimulated cytokine production, presumably via activation of the $P2Y_6$ receptor (19).

In the present study, we have demonstrated that ATP and the ATP derivatives, ATPγS, BzATP, and AR-C67085, synergized with TNF-α to induce de novo expression of CD83 Ag and production of high levels of IL-12 in human monocyte-derived DC.

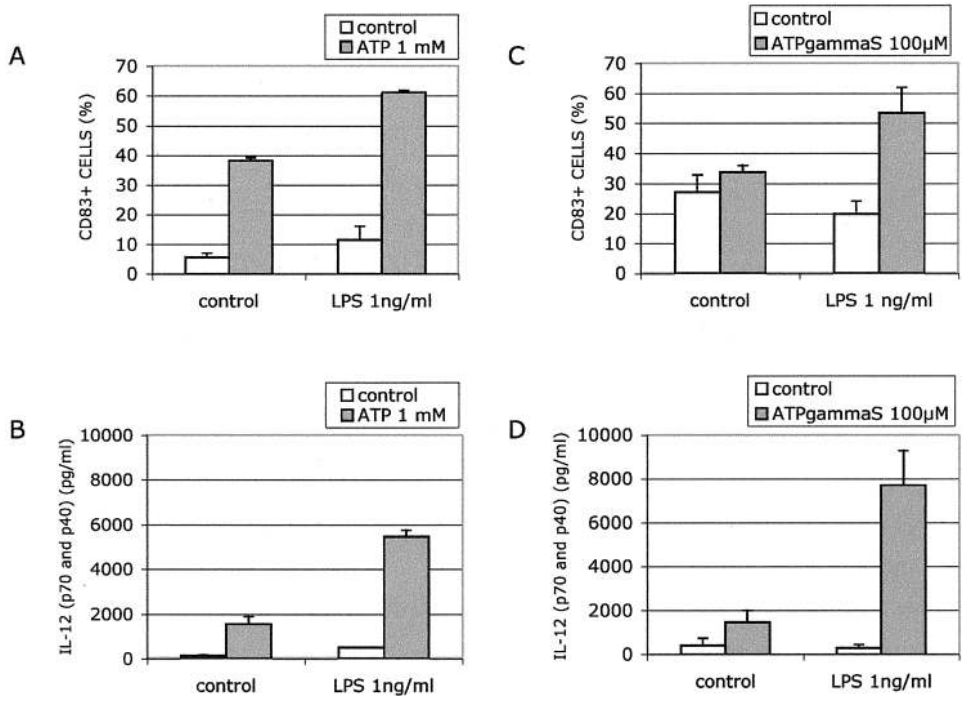

FIGURE 5. Effects of the combination of LPS with ATP (*A* and *B*) and ATPγS (*C* and *D*) on CD83 expression and IL-12 secretion in human DC. DC were incubated with 1 mM ATP or 100 μM ATPγS, either alone or in combination with 1 ng/ml LPS. *A* and *C*, After 48 h, cells were harvested and CD83 expression was measured by flow cytometry. *B* and *D*, After 48 h, supernatants were harvested for IL-12 ELISA. Data are given as the mean ± range of duplicate points and are representative of at least three independent experiments.

This is consistent with previous reports that demonstrated additive or synergistic effects of ATP and TNF-α on up-regulation of the cell surface markers, CD80, CD83, and CD86, during the maturation of DC (9). Since these authors have tested only ATP and shown that DC express an important ecto-nucleotidase activity, the effect of ATP might have resulted from its degradation into adenosine and activation of adenosine rather than P2 receptors. Schnurr et al. (10) have shown that ATP induces CD86 expression, increases IL-12 production, and enhances the T cell stimulatory capacity in human DC. The lack of adenosine action, the reproduction of the ATP effect by hydrolysis-resistant analogs, and the inhibition by suramin supported the involvement of P2 receptors, but the particular P2 subtype involved was not identified.

Knowing that PGE_2, forskolin, or db-cAMP enhances the maturation of human DC at least with TNF-α as maturation factor (4), we made the hypothesis that the $P2Y_{11}$ receptor might be involved in DC maturation. Indeed, among P2 receptors, the $P2Y_{11}$ receptor has the unique feature of being dually coupled to phospholipase C and adenylyl cyclase stimulation. The presence of $P2Y_{11}$ mRNA in DC, either immature or mature, as well as in the monocyte precursors, was indeed demonstrated by quantitative RT-PCR. Expression of functional $P2Y_{11}$ receptors on human DC was confirmed by the observation that known agonists of the human recombinant $P2Y_{11}$ receptor, AR-C67085, ATPγS, BzATP, and ATP (16), increased cAMP in DC in a xanthine-insensitive way, thus excluding the role of adenosine receptors. The rank order of potency with which nucleotides enhanced the effect of TNF-α on CD83 expression and IL-12 production was similar to that characterizing the recombinant human $P2Y_{11}$ receptor: AR-C67085 > ATPγS = BzATP > ATP > 2-MeSATP (16). On the other hand, Schnurr et al. (10) have shown that the activating effect of nucleotides on DC was abolished by suramin, which is a potent competitive antagonist of the recombinant $P2Y_{11}$ receptor (16). The involvement of other P2Y receptors can be excluded. Indeed, the lack of a UTP effect is inconsistent with a role of either $P2Y_2$ or $P2Y_4$ receptor, the inactivity of UDP excludes the $P2Y_6$ receptor, and the low potency of 2-MeSATP as well as the variable effect of ADP and its sensitivity to 8-p-SPT are incompatible with a role for the $P2Y_1$ receptor (6, 11). On the other hand, AR-C67085, the most potent activator of DC found in this study, behaves as a weak partial agonist in tissues containing P2X receptors (20).

Rieser et al. (4) have shown that db-cAMP as well as agents increasing the endogenous cAMP level, such as forskolin and PGE_2, enhance the maturation of human DC. Our results with $P2Y_{11}$ agonists are consistent with the concept that the cAMP and TNF-α signal transduction pathways cooperate in the maturation of DC. Similarly, in human monocytes, elevation of cAMP synergized with TNF-α to up-regulate the synthesis of IL-1β (21). In contrast to the synergism between PGE_2 and TNF-α, Rieser et al. (4) have reported that PGE_2 inhibited the LPS-induced synthesis of IL-12 by human DC. In contrast with these results, we have observed a synergism between ATPγS and LPS. This discrepancy could be due to concentration of LPS, as we did not observe such synergism when 10 ng/ml LPS was used (data not shown).

In conclusion, our results suggest that the $P2Y_{11}$ receptor plays an immunomodulatory role by activating DC.

Acknowledgments

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