Extracellular nucleotide signaling by P2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the cAMP pathway

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The interleukin-12 (IL-12) cytokine family plays important roles in the orchestration of innate and adaptive immunity by dendritic cells (DCs). The regulation of IL-12 expression has been thoroughly studied, but little is known about factors governing the expression of IL-23 and IL-27, 2 novel IL-12 family members acting on memory and naive T cells, respectively. We report that the expression of these cytokines by DCs was critically dependent on the mode of activation. DC activation by CD40L predominantly induced IL-12. Ligands of the Toll-like receptor (TLR) 3 and TLR4 induced IL-12 and IL-27, whereas exposure to intact Escherichia coli resulted in high expression of IL-12, IL-27, and IL-23. The nucleotide adenosine triphosphate (ATP) has been shown to inhibit IL-12 production by P2 receptors. We found that ATP also inhibited IL-27 expression but enhanced IL-23 expression. Interestingly, the reciprocal regulation of IL-12/IL-27 and IL-23 by ATP was mediated by 2 distinct P2 receptors and was also induced by prostaglandin E2 by cyclic adenosine monophosphate (cAMP)–elevating EP2/EP4 receptors. As a consequence, DCs were selectively impaired in their ability to induce interferon-γ (IFN-γ) in naive T cells but continued to promote IFN-γ and IL-17 production in memory T cells. These studies identify P2 receptors as promising targets for the design of novel strategies to manipulate specific stages of T-cell responses and to treat IL-12- and IL-23–mediated disorders. (Blood. 2005;105:1582-1589)
adenosine monophosphate (cAMP).25 These include EP2/EP4 receptors for prostaglandin E2 (PGE2), the histamine receptor H2, and the adenosine receptor A2a. The efficacy of cAMP signaling as a negative regulatory mechanism of IL-12 production can also be demonstrated with cAMP analogs and cholera toxin, a potent activator of Galpha. Potent inhibition of IL-12 production has also been reported for extracellular nucleotides, such as adenosine triphosphate (ATP), through the activation of membrane-bound P2 receptors.26 P2 receptors are subdivided into P2X, a family of ligand-gated ion channels, and G-protein–coupled P2Y receptors. We and others have previously reported that human DC types express several subtypes of P2X and P2Y receptors.7,27-31 Initial studies reported an induction of IL-12p40 expression by ATP, especially in synergy with TNF-α, with pharmacologic data using the synthetic nucleotides ATPγS and AR-C67085 pointing to the P2Y11 receptor and cAMP signaling.32,33 With the availability of the P2Y11 receptor and by cAMP signaling,34 However, an interaction with other P2 receptors and signaling pathways cannot be excluded because ATP activates multiple P2 receptor subtypes in DCs.31

To date, little is known about the regulation of IL-23 or IL-27 expression by human DCs. Expression of IL-23 and IL-27 subunits has been reported for monocytic-derived DCs (MoDCs) activated with combinations of cytokines, CD40L and TLR ligands, or intact bacteria,7,16,35-37 but systematic studies comparing the influence of specific stimuli are lacking. Therefore, we have studied how these molecules influence the ability of DCs to activate naive and memory CD4+ T-cell subsets.

Materials and methods

DC culture and activation

Peripheral blood mononuclear cells (PBMCs) from healthy blood donors (Red Cross Blood Bank, Melbourne, Australia) were prepared by Ficoll–Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). To generate MoDCs, CD14+ monocytes were isolated using MACS magnetic beads (Miltenyi Biotech, Auburn, CA) and were cultured in RPMI/10% fetal calf serum (FCS) supplemented with 20 ng/mL granulocyte macrophage–colony-stimulating factor (GM-CSF) and 500 U/mL IL-4 for 6 days, as reported.19 MoDCs were replated at 5×10^5 cells/mL in their conditioned media and were stimulated with the following combinations of stimuli: CD14+ monocytes were cultured with intact bacteria (5×10^7/mL), ATP (50 μM), and IL-12 (50 pg/mL). After activation and were stimulated with CD3/CD28 T-cell Expander Dynabeads (Dynal Biotech, Oslo, Norway) according to the manufacturer’s instructions. Supernatant was collected from the T-cell cultures after 4 days for IFN-γ and IL-17 ELISA.

Isolation and activation of mouse T cells

Human CD4+ T cells were positively selected from PBMCs using MACS magnetic beads (Miltenyi Biotech). CD4+ T cells were stained with fluorochrome-labeled anti-CD45RA and anti-CD45RO mAb (BD PharMingen), and cells were sorted using a MoFlo cell sorter (Cytomation, Fort Collins, CO). T cells (5×10^5/mL) were cultured in RPMI 1640/10% FCS with 1% to 20% (vol/vol) MoDC-derived supernatant. Blocking mAbs against IL-12p70 (clone 20C2; BD PharMingen), IL-12p40/p70 (clone C8.6; BD PharMingen), and an IL-23 receptor/Fc chimeric protein (R&D Systems) were all used at 10 μg/mL. Reagents containing azide were diazoyzed with saline before use.

RNA was isolated from MoDCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized as previously described.33 One microliter cDNA was used as template for quantitative real-time polymerase chain reaction (RT-qPCR). Gene expression levels were quantified using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Predeveloped assay reagents (PDARs) for IL-12p35 and IL-12p40 were obtained from Applied Biosystems and were used in multiplex reactions with 18S rRNA PDAR (Applied Biosystems) for normalization. Primers and probes were as follows: IL-23p19, forward 5′-GGGCTGTCTTGCCATG-3′, reverse 5′-GAGTTGCCATCCTTGAGCTGC-3′; EB1, probe 6FAM-TGGGGCCTAAGGCTCAGTAC-TAMRA, forward 5′-CCGAGGAGCTGCTCAC-3′, reverse 5′-CCAGCTCACTAGTCTCCGG-3′, IL-27p28, probe 6FAM-AGT-GAACGTATACGCTGGCTGT-3′, forward 5′-GCCGAATCTCACCTGCACA-3′, reverse 5′-GGAAACATCGGAGACGT-3′. PCR reactions were set up in 96-well plates and were analyzed using SDS program version 1.9 (Applied Biosystems, Foster City, CA). Relative expression was calculated using the ΔCt method and was expressed relative to a calibrator, in this case MoDCs cultured in the presence of GM-CSF and IL-4. ΔCt = Ctprobe – CtE18S; ΔCtSample = ΔCtSample – ΔCt GM-CSF; relative expression = 2 –ΔΔCtSample.

Cytokine ELISA

Cytokine enzyme-linked immunosorbent assay (ELISA) kits (BD OptiEIA; BD Biosciences, San Diego, CA) were used to quantify IL-12p70, IL-12p40, and IFN-γ (BD Biosciences). Human IL-17 was detected using the IL-17 Cytoscreen kit (Biosource Europe SA, Nivelles, Belgium) and mouse IL-17 with the Quantikine M kit (R&D Systems, Minneapolis, MN).

Isolation and activation of mouse splenocytes

Splenocytes were isolated from C57BL/6J mice using Thy 1.2+ (CD90) MACS magnetic beads (Miltenyi Biotech). T cells (10^6/mL) were cultured for 6 days in 96-well flat-bottom plates with RPMI 1640/10% FCS supplemented with 100 U/mL IL-2 and 1% to 20% (vol/vol) MoDC-derived supernatant. Blocking mAbs against IL-12p70 (clone 20C2; BD PharMingen), IL-12p40/p70 (clone C8.6; BD PharMingen), and an IL-23 receptor/Fc chimeric protein (R&D Systems) were all used at 10 μg/mL. Reagents containing azide were diazoyzed with saline before use.

Results

IL-12, IL-23, and IL-27 production by MoDCs in response to specific stimuli

To assess the potency of different classes of stimuli to induce IL-12 production, MoDCs were cultured with intact bacteria (E coli), influenza virus (strain PR-8), Pam3Cys (TLR2 ligand), poly(I:C) (TLR3 ligand), LPS (TLR4 ligand), R-848 (TLR7/8 ligand), CpG ODN2006 (TLR9 ligand), proinflammatory mediators (combination of TNF-α, IL-1β, IL-6, and PGE2), ATP, or CD40L. Secretion

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of IL-12p40 (Figure 1A) and bioactive IL-12p70 (Figure 1B) was analyzed by ELISA. Striking differences between these stimuli were observed. Stimulation of MoDCs with intact E coli induced the highest levels of IL-12p70 (10.89 ± 4.34 ng/mL). Moderate levels of IL-12p70 were induced by CD40L (0.75 ± 0.30 ng/mL) and LPS (0.33 ± 0.25 ng/mL), whereas low levels were induced by poly(I:C) (0.09 ± 0.09 ng/mL) and R-848 (0.06 ± 0.11 ng/mL). Consistently, the IL-12-40 subunit was produced in excess of IL-12p70. Activation of MoDCs with proinflammatory cytokines induced high levels of IL-12p40 (19.41 ± 4.95 ng/mL) but no detectable IL-12p70. Similarly, ATP induced only IL-12p40 (1.70 ± 0.80 ng/mL). Finally, Pam3Cys (and other TLR2 ligands, such as peptidoglycan; data not shown) and CpG ODN failed to induce significant levels of IL-12p40.

Excessive IL-12p40 levels could reflect the presence of inactive monomers, homodimers, or IL-23p40/p19 heterodimers. To assess whether high levels of p40 induced by some stimuli, such as proinflammatory cytokines, reflected IL-23 production, we analyzed IL-23p19 expression during the 48 hours (Figure 4D-E). Inhibitory and stimulatory effects of ATP on IL-12, IL-27, and IL-23 expression mirrored the influences the expression pattern of IL-12–related cytokines, with IL-23 and IL-27 expression strongly induced by intact microbes.

**Regulation of IL-12, IL-23, and IL-27 expression by ATP**

Having identified E coli as a potent inducer of IL-12, IL-23, and IL-27 expression enabled us to investigate the regulation of these 3 cytokines by physiologic modulators of cytokine production. The nucleotide ATP has inhibitory effects on IL-12p70 expression through P2 receptors. To investigate whether ATP also affects other IL-12–related cytokines, we stimulated MoDCs with E coli in the absence or presence of ATP. As assessed by RT-qPCR and ELISA, ATP inhibited the expression of IL-12p35 and IL-12p40 (Figures 3A, 4A-B), resulting in reduced levels of IL-12p70 (Figure 3B). In contrast, IL-23p19 expression was enhanced (Figure 3C). Additionally, ATP suppressed IL-27p28 expression (Figure 3D) but had only a minor effect on EBI3 expression (Figure 3E). Thus, the expression of IL-12 and IL-27 is regulated in a manner reciprocal to that of IL-23 by ATP. This regulation pattern of IL-12–related cytokines by ATP was also observed for MoDCs stimulated with CD40L or LPS (data not shown). To exclude that these observations were attributed to disparities in the kinetics of cytokine synthesis, we analyzed cytokine expression over a 48-hour time period (Figure 4). The expression of IL-12p40, IL-12p35, and IL-23p19 was up-regulated within the first 6 hours after activation with E coli, peaking at approximately 9 to 12 hours and decreasing to background levels by 24 to 48 hours (Figure 4A-C). In contrast, the up-regulation of IL-27p28 expression was more rapid, peaking at 6 hours and rapidly decreasing thereafter, whereas EBI3 expression increased gradually and was sustained during the 48 hours (Figure 4D-E). Inhibitory and stimulatory effects of ATP on IL-12, IL-27, and IL-23 expression mirrored the
kinetic profile observed in the absence of ATP, arguing against disparities in the kinetics of cytokine synthesis.

Regulation of IL-12 and IL-23 expression by ATP is mediated by different P2 receptors

DCs express different subtypes of P2 receptors with distinct pharmacologic properties. To identify the P2 receptors involved in the regulation of IL-12, IL-23, and IL-27 expression, we assessed the rank order of potency of various nucleotides on cytokine expression. ATP inhibited IL-12p40 and IL-12p70 production of E. coli–stimulated MoDCs in a dose-dependent fashion (concentration producing 50% of the maximum rate of inhibition [EC50], approximately 100 μM) (Figure 5A-B). Less efficient IL-12 inhibition was seen for ADP (EC50, approximately 250 μM), whereas UTP was ineffective. Nucleotides with a high affinity for the P2Y11 receptor, such as AR-C67085 (EC50, approximately 0.4 μM) and ATPγS (EC50, approximately 3 μM), had the highest inhibitory potency (Figure 5A-B). Next, we assessed the influence of the P2 receptor antagonists suramin and PPADS on cytokine expression. No cytokine induction was seen for these inhibitors (data not shown). The ATP effect on IL-12 expression was sensitive to the P2 receptor antagonist suramin but not to PPADS (Figure 6A-B). This pharmacologic profile is characteristic for the P2Y11 receptor. As with the IL-12 subunits, expression of the IL-27 subunit p28 was inhibited most effectively by nucleotides with high affinity for the P2Y11 receptor (Figure 5D-E). In contrast, IL-23p19 expression was up-regulated by ATP and ADP in dose-dependent...
fashion, whereas specific P2Y11 receptor agonists and UTP were ineffective (Figure 5C). Furthermore, the ATP effect on IL-23p19 expression was not antagonized by suramin but was antagonized by PPADS ($P < .01$) (Figure 6C). The same effect was induced by the EP2/EP4 agonist 11-deoxy-PGE1, but not by the EP1/EP3 agonist sulprostone (data not shown). The role of cAMP signaling in the reciprocal regulation of IL-12 and IL-23 expression was further supported using forskolin (Figure 6A-C).

**Reciprocal regulation of IL-12 and IL-23 expression is mediated by the cAMP pathway**

GPCRs linked to $G_{q/o}$ inhibited IL-12 production by enhancing levels of intracellular cAMP. To assess whether cAMP mediated the up-regulation of IL-23p19, we examined p35, p40, and p19 expression of *E. coli*–activated MoDCs in response to PGE2, which enhances cAMP levels through EP2/EP4 receptors. As previously reported, PGE2 effectively inhibited IL-12p40 and p70 production induced by *E. coli* (Figure 6A-B).$^{39}$ Interestingly, as observed for ATP, PGE2 enhanced IL-23p19 expression (Figure 6C). The same effect was induced by the EP2/EP4 agonist 11-deoxy-PGE1, but not by the EP1/EP3 agonist sulprostone (data not shown). The role of cAMP signaling in the reciprocal regulation of IL-12 and IL-23 expression was further supported using forskolin (Figure 6A-C).

**MoDCs activated with *E. coli* and ATP secrete high levels of bioactive IL-23**

Next, we attempted to confirm whether ATP-mediated up-regulation of IL-23p19 expression in MoDCs, as detected by RT-qPCR, correlated with the secretion of bioactive IL-23. Because an IL-23–specific ELISA was unavailable, we assessed IL-23 bioactivity by the induction of IL-17 by mouse MoDC supernatants, and murine IL-17 was measured by ELISA. Supernatants of unstimulated MoDCs (cultured with GM-CSF and IL-4) did not induce detectable levels of IL-17, correlating with the lack of IL-23p19 expression found by RT-qPCR (Figure 7A, left graph). In contrast, MoDCs stimulated with *E. coli* and ATP induced high levels of IL-17, which were in the range of those maximally obtained with recombinant human IL-23 (rHL-23) added to T-cell cultures (Figure 7A, right graph). To examine whether IL-17 production was IL-23 specific, we performed experiments in the presence of neutralizing mAbs against IL-12p70 or IL-12p40, the latter blocking IL-12 and IL-23. Blocking antibodies against p40, but not p70, abrogated IL-17 production. Furthermore,
adding an IL-23R/Fc chimeric protein (with IL-23–neutralizing activity) also inhibited IL-17 production (Figure 7A, left graph). Thus, MoDCs activated with E coli and ATP produced high levels of bioactive IL-23.

To examine whether MoDC-derived IL-23 also induces IL-17 production by human T cells, we sorted human CD4+ T cells into naive (CD45RA+) and memory (CD45RO+) T-cell populations and stimulated these with beads coated with anti-CD3/CD28 mAbs in the absence or presence of MoDC supernatants. No IL-17 was detected in cultures of naive CD4+ T cells. In contrast, bead-stimulated memory T cells produced significant levels of IL-17 even in the absence of MoDC supernatant (Figure 7B), indicating that, in contrast to production by mouse T cells, IL-17 production by human T cells was not strictly dependent on IL-23. Supernatants of MoDCs activated with E coli in the absence or presence of ATP strongly enhanced IL-17 production (Figure 7B). In contrast again to mouse cells, IL-17 production by human memory T cells was not neutralized by p40- or p70-blocking mAbs (Figure 7B). Thus, once more in contrast to mouse T cells, IL-23 does not appear to be required for IL-17 production by human memory T cells.

ATP reduces the ability of E.coli–activated MoDCs to induce IFN-γ by naive T cells but not by memory T cells

In humans, naive T cells produce IFN-γ in response to IL-12 and IL-27, whereas memory T cells produce IFN-γ in response to IL-12 and, to a lesser extent, in response to IL-23 (for a review, see Trinchieri et al27). Because ATP reduced the expression of IL-12 and IL-27 in E.coli–activated MoDCs, we speculated that the ability of these DC to activate naive T cells would be impaired. The impact of ATP on memory T-cell activation was difficult to predict because both IL-12 and IL-23 target this T-cell subset in humans. To examine the impact, naive and memory CD4+ T cells were stimulated with anti-CD3/CD28 mAb-coated beads in the absence or presence of MoDC supernatant, and IFN-γ induction was analyzed. Supernatants from E.coli–activated MoDCs (but not unstimulated MoDCs) induced high levels of IFN-γ by naive and memory T cells. MoDCs exposed to ATP showed a reduced capacity for inducing IFN-γ production by naive T cells (Figure 8A-B). The importance of DC-derived IL-12 on naive T-cell IFN-γ was confirmed by blocking IFN-γ induction with anti–IL-12p70 mAb (Figure 8B). In contrast, the ability of MoDCs to induce memory T-cell IFN-γ was not significantly inhibited by ATP. Even when the DC supernatant was diluted to 1%, memory T-cell activation was still highly efficient (Figure 8A). The influence of IL-12 and IL-23 on memory T-cell IFN-γ induction was next assessed. Both p40- and p70-blocking mAbs effectively blocked memory T-cell IFN-γ. Thus, as observed for naive human T cells, memory human T-cell IFN-γ production depended primarily on IL-12.

Discussion

It has recently become evident from mouse studies that 2 novel members of the IL-12–related cytokine family, IL-23 and IL-27, play crucial roles in the regulation of innate and adaptive immune responses. We investigated how the expression of these cytokines is regulated in human DCs and found that the expression of IL-12, IL-23, and IL-27 was critically dependent on the mode of DC activation. A mixture of proinflammatory mediators (IL-1β, IL-6, TNF-α, and PGE₂), commonly used to mature MoDCs, induced the expression of the shared IL-12/IL-23 p40 subunit but not IL-12p70 or IL-23p19. CD40L and specific TLR ligands induced either predominantly IL-12 or IL-12 and IL-27, whereas intact E.coli induced IL-12, IL-27, and IL-23. Expression of these 3 IL-12–related cytokines was recently reported for MoDCs stimulated with intact Streptococcus pyogenes.33 Thus, IL-23 production by MoDCs can be induced by Gram-negative and Gram-positive bacteria. In line with a previous report,35 to exhibit this activity the bacteria had to be intact, indicating that DC activation by bacteria differs from DC activation by TLR ligands, possibly because of a combined effect of engaging multiple TLR or of as yet unidentified mechanisms requiring active bacterial metabolism.

MoDCs activated with E.coli were used to study the regulation of IL-12–related cytokine expression by physiologic modulators of cytokine production. ATP has previously been shown to influence IL-12 production of MoDCs in 2 ways: in synergy with TNF-α, ATP up-regulates IL-12p40 expression32,33 but inhibits IL-12p70 production in response to LPS or CD40L.26,34 In this respect, ATP has similarities with PGE₂.39-41 Our study confirmed that ATP inhibits IL-12p70 production in response to potent IL-12–inducing stimuli, such as E.coli, LPS, or CD40L (data for CD40L and LPS not shown). In addition, we found that ATP suppressed IL-27, but enhanced IL-23, expression. The production of high levels of bioactive IL-23 heterodimers was confirmed by the ability of DC supernatants to induce IL-17 in mouse splenocytes in an IL-23–specific manner. The lack of IL-23–specific mAbs for use in ELISA did not allow exact IL-23 quantification; however, IL-23 levels can be estimated by the bioactivity of DC supernatants, which induced IL-17 levels similar to those maximally obtained with rhIL-23 (greater than 50 ng/mL). Thus, ATP had a selective effect on the expression profile of IL-12–related cytokines, shifting the ratios of IL-12/IL-23 and IL-27/IL-23 toward IL-23.

What is the physiologic relevance of our findings in vivo? ATP is stored in the cytosol at millimolar concentrations and is released...
ATP to AMP. Extracellular ATP binds to P2 receptors expressed by MoDC. Interestingly, forskolin and PGE2, which influenced by P2Y11 receptor agonists but was strongly up-regulated by the P2Y11 receptor. Interestingly, IL-23p19 expression was not enhanced cAMP levels through EP2/EP4 receptors, also shifted the paired DC function and altered cellular immune responses. Human DCs express several P2 receptor subtypes, and signaling through these receptors modulates DC functions. ATP enhances antigen uptake, the expression of activation markers, and the T-cell regulation of IL-12 family members shed light on the orchestration of Th1 responses. Trends Immunol. 2003;24: 207-212.

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