Stimulation of Purinergic Receptors Modulates Chemokine Expression in Human Keratinocytes

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ATP is abundantly released from stressed or damaged cells in response to mechanical stimulation, bacteria, or noxious agents. In this study, we have investigated the possible involvement of P2 receptors (receptor for extracellular nucleotides) in the expression and release of inflammatory mediators by human keratinocytes. Notably, extracellular ATP displayed a complex regulation of IFN-γ-stimulated chemokine expression, with upregulation of CC chemokine ligand 2 (CCL2), CCL5 and CXC chemokine ligand 8 (CXCL8), and suppression of the receptor CXC chemokine receptor 3 (CXCR3), CXCL9, CXCL10, and CXCL11. The effect of ATP was mimicked by ADP and adenosine-5′-*O*-3-thiotriphosphate, whereas 2′,3′-*O*-(4-benzoylbenzoyl) ATP (BzATP) down-modulated all chemokines investigated. UTP had no effect on IFN-γ-stimulated chemokine secretion. The broad-spectrum P2 receptor antagonist suramin and the selective $P2Y_1$ inhibitor adenosine 3′-phosphate 5′-phosphosulfate counteracted the effect of ATP on secretion of all the chemokines examined, whereas pyridoxal phosphate 6-azophenyl 2′,4′-disulfonic acid and KN62 (1-[*N*,*O*-bis(5-isoquinoline sulfonyl)-*N*-methyl-L-tyrosyl] 4 phenylpiperazine) partially prevented the inhibitory effect of ATP on CXCL10 secretion, but on the other hand potentiated the ATP-stimulatory effect on CCL5, CCL2, and CXCL8 release. In lesional skin of psoriasis and atopic dermatitis patients, intense $P2X_7$ reactivity was confined to the cell membrane of the basal layer, whereas diffuse $P2Y_1$ immunostaining was found throughout the epidermis. Collectively, our data suggest that the orchestrated activation of distinct P2Y and P2X receptors modulates skin inflammation.

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INTRODUCTION

The skin is a highly specific immune defense organ. Physical, chemical, or immune-specific insults rapidly evoke the response of resident cells of the epidermis, characterized by the increased expression of a wide range of proinflammatory mediators. In particular, keratinocytes can release high amounts of (CC ligand), chemokine of the CC subfamily (CCL)2/monocyte chemoattractant protein-1 and CCL5/RANTES (regulated upon activation, normal T cell expressed and secreted), active in the recruitment of monocytes/macrophages, dendritic cells and T cells, and (CXC ligand), chemokine of the CXC subfamily (CXCL)8/IL-8, a selective neutrophil chemoattractant (Pastore *et al.*, 2004). Type 1 T helper (Th1) cells, crucial effectors in chronic inflammatory skin disorders, are attracted into the skin by the release of CXCR3 ligands, namely CXCL9/monokine-induced by IFN-γ (MIG), CXCL10/IFN-γ-induced protein of 10 kDa (IP-10), CXCL11/IFN-γ-induced T cell α chemoattractant (ITAC). The expression of these chemokines is strongly upregulated by IFN-γ, abundantly released by Th1 cells (Albanesi *et al.*, 2005).

Growing evidence suggests that ATP is not only a source of energy for the cell but also an important intercellular mediator in both physiological and pathological conditions. In particular, extracellular nucleotides may act as early danger signals, alerting immune cells of tissue injury or stress. In response to mechanical stimulation, bacterial infections or cell damage, ATP, and other nucleotides including ADP and UTP may be released into the extracellular space (Ferrari *et al.*, 1997; Pellegatti *et al.*, 2005), and once released, they activate a family of plasma membrane receptors known as purinergic (P2) receptors (receptor for extracellular nucleotides). ATP and UTP can be released via lytic or non-lytic mechanisms involving vesicle-mediated secretion,

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The experimental work was carried out in Roma and in Ferrara, Italy.

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Abbreviations: ATPγS, adenosine-5′-O-3-thiotriphosphate; BzATP, 2′,3′-O-(4-benzoylbenzoyl) ATP; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; KN62, 1-[N,O-bis(5-isoquinoline sulfonyl)-N-methyl-L-tyrosyl] 4 phenylpiperazine; P2 receptor, receptor for extracellular nucleotides; PPADS, pyridoxal phosphate 6-azophenyl 2′,4′-disulfonic acid; TNF-α, tumor necrosis factor-alpha

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carrier-mediated transport, or plasma membrane channels (Filippini *et al.*, 1990; Jiang *et al.*, 1998; Dixon *et al.*, 1999; Burrell *et al.*, 2005). P2 receptors can be classified according to the molecular structure into two subfamilies, P2Y and P2X (Dubyak and el-Moatassim 1993; Abbracchio and Burnstock, 1994). P2Y receptors are seven-membrane-spanning proteins (Von Kugelgen and Wetter, 2000). To date, eight P2Y subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) have been cloned. P2X receptors are cation-selective, ligand-gated channels (North, 2002). Seven P2X receptor subtypes have been cloned so far ($P2X_{1-7}$). P2X subunits aggregate to form homomeric or heteromeric channels.

A number of experimental observations indicate that ATP may play a relevant role in skin inflammation (Holzer and Granstein, 2004). However, the detailed characterization of its effects on keratinocyte proinflammatory activation is still missing. In this study, we demonstrate that ATP affects chemokine expression in cultured normal human keratinocytes with a relevant, dose-dependent downregulation of CXCR3 ligands and upregulation of CCL2, CCL5, and CXCL8. In keeping with previous evidence, we found that human keratinocytes express P2Y and P2X receptor subtypes (Burrell *et al.*, 2003; Greig *et al.*, 2003b; Inoue *et al.*, 2005), and their stimulation is coupled to $[Ca^{2+}]_i$ transients (Pillai and Bikle, 1992; Dixon *et al.*, 1999; Burrell *et al.*, 2003). Of note, only $P2X_7$ and $P2Y_1$ were found upregulated by IFN-γ. Chronic lesions from psoriasis and atopic dermatitis patients stained intensely with specific anti-$P2X_7$ and anti-$P2Y_1$ antisera. These data emphasize the multifaceted roles of extracellular adenine nucleotides during skin inflammation.

RESULTS

Adenine nucleotides differentially affect expression of proinflammatory mediators in keratinocytes

ATP is a potent cytotoxic effector in several cell types (Di Virgilio *et al.*, 1998). In monocyte-derived dendritic cells, 90 minutes incubation in the presence of 1 mM ATP causes cytolysis of over 20% of the cell population (Ferrari *et al.*, 2000). In contrast, adherent human keratinocytes were much more resistant to ATP-triggered cytotoxicity. Following 24 hours incubation in the presence of 1 mM ATP, 2′,3′-*O*-(4-benzoylbenzoyl) ATP (BzATP), adenosine-5′-*O*-3-thiotriphosphate (ATPγS), or UTP, only 2–5% of the cells were killed (Figure 1). The addition of IFN-γ or tumor necrosis factor-alpha (TNF-α) together with ATP did not affect this striking insensitivity to nucleotide-mediated cytotoxicity (not shown). Chronic incubation in the presence of low concentrations of ATP reduced the intracellular accumulation of IL-1α and IL-1β, but not IL-1ra, either in the presence or absence of IFN-γ or TNF-α (Figure S1, Supplementary Material). ATP had no effect on the very low extracellular levels of these cytokines (data not shown). These data suggested that chronic ATP signalling downmodulated the proinflammatory function of epidermal keratinocytes. To further investigate this hypothesis, we examined the effects of nucleotide signalling on the expression of a cluster of chemokines including the CXCR3 ligands, CCL2, CCL5, and CXCL8.

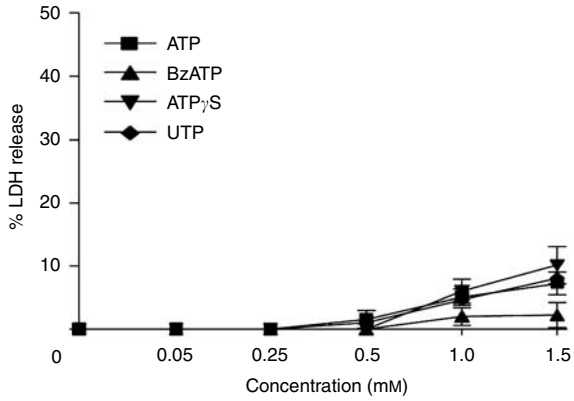

Figure 1. Lack of cytotoxic effect of P2 agonists in human keratinocytes. Keratinocyte cultures were incubated with increasing nucleotide concentrations and their medium was collected for lactic dehydrogenase analysis after 24 hours. Data were normalized per 10^6 cells and are expressed as means ± SD of triplicate determinations.

Chronic exposure to ATP dose-dependently downregulated IFN-γ-induced release of CXCR3 ligands (Figure 2a) and downmodulated their mRNA levels, as shown by real-time reverse transcriptase (RT)-PCR (Figure 2b–d). In contrast, ATP stimulated CCL5 and CXCL8 mRNA levels at the longest time point tested (16 hours) (Figure 3a). The CCL2 transcript was also upregulated by ATP at 4 and 8 hours time points. Downmodulation of CXCL10 gene expression by ATP is also shown for comparison. Dose-dependency curves of nucleotide-dependent modulation of IFN-γ-driven chemokine secretion showed that maximal effect was reached at about 500 μM ATP, and that ADP was also effective. ATPγS was a more potent inducer of CCL5 and CXCL8 release than ATP, whereas BzATP had a generalized inhibitory activity and UTP lacked any effect whatsoever (Figure 3b–e). Neither ATP nor ADP or ATP synthetic analogs significantly affected TNF-α-induced chemokine expression or release (data not shown).

P2 receptor expression in human keratinocytes

Many responses elicited by extracellular nucleotides are mediated by an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), thus we investigated Ca^{2+} signalling in nucleotide-stimulated keratinocytes. Human keratinocyte monolayers were loaded with the fluorescent Ca^{2+} indicator fura-2/AM, and intracellular Ca^{2+} changes were monitored by fluorometric analysis. In the presence of extracellular Ca^{2+}, ATP (1 mM) triggered a fast $[Ca^{2+}]_i$ increase followed by a sustained plateau (Figure 4a, continuous line). ATP threshold concentration for the $[Ca^{2+}]_i$ rise was 30 nM, while plateau was reached at 30 μM (Figure 4b). At the concentration of 30 nM, ATP caused a slow and long-lasting increase in the $[Ca^{2+}]_i$ (Figure 4a, dashed line). In the absence of extracellular Ca^{2+}, ATP triggered a spiking increase in $[Ca^{2+}]_i$, which was not followed by a sustained plateau (Figure 4c, continuous line). Under these conditions, the ATP threshold was shifted to 300 nM, while ATP concentration for plateau was 100 μM (see Figure 4d and c, dashed line). The

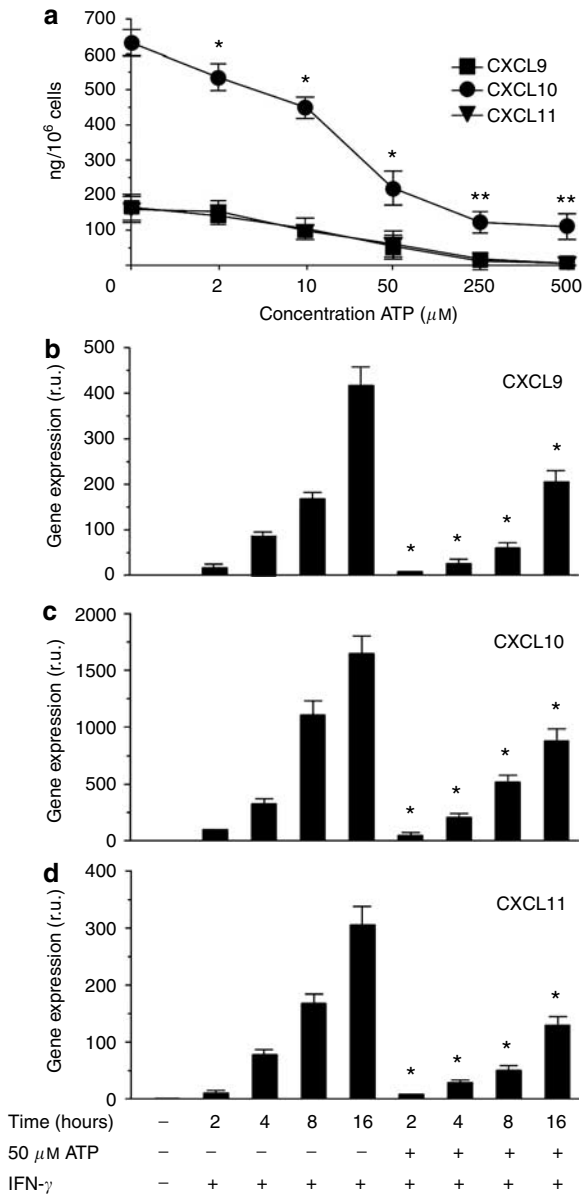

Figure 2. ATP dose-dependently downregulates CXCR3 ligands. (**a**) Chemokines were measured in cell supernatants after 24 hours co-treatment with IFN-γ and increasing doses of ATP. Results are expressed as the mean of three independent experiments ± SD. For the three curves, *$P<0.05$ and **$P<0.01$ versus culture condition in the absence of ATP. (**b–d**) Time course of CXCR3 ligands transcript levels as obtained by SYBR Green-based quantitative real-time RT-PCR. Data are representative of four independent experiments. *$P<0.01$ versus culture conditions in the absence of ATP at the same time point.

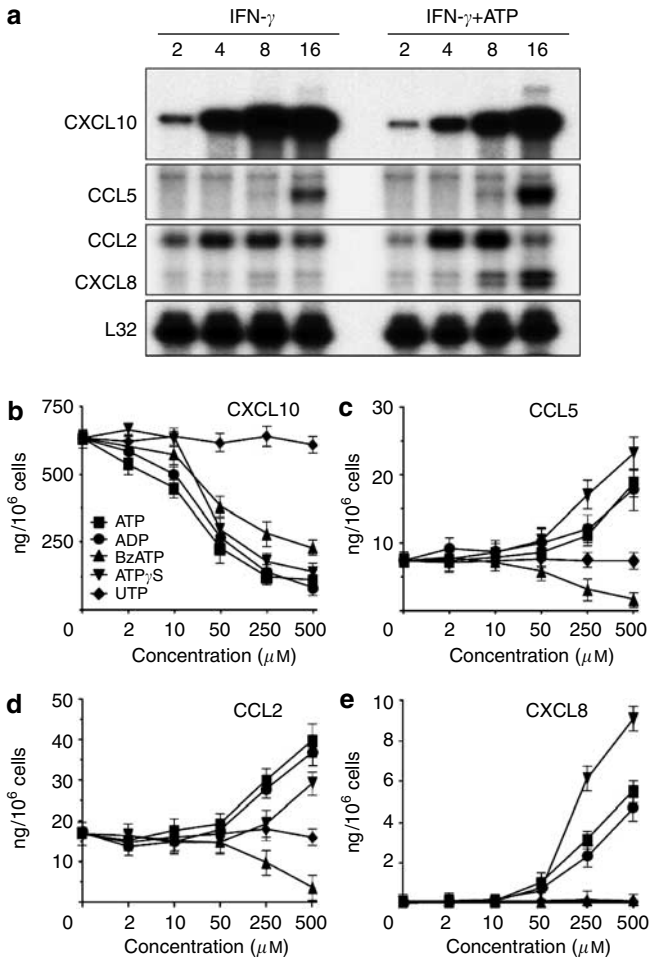

Figure 3. ATP increases CCL2, CCL5, and CXCL8 expression in keratinocytes. (**a**) Chemokine gene expression was measured by RNase Protection Assay in total RNA (10 μg) extracted from keratinocytes stimulated with IFN-γ with or without 50 μM ATP for the time points indicated. (**b–e**) ELISA of IFN-γ-induced chemokine levels after 24 hours co-treatment with escalating doses of ATP, UTP, or the ATP synthetic analogs ATPγS and BzATP. Results are expressed as mean ± SD of three independent experiments.

$P2Y_2$ and $P2Y_4$ agonist UTP was also a potent stimulus for $[Ca^{2+}]_i$ increase (Figure 4e and f). Figure 4f shows UTP dose-dependency curve; the most effective concentration for the $[Ca^{2+}]_i$ rise was about 1 mM UTP. BzATP induced a spiking $[Ca^{2+}]_i$ increase both in the presence or absence of extracellular Ca^{2+} (Figure 4g). Figure 4h shows the dose-dependency curve in keratinocytes stimulated with BzATP in the presence of external Ca^{2+}. ADP and ATPγS were also potent stimuli for $[Ca^{2+}]_i$ increases in keratinocytes (not shown). No response to UTP-glucose was observed. The wide range of nucleotides acting as Ca^{2+}-mobilizing agonists indicated expression of multiple P2Y receptors, among which are $P2Y_2$ and $P2Y_4$ (activated by UTP), $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ (activated by ADP), and $P2Y_{11}$ (activated by BzATP and ATPγS). Indeed, the specific transcripts for $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, and $P2X_7$ subtypes, and $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ subtypes, but not $P2Y_{14}$ (not shown), were detected by RT-PCR (Figure S2, Supplementary Material). Among P2 receptors subtypes, only $P2X_7$ and $P2Y_1$ were reproducibly upregulated by IFN-γ, whereas TNF-α downregulated $P2X_7$ at 6 h time point (Figure 5).

Differential contribution of P2 receptors to chemokine expression

To identify the P2 receptor subtypes responsible for nucleotide-dependent modulation of IFN-γ chemokine release, we tested several P2 blockers. Suramin was used as a

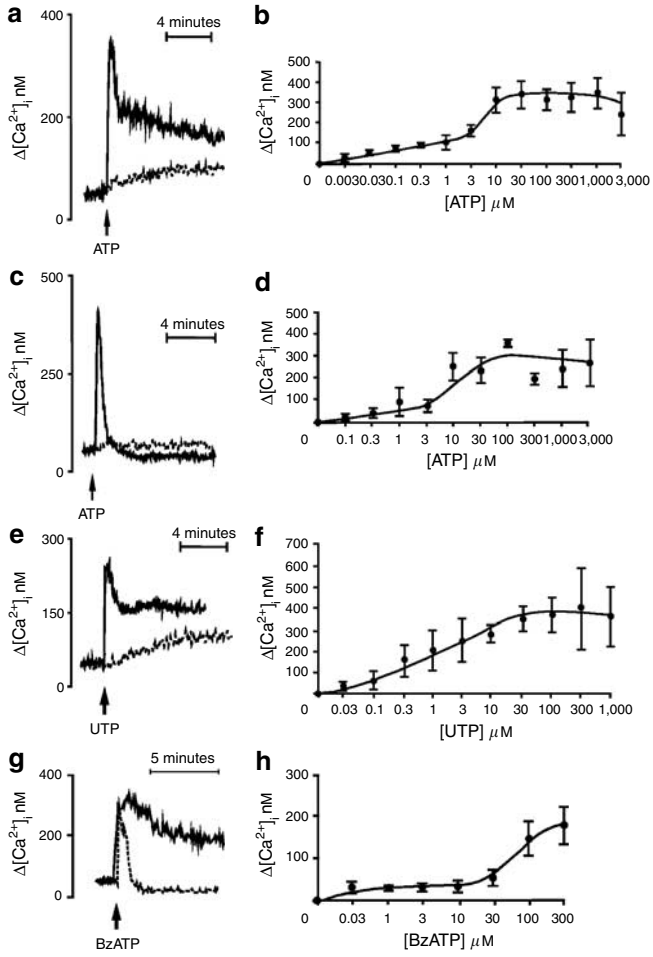

Figure 4. Extracellular nucleotides induce $[Ca^{2+}]_i$ increase in human keratinocytes. Cells were seeded onto glass coverslips, loaded with the Ca^{2+} indicator fura-2/AM as reported in Materials and Methods, and stimulated with (**a–d**) ATP; (**e, f**) UTP, or (**g, h**) BzATP. In panels a–d, 1 mM ATP, continuous line; 30 nM ATP dashed line. In panel e, 1 mM UTP, continuous line; 30 nM UTP, dashed line. In panel g, 300 μM BzATP, in the presence of extracellular Ca^{2+}, continuous line; in the absence of extracellular Ca^{2+}, dashed line. Panels a, b, e, and f, presence of extracellular Ca^{2+}. Panels c and d, absence of extracellular Ca^{2+}. For Ca^{2+}-free conditions, 0.5 mM EGTA was added to Ca^{2+} free saline solution to chelate contaminating Ca^{2+}. (**b, d**) ATP, (**f**) UTP, and (**h**) BzATP dose-dependency curves. Data are means + SD of four independent experiments.

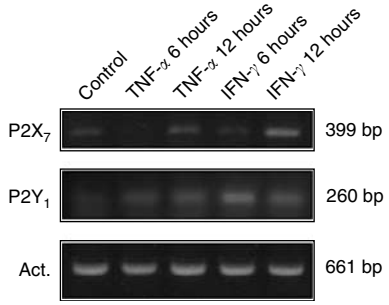

Figure 5. IFN-γ upregulates $P2X_7$ and $P2Y_1$ mRNA expression. Keratinocytes were collected, lysed, and mRNA extracted and amplified as described in Materials and Methods. β-Actin mRNA (Act.) was used as an internal control. Representative RT-PCRs are shown. Samples obtained from three different keratinocyte donors gave very similar results.

wide-spectrum P2 antagonist (Ishii-Watabe *et al.*, 2000), pyridoxal phosphate 6-azophenyl 2′,4′-disulfonic acid (PPADS) as a blocker, although not specific of the P2X receptors (Denda *et al.*, 2002; Inoue *et al.*, 2005), KN62 (1-[*N*,*O*-bis(5-isoquinoline sulfonyl)-*N*-methyl-L-tyrosyl] 4 phenylpiperazine) as a potent and selective $P2X_7$ antagonist (Gargett and Wiley, 1997; Di Virgilio *et al.*, 2001; Idzko *et al.*, 2003), and adenosine 3′-phosphate 5′-phosphosulfate (A3P5PS) as a selective $P2Y_1$ antagonist (Hibell *et al.*, 2001). For each blocker, the following concentrations were tested: 50 and 100 μM suramin, 50 and 100 μM PPADS, 25 and 50 nM KN62, and 50 and 100 μM A3P5PS with very similar results; thus only data obtained with the higher concentrations are shown.

Chemokine secretion was affected by these antagonists whether in the absence or presence of extracellular ATP. In the absence of added ATP, the most relevant effect was observed on CCL5, CCL2, and CXCL8 release that was significantly reduced by suramin or A3P5PS (Figure 6b–d, left panels). PPADS and KN62 had no effect on the release of these chemokines. All the blockers caused a slight upregulation of IFN-γ-driven CXCL10 release that, however, was not statistically significant (Figure 6a, left panel). In the presence of added ATP, all the antagonists markedly influenced chemokine expression. ATP-dependent downregulation of CXCL10 expression was strongly counteracted by suramin and much less so by the other antagonists (Figure 6a, right panel). Suramin also strongly inhibited ATP-mediated stimulation of CCL5, CCL2, and CXCL8 secretion (Figure 6b–d, right panels). A3P5PS was also effective in reducing the production of these chemokines, whereas PPADS and KN62 further enhanced ATP-dependent secretion.

$P2X_7$ and $P2Y_1$ upregulation in the lesional skin of atopic dermatitis and psoriasis

The observation that extracellular nucleotides modulate IFN-γ-stimulated chemokine secretion and that $P2Y_1$ and $P2X_7$ expression is stimulated by IFN-γ treatment prompted us to investigate their possible involvement in skin inflammation. At variance with previous results obtained with fluorescence-based immunodetection (Greig *et al.*, 2003a, b), chromogen-based immunohistochemistry did not allow to detect $P2X_7$ or $P2Y_1$ in normal human skin (Figure 7a and e). By contrast, strong $P2X_7$ immunoreactivity was detected in the epidermal basal layer of chronically inflamed skin from patients with atopic dermatitis (Figure 7b) and psoriasis (Figure 7c), with selective localization on keratinocyte membrane. Weak, specific membrane $P2X_7$ staining could also be clearly observed throughout the epidermis in atopic dermatitis (Figure 7b). A control run with an isotype-matched Ig as primary antibody confirmed staining specificity (Figure 7d). In skin lesions from atopic dermatitis and psoriasis, we also detected a diffuse reactivity using a selective anti-$P2Y_1$ antibody, with a peri/nuclear localization particularly evident in the suprabasal keratinocytes of atopic dermatitis lesions

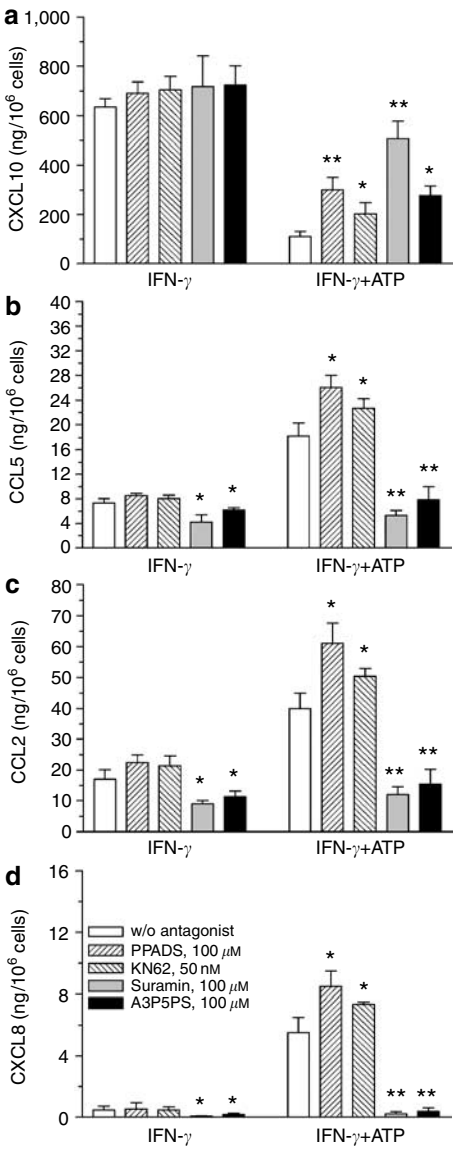

Figure 6. Purinergic antagonists display differential effects on IFN-γ-induced chemokine expression in keratinocytes. (**a–d**) Cells were treated with the indicated doses of P2 antagonists for 1 hour before the addition of IFN-γ, either in the presence or absence of ATP. Cell supernatants were then collected after 24 hours and chemokine secretion measured by ELISA. Data are means ± SD of triplicates. $*P<0.05$ and $**P<0.01$ versus the corresponding culture condition in the absence of antagonists.

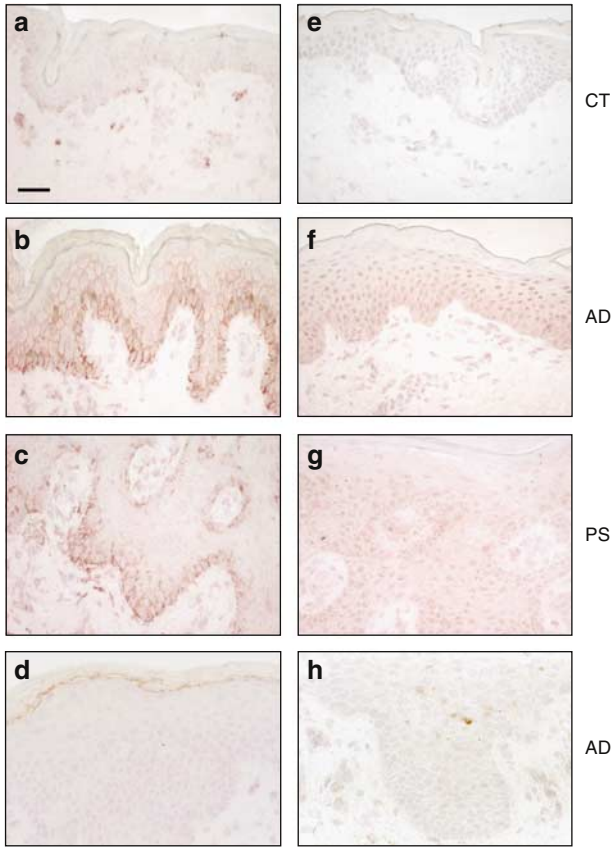

Figure 7. $P2X_7$ and $P2Y_1$ are overexpressed in chronically inflamed skin of patients with psoriasis or atopic dermatitis. (**a** and **e**) P2 receptor expression in the epidermis of a healthy donor (CT), (**b** and **f**) in lesional skin of atopic dermatitis (AD) , and (**c** and **g**) chronic plaque psoriasis (PS). (**d**) As staining specificity control, skin sections from patients with AD were incubated with an isotype-matched antibody in the place of monoclonal anti-$P2X_7$ antibody. (**h**) Anti-$P2Y_1$ antibody was pre-adsorbed with the corresponding antigen before staining of atopic dermatitis sections. Representative immunohistochemistry pictures obtained from four healthy donors, four patients with AD and four patients with psoriasis are shown. (**a–d**) $P2X_7$, (**e–h**) $P2Y_1$. Original magnification, ×200. Bar = 40 μm.

(Figure 7f and g). Preadsorption, with the epitope against which the anti-$P2Y_1$ antibody was specifically raised, abrogated tissue staining (Figure 7h).

DISCUSSION

Keratinocytes are the dominant cell population of the skin and the major contributors to the establishment of the local cytokine and chemokine milieu during skin inflammatory responses (Grone, 2002; Pastore *et al.*, 2004). In this study, we investigated the potential role of P2 receptors in the modulation of chemokine production in human keratinocytes. Of note, ATP, but not UTP, dose-dependently downregulated the expression of IFN-γ-driven CXCR3 ligands CXCL9, CXCL10, and CXCL11 both at the mRNA and protein level. ATP also caused upregulation of IFN-γ-driven secretion of CCL2, CCL5, and CXCL8. ADP and the non-hydrolysable synthetic ATP analog ATPγS mimicked ATP in its differential effect on chemokine expression by causing downregulation of CXCL10 and enhanced expression of CCL5, CCL2, and CXCL8. By contrast, the synthetic nucleotide BzATP, well known as a potent, although not selective, agonist at $P2X_7$ (Di Virgilio *et al.*, 2001) displayed a distinct activity profile, characterized by downregulation of all the chemokines investigated.

In keeping with previous evidence, we found that human keratinocytes expressed all P2X and P2Y receptor subtypes so far cloned, except $P2Y_{14}$ (Burrell *et al.*, 2003; Greig *et al.*, 2003b; Inoue *et al.*, 2005), and that their stimulation was coupled to $[Ca^{2+}]_i$ transients, as expected (Pillai and Bikle,

1992; Dixon *et al.*, 1999; Burrell *et al.*, 2003). However, only $P2X_7$ and $P2Y_1$ were upregulated by IFN-γ, suggesting a possible involvement in keratinocyte responses to this cytokine. In the past decade, several reports have highlighted the involvement of P2 receptors, especially $P2Y_1$ and $P2X_7$, in keratinocytes survival and proliferation (Cook *et al.*, 1995; Groschel-Stewart *et al.*, 1999). In particular, Greig *et al.* (2003a, b) showed that $P2Y_1$ receptors colocalize with the proliferation marker Ki-67 in a sub-population of basal and para-basal keratinocytes in human adult and fetal epidermis. Colocalization of $P2Y_2$ and the proliferation marker proliferating cell nuclear antigen was also observed, suggesting that these two P2Y receptors might participate in keratinocyte proliferation. On the other hand, $P2X_5$ and $P2X_7$ were colocalized with cytokeratin K10 (a differentiation marker) and TUNEL-positive (apoptotic) cells, respectively, suggesting a role for $P2X_5$ and $P2X_7$ in keratinocyte differentiation and apoptosis. A recent report by Georgiou and collaborators showed that $P2X_7$ activation in monocyte-derived dendritic cells, Langerhans cells, and keratinocytes induced permeabilization of plasma membrane to fluorescent cations such as ethidium or propidium (Georgiou *et al.*, 2005). In our hands, ATP or BzATP did not show cytotoxicity on adherent keratinocytes, and this held true also in the simultaneous presence of IFN-γ.

Inhibitor studies suggest a complex interplay between P2X and P2Y receptors in the modulation of IFN-γ-driven chemokine secretion. The wide range, but poorly selective, P2 blocker suramin abrogated all ATP-mediated effects on chemokine secretion, and also showed an effect in the absence of ATP treatment, especially on CCL5, CCL2, and CXCL8. The wide range P2X antagonist PPADS partially abrogated ATP-dependent downregulation of CXCL10, while it enhanced the expression of CCL5, CCL2, and CXCL8. This suggests that P2X stimulation may have a generalized inhibitory effect on chemokine release, and that this inhibition is released when the P2X receptors are blocked. This interpretation is supported by the similar effect of KN62, which, like PPADS, increased IFN-γ-stimulated chemokine secretion in the presence of ATP. Although inhibitor studies do not allow to identify the P2X receptor involved, the finding that the potent $P2X_7$ agonist BzATP was the only nucleotide that invariably downregulated these chemokines support a role for $P2X_7$ in the ATP-dependent inhibition of chemokine release. The $P2Y_1$-selective antagonist A3P5PS partially relieved ATP-mediated suppression of CXCL10, and caused a large reduction of ATP-stimulated potentiation of IFN-γ-driven CCL5, CCL2, and CXCL8 secretion. These results point to $P2Y_1$ as the main P2 receptor responsible for ATP-mediated potentiation of CCL5, CCL2, and CXCL8 release. This conclusion is also supported by the pharmacological profile of nucleotide-specific effects on keratinocyte response IFN-γ stimulation. Indeed, ATP effects were fully mimicked only by ADP (equipotent as ATP at $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$) and ATPγS (a potent agonist at $P2Y_1$). In contrast, UTP was inactive (thus ruling out $P2Y_2$, $P2Y_4$, and $P2Y_6$) and BzATP (a potent agonist at P2X receptors and at the $P2Y_{11}$) caused a generalized inhibition.

The complex modulation of skin inflammation *in vivo* is epitomized by a report on mice deficient in CD39, the main ecto-ATPase of Langerhans cells (Mizumoto *et al.*, 2002). The exaggerated acute inflammatory response to irritant agents observed in $CD39^{-/-}$ mice was taken as the evidence that accumulation of ATP and/or ADP released by damaged keratinocytes in the epidermis acted as a proinflammatory stimulus. In keeping with this hypothesis, injection of ATP into mouse ears triggered inflammation. In contrast, the induction of hapten-specific contact hypersensitivity was severely decreased in CD39-deficient mice, indicating that endogenously released ATP might be involved in the down-regulation of Th1 cell recruitment and/or activation. The *in vitro* demonstration that chronic exposure to low ATP doses suppresses DC-driven Th1 activation and downregulates CXCR3 ligands both in DCs and in keratinocytes (la Sala *et al.*, 2003 and present data) provides a mechanism for these *in vivo* observations. By contrast, a clearcut proinflammatory role was recently proposed for purinergic agonism in the skin, with intradermal administration of ATPγS resulting in enhanced activation of antigen-presenting cell functions (Granstein *et al.*, 2005). Quite similarly to what we observed in keratinocytes, ATPγS was shown to enhance the production of a number of proinflammatory mediators, including CCL2 and CXCL8, by human dermal endothelial cells (Seiffert *et al.*, 2006). Both suramin and PPADS effectively opposed chemokine expression by endothelial cells, with suramin much more effective than PPADS. These authors proposed that $P2Y_{11}$, strongly activated by ATPγS and effectively antagonized by suramin, could be among the P2 receptors critically implicated in the enhanced expression of pro-inflammatory mediators by endothelial cells (Seiffert *et al.*, 2006).

Finally, participation of $P2Y_1$ and $P2X_7$ to inflammation in the skin is also supported by the finding that they are the only P2 receptors upregulated by IFN-γ and that immunoreactivity for these receptors was enhanced in the lesional skin of patients with psoriasis and atopic dermatitis. Strong $P2X_7$ staining was localized at the membrane of all basal keratinocyte layers, which are actively proliferating in these disorders, whereas $P2Y_1$ was found throughout the epidermis. While the real contribution of P2 receptors to skin inflammation and/or hyperproliferation awaits future confirmation by *in vivo* experiments, our data suggest that P2 receptors play an important role in skin inflammation.

MATERIALS AND METHODS

Subjects

Four-mm punch biopsies were taken from lesional skin of adult patients with atopic dermatitis ($n=4$, two females and two males, age 25–40 years), chronic plaque psoriasis ($n=4$, two females and two males, age 35–45 years), and from normal skin of healthy subjects ($n=4$, two females and two males, age 30–45 years). None of the patients had received oral corticosteroids within 1 month of skin explant, and topical corticosteroids were not allowed for a period of at least 2 weeks before enrollment. Epidermal sheets for keratinocyte cultures were obtained from healthy individuals

undergoing plastic surgery ($n = 4$, two females and two males; age 25–45 years). These studies were conducted according to the Declaration of Helsinki Principles and approved by the review board of the Istituto Dermopatico dell'Immacolata. All subjects gave written informed consent before enrollment.

Reagents and cytokines

ADP, ATP, and UTP were from Roche (Milan, Italy). ATPγS, BzATP , PPADS, KN62, suramin, and A3P5PS were from Sigma Chemicals (Milan, Italy). Fura-2/AM was from Molecular Probes (Leiden, The Netherlands). Recombinant human TNF-α and IFN-γ were from R&D Systems (Abingdon, UK).

Keratinocyte cultures and treatments

Primary cultures were established as described (Pastore *et al.*, 1997). Keratinocytes were sub-cultured in serum-free medium Keratinocyte Growth Medium (Cambrex, San Diego, CA), prepared from the essential solution supplemented with 10 ng/ml EGF, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 2 ml bovine pituitary extract, and antibiotics. Second- or third-passage keratinocytes were used in all experiments. Cultures at 80% confluence were treated with 100 ng/ml TNF-α or 100 U/ml IFN-γ alone, or together with a P2 agonist. In some experiments, keratinocytes were pretreated with P2 antagonists for 1 hour before stimulation with IFN-γ with or without ATP. All experiments were repeated with keratinocytes from at least three different donors.

Lactic dehydrogenase assay

Lactic dehydrogenase activity was measured with a commercial kit from Roche Diagnostics (Mannheim, Germany). Briefly, keratinocytes (2.5×10^5) were plated in six-well plates until they reached subconfluence. They were subsequently incubated in the presence of known concentrations of nucleotides, with or without IFN-γ, for a period of 24 hours. Supernatants obtained from keratinocyte cultures that underwent total cell lysis with 0.1% Triton X-100 provided 100% of lactic dehydrogenase activity.

ELISA

Cytokines and chemokines were measured in total cell extracts and/or supernatants with dedicated kits from BD Biosciences (San Diego, CA).

Chemokine mRNA determination by RNase protection assay

Total RNA was extracted from keratinocytes using the TRIzol Reagent, according to the manufacturer's instructions (Invitrogen SRL, Carlsbad, CA). The templates of the human chemokines, the housekeeping molecule L32, and the RNase Protection Assay kit were purchased from BD Biosciences.

Chemokine mRNA determination by quantitative real-time RT-PCR analysis

Total RNA was treated with DNase I (Qiagen, Valencia, CA) to eliminate DNA traces. cDNA was prepared by reverse transcription of 1 μg total RNA. The primers used to detect the expression levels of CXCL9/MIG, CXCL10, and CXCL11/ITAC transcripts (Table S1, Supplementary Material) were designed with ABI PRISM Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and synthesized at MWG (Milan, Italy). Continuous quantitative measurement of the PCR products was achieved by incorporation of SYBR Green fluorescent dye (Applied Biosystems), using the ABI PRISM 7000 sequence detection system (Applied Biosystems). The transcript level of each gene was normalized to the corresponding β-actin.

RT-PCR of P2 receptors

Two hundred nanograms of total RNA were reverse transcribed. RT-PCR reaction was performed to amplify both P2Y and P2X receptor subtypes, whereas the amplification of β-actin was carried out as an internal positive control. The specific primers used for RT-PCR amplification were purchased from M-Medical (Milan, Italy), and their sequence and annealing conditions can be found in Table S2, Supplementary Material. The conditions for PCR amplification were 30 cycles, with denaturation at 95°C for 45 seconds and final extension was at 72°C for 30 seconds. The PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining.

Cytosolic Ca^{2+} concentration measurements

Keratinocytes were seeded onto glass coverslips. After 24–48 hours, cells were loaded with the Ca^{2+} indicator fura-2/AM (2 μM) for 20 minutes at 37°C in a saline solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 20 mM HEPES, 5.5 mM glucose, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, and 250 μM sulfinpyrazone, pH 7.4). Coverslips were mounted in a temperature-controlled magnetically stirred cuvette at 37°C. Fluorescence was measured with a Perkin-Elmer fluorometer (Perkin-Elmer Ltd., Beaconsfield, UK). The excitation was at 340–380 nm, and emission at 510 nm. Ca^{2+} concentration was calculated using the FLwinlab software (Perkin-Elmer) (Grynkiewicz *et al.*, 1985).

Immunohistochemistry

Four-μm cryostatic sections were fixed with 4% paraformaldehyde for 10 minutes, treated with 0.3% hydrogen peroxide, permeabilized with 0.05% Triton X-100, and then incubated for 1 hour at room temperature with the primary antibody. Anti-human $P2X_7$ receptor monoclonal antibody (1.15 mg/ml) was a kind gift from Dr Gary Buell, and was finally used 100-fold diluted. As negative controls, anti-$P2X_7$ antibody was omitted or replaced with isotype-matched Ig (IgG2b). Polyclonal anti-$P2Y_1$ antibody (0.6 mg/ml) including its control antigen was obtained from Alomone Laboratories (Jerusalem, Israel), and was finally used at a 80-fold dilution. For control of $P2Y_1$ specificity, preadsorption with the corresponding peptide was performed per the data sheet instruction. Secondary biotinylated antibodies and staining kits were from Vector Laboratories (Burlingame Laboratories, CA). Immunoreactivity was revealed using avidin–biotin–peroxidase system and 3-amino-9-ethylcarbazole as chromogen. Sections were counterstained with Mayer's hematoxylin.

Statistical analysis

The Wilcoxon signed-rank test was applied to compare differences between groups of data. Significance was assumed as a *P*-value of 0.05 or less.

CONFLICT OF INTEREST

The author states no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. ATP downregulates the intracellular levels of IL-1α and β.

Figure S2. P2X and P2Y mRNA expression in keratinocytes.

Table S1. Sequence of primers used in real-time RT-PCR.

Table S2. Sequence of primers used in RT-PCR and annealing conditions.

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