P2Y receptor signaling regulates phenotype and IFN-α secretion of human plasmacytoid dendritic cells

Amanda Shin,[1] Tracey Toy,[2] Simon Rothenfusser,[3] Neil Robson,[2] Julia Vorac,[3] Marc Dauer,[1] Moritz Stuplich,[3] Stefan Endres,[3] Jonathan Cebon,[2] Eugene Maraskovsky,[4] and Max Schnurr[1]

[1]Department of Internal Medicine, University of Munich, Munich, Germany; [2]Division of Clinical Pharmacology, University of Munich, Munich, Germany; [3]Ludwig Institute for Cancer Research, Melbourne Centre for Clinical Sciences, Austin Health, Heidelberg, Australia; and [4]CSL Limited, Parkville, Australia

Plasmacytoid dendritic cells (PDCs) play powerful regulatory roles in innate and adaptive immune responses and are a major source of type I interferon (IFN) following viral infection. During inflammation and mechanical stress, cells release nucleotides into the extracellular space where they act as signaling molecules via G protein–coupled P2Y receptors. We have previously reported on the regulation of myeloid dendritic cell (DC) function by nucleotides. Here, we report that human PDCs express several subtypes of P2Y receptors and mobilize intracellular calcium in response to nucleotide exposure. As a functional consequence, PDCs acquire a mature phenotype that is further enhanced in the context of CD40 ligation. Strikingly, nucleotides strongly inhibit IFN-α secretion induced by influenza virus or CpG-A. This effect is most pronounced for the uridine nucleotides UDP and UTP and the sugar nucleotide UDP-glucose, ligands of $P2Y_6$, $P2Y_4$, and $P2Y_{14}$, respectively. Nucleotide-induced inhibition of IFN-α production is blocked by suramin, a P2Y receptor antagonist. Pharmacological data point toward a role of protein kinase C in the negative regulation of type I IFN. Manipulating PDC function with P2Y receptor agonists may offer novel therapeutic strategies for autoimmune diseases or cancer. (Blood. 2008; 111:3062-3069)

Introduction

Dendritic cells (DCs) are highly specialized antigen-presenting cells and play key roles in the regulation of immune responses. Two populations of DCs are found in human peripheral blood. Myeloid DCs that express CD11c are highly phagocytic, produce a variety of chemokines and cytokines including IL-12, and are uniquely capable of presenting exogenous antigen on MHC-I molecules to $CD8^+$ T cells. In contrast, plasmacytoid DCs (PDCs) express the IL-3 receptor (CD123), have a low phagocytic capacity, and secrete high amounts of type I IFN (IFN-α, β, ω, δ) in response to various viruses.[1] PDCs owe their ability to detect and respond to microbes to a specific repertoire of Toll-like receptors (TLRs). In particular, PDCs express high levels of TLR7 and TLR9, which are located in the endosomes and recognize certain nucleotide motifs in viral RNA and DNA, respectively.[2] PDCs thus play an important role in viral immunity and in linking innate and adaptive immune responses.

Activation of DCs generally occurs in a situation of "danger" and can be induced either by exogenous danger signals derived from pathogens, or by endogenous danger signals, released by tissues undergoing stress, damage, or abnormal death.[3] Nucleotides, such as ATP, fit well the role of endogenous danger signals. They are ubiquitously present at high concentrations inside cells and are released into the extracellular space only upon cell activation, stress, or damage. This can be by lytic as well as nonlytic mechanisms caused by host- or pathogen-derived factors. At cell surfaces, nucleotides activate membrane-bound P2 receptors, which are widely distributed in tissues and mediate diverse biologic effects.[4] Currently, 15 members of the P2 receptor family have been cloned and are classified as either G protein–coupled P2Y receptors or ligand-gated ion channels termed P2X.

We and others have previously reported that human myeloid DCs express P2 receptors of both subfamilies and that nucleotides regulate key cell functions, such as antigen uptake, maturation marker expression, migration to draining lymph nodes, cytokine production, and T-cell stimulation.[5-9] In this paper, we report that human PDCs express a unique repertoire of P2Y receptors that regulate critical aspects of PDC function.

Methods

Media and reagents

Human PDCs were cultured in RPMI 1640 medium (Biochrom, Berlin, Germany), supplemented with 2% human AB serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine (PAA, Linz, Austria), 100 U/mL penicillin, 100 μg/mL streptomycin (PAA), and 10 ng/mL IL-3. Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), uridine 5′-triphosphate (UTP), uridine 5′-diphosphate (UDP), inosine 5′-diphosphate (IDP), uridine 5′-diphosphoglucose (UDP-glucose), adenosine, phorbol 12-myristate 13-acetate (PMA), and suramin were obtained from Sigma-Aldrich (St Louis, MO). The $P2Y_{11}$ receptor agonist AR-C67085 was a generous gift from AstraZeneca UK (London, United Kingdom). CpG ODN 2216 and ODN 2006 were provided by Coley Pharmaceutical Group (Wellesley, MA) and used at 6 μg/mL. R-848 was from InvivoGen (San Diego, CA). Soluble CD40L-trimer (CD40L) was a gift from Amgen (Seattle, WA) and was used at 1 μg/mL. Influenza virus PR8 (Puerto

Submitted February 1, 2007; accepted October 21, 2007. Prepublished online as *Blood* First Edition paper, November 9, 2007; DOI 10.1182/blood-2007-02-071910.

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Table 1. Primer sequences

Gene	Forward primer	Reverse primer	Probes
P2Y1	atgttctgtgtccccttggt	aaatcaaagctctcacaattaatcc	ctgggctg
P2Y2	agtgaggaacccgtgcag	cctccaagctctggaacct	cctggaga
P2Y4	ggccattggtcctacaatctt	aaatgggacagggaaagagg	cttcccca
P2Y6	ctgcccacagccatcttc	tgaggtcatagcagacagtgc	catccagc
P2Y11	gggaactgggtagcagacac	agttggcagggcaggact	gctgagga
P2Y12	tttgcctaacatgattctgacc	ggaaagagcatttcttcacattct	caggcagc
P2Y13	ttttcttgaccggcatcc	agctggggatgtgaacaaac	tggctctg
P2Y14	cggtatatgaaagaattcactctgc	aagaaataaataatagggtccaagca	tctgctgc
HPRT	gactttgctttccttggtca	ggctttgtattttgcttttcc	gctgagga

Rico/8/34, H1N1) was propagated in 10-day-old embryonated chicken eggs and used at 25 HAU.

Monoclonal antibodies, cytokines, and enzyme-linked immunosorbent assay

Fluorochrome-conjugated mAbs against CD39, CD62L, CD83, CD86, CD123, CD197 (CCR7), and HLA-DR were purchased from BD PharMingen (San Diego, CA); mAb against BDCA-4 was from Miltenyi Biotech (Bergisch-Gladbach, Germany). IL-3 was obtained from Peprotech (Rocky Hill, NJ). The human enzyme-linked immunosorbent assay (ELISA) for IFN-α (Bender Med Systems, Vienna, Austria), TNF-α, IL-6, and IL-10 (BD PharMingen) were used according to the manufacturers' protocol.

Isolation and culture of PDCs

Peripheral blood mononuclear cells were isolated from buffy coats of healthy volunteers by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). PDCs were isolated using the BDCA-4 isolation kit (Miltenyi Biotech) by repeated positive selection until purity of $CD123^+$ $HLA\text{-}DR^+$ cells was more than 95%. PDCs were cultured in 96-well plates at 2×10^5 cells/mL in the presence of IL-3 (10 ng/mL) and after a short resting period in calcium-containing medium incubated with the indicated stimuli. Supernatants were harvested after 48 hours for analysis of cytokine production by ELISA.

RNA isolation and reverse transcriptase–polymerase chain reaction

Total RNA was isolated from PDCs using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). RNA level was measured by spectrophotometer (Ultro Spec 3000; Pharmacia Biotech, Freiburg, Germany) at 260 nm. RT-PCR was carried out using 1 μg total RNA and 20 to 100 pmol oligo p(DT)15 primer (Roche Diagnostics, Mannheim, Germany). After denaturation at 65°C for 10 minutes, a mixture containing 200 mM Tris-HCl (pH 7.5), 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine, 100 mM dithiothreitol, 10 mM deoxyribonucleoside triphosphates, 40 U/μL RNasin ribonuclease inhibitor, and 50 U/μL expand reverse transcriptase (Roche Diagnostics) was added. cDNA synthesis was carried out at 37°C for 60 minutes in a 20-μL reaction volume. Aqua dest (40 μL; Promega, Madison, WI) was added for a concentration of 16.7 ng/μL. Subsequently, 3 μL of the reaction was analyzed by quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) in a LightCycler device using the Universal ProbeLibrary Set, Human (Roche Diagnostics), in the presence of 10 pmol/μL of forward and reverse primers. Primers were synthesized by Metabion (Martinsried, Germany). Forty-five cycles were carried out at conditions indicated in Table 1, after DNA extension time. Specific amplification was proceeded at 60°C. Hypoxanthine guanine phosphoribosyltransferase (HPRT) was used as the housekeeping gene to normalize for differences in total RNA among samples.

Intracellular Ca^{2+} measurements

Ca^{2+} signaling was measured by flow cytometry as described.[9] In short, PDCs were incubated with fluo-3 acetoxymethyl ester (fluo-3/AM, 4 μg/mL) and Fura Red (Fura Red/AM, 10 μg/mL) in the presence of 0.02% pluronic F-127 (all from Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Cells were washed with assay buffer containing 1 mM $CaCl_2$. For analysis, cells were transferred into tubes containing assay buffer at 37°C. Flow rate was adjusted to 100 to 150 events/sec, and P2Y receptor agonists were added. Data were analyzed by plotting the LFL1/LFL3 ratios versus time using FlowJo software (version 3.4; Tree Star, San Carlos, CA) and are expressed as relative values to a maximal signal obtained with a standard dose of ionomycin.

Statistical analysis

Data are expressed as means plus or minus SEM. Statistical significance was determined by the paired 2-tailed Student *t* test. Differences were considered statistically significant for *P* values less than .05.

Results

Human PDCs express mRNA for several P2Y receptors

PDCs were isolated from healthy donors to assess the expression pattern of P2Y receptors by real-time RT-PCR in primary immature PDCs as well as mature PDCs activated with IL-3 and CD40L for 24 hours. Figure 1 shows that immature PDCs express mRNA for several P2Y receptor subtypes, that is, receptors with high affinity to UTP ($P2Y_4$), UDP ($P2Y_6$), and ADP ($P2Y_{12}$, $P2Y_{13}$). Interestingly, highest expression levels were found for $P2Y_{14}$, a receptor for sugar nucleotides, such as UDP-glucose. In contrast, PDCs expressed no mRNA for receptors with high affinity for ATP (ie, $P2Y_2$ and $P2Y_{11}$). Expression of all P2Y receptor subtypes was found in PBMCs, which served as positive controls. Figure 1 also

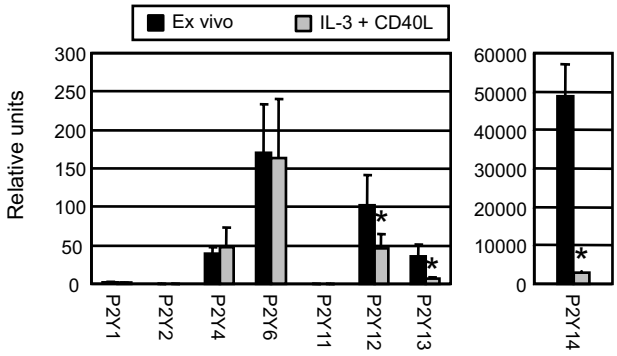

Figure 1. Expression of P2Y receptor mRNA in human PDCs. PDCs were isolated from PBMCs at high purity (> 95%) and mRNA was extracted immediately after isolation (ex vivo) or after 24-hour culture in the presence of IL-3 and CD40L to induce phenotypic PDC maturation. Profiles of P2Y receptor expression were generated by real-time RT-PCR. Results (mean ± SEM) of 3 independent experiments are given as relative units after normalization for differences to expression of the housekeeping gene HPRT. *$P < .05$.

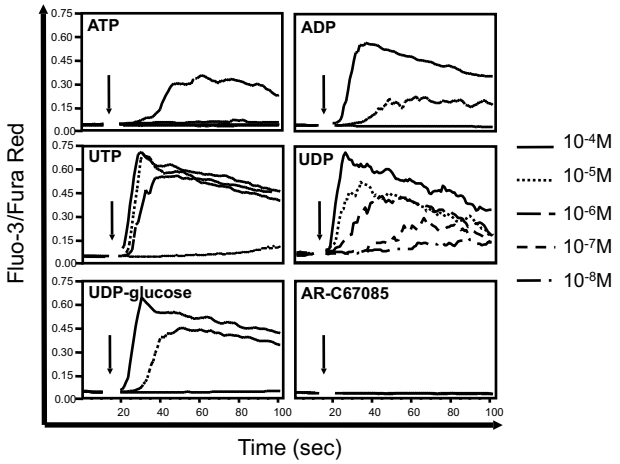

Figure 2. Ca^{2+} signaling in PDCs in response to various P2Y receptor agonists. Ca^{2+} ion transients in freshly isolated PDCs exposed to ATP, ADP, UTP, UDP, UDP-glucose, and the $P2Y_{11}$ agonist AR-C67085 were analyzed by flow cytometry. After establishing a baseline for 10 seconds, nucleotides were added at the indicated concentrations. Note: Only for UDP, the concentration was titrated down to 0.01 μM. Data were normalized to the peak calcium signal induced by a standard dose of ionomycin (arbitrarily set to 1.0). Representative data of 4 different experiments are shown.

shows that upon maturation, PDCs down-regulate mRNA expression for $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$, whereas $P2Y_4$ and $P2Y_6$ were unaffected.

Effect of nucleotides on the intracellular free Ca^{2+} concentration of PDCs

We previously observed that mRNA expression of P2 receptors in DCs does not necessarily reflect the expression of functional membrane-bound receptors.[9] Since P2Y receptors are linked to phospholipase C, receptor signaling can be studied by measuring intracellular Ca^{2+} transients in response to exposure of viable cells to nucleotides. P2Y receptor subtypes exhibit characteristic binding affinities to various nucleotides. ATP and/or ADP interact with $P2Y_1$, $P2Y_2$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$, whereas uridine nucleotides, such as UTP and UDP, activate the pyrimidine receptors $P2Y_2$, $P2Y_4$, and $P2Y_6$.[10] The recently cloned $P2Y_{14}$ responds to UDP-glucose and related sugar nucleotides.[11] As shown in Figure 2, primary PDCs are highly sensitive to UDP, UTP, UDP-glucose, and, to a lesser extent, ADP. This functional profile corresponds to the mRNA expression pattern obtained with RT-PCR (Figure 1). Thus, $P2Y_4$, $P2Y_6$, $P2Y_{14}$ $P2Y_{12}$, and $P2Y_{13}$ receptors are functional in PDCs. No signaling was observed in response to AR-C67085, a specific $P2Y_{11}$ agonist. Moreover, in accordance with low mRNA expression for the high-affinity ATP receptors, $P2Y_2$ and $P2Y_{11}$ by RT-PCR, PDCs were less sensitive to ATP, with signaling detected only at concentrations of 100 μM or higher.

Nucleotides induce phenotypic maturation of PDCs

Since the presence of extracellular nucleotides may confer danger to immune cells, thereby inducing activation, we investigated the effect of ATP and other nucleotides on the expression of maturation markers in PDCs. We found that in response to ATP exposure, PDCs moderately up-regulated the expression of CD86 and CD83 (Figure 3), CCR7, and HLA-DR surface expression, the latter being expressed at high levels by PDCs cultured with IL-3 alone. Induction of maturation marker expression was also observed for UTP, UDP, UDP-glucose, and ADP (Figure 3 and data not shown). Since DC activation likely occurs in the context of other stimulatory signals present at sites of inflammation, we analyzed whether ATP (or UDP) synergizes with CD40L, a TNF family member of membrane molecules expressed by activated T cells. These experiments revealed an additive effect between CD40L and nucleotides in regard to induction of CD83, CD86, and CCR7 expression

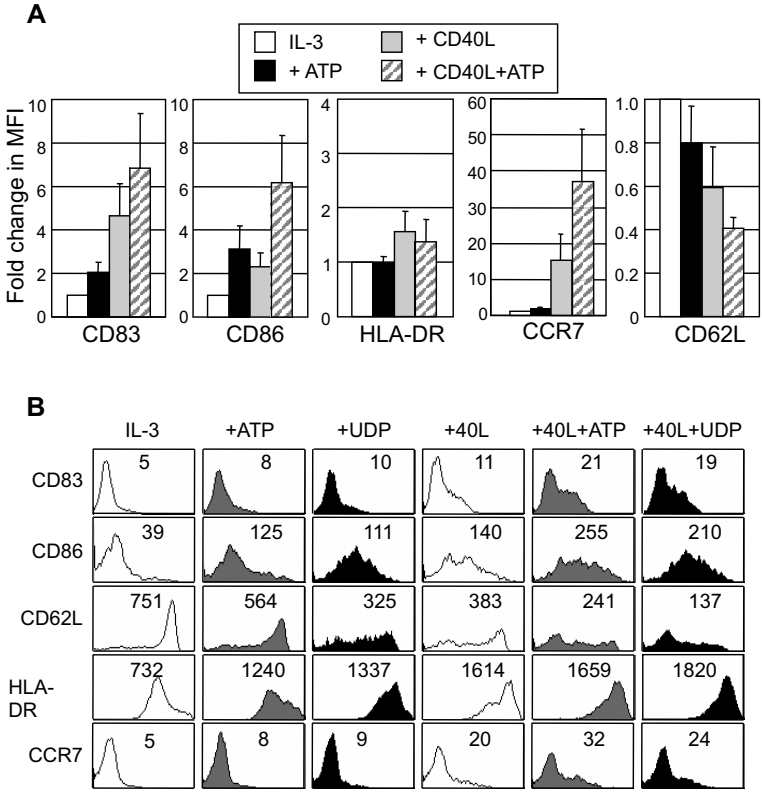

Figure 3. Surface activation marker expression of PDCs after exposure to extracellular nucleotides. PDCs were cultured with IL-3 in the absence or presence of 250 μM ATP or UDP. Where indicated, 1 μg/mL CD40L trimers was added. After a culture period of 36 hours, surface expression of CD83, CD86, HLA-DR, CCR7, and CD62L was assessed by flow cytometry. In the upper panel, data are expressed as n-fold change of mean fluorescence intensity (MFI) with expression levels of IL-3–cultured PDCs normalized to 1. Means of x-fold changes (± SEM) of 4 to 6 different donors are shown. In the lower panel, histogram plots with MFI of PDCs from a representative donor are shown.

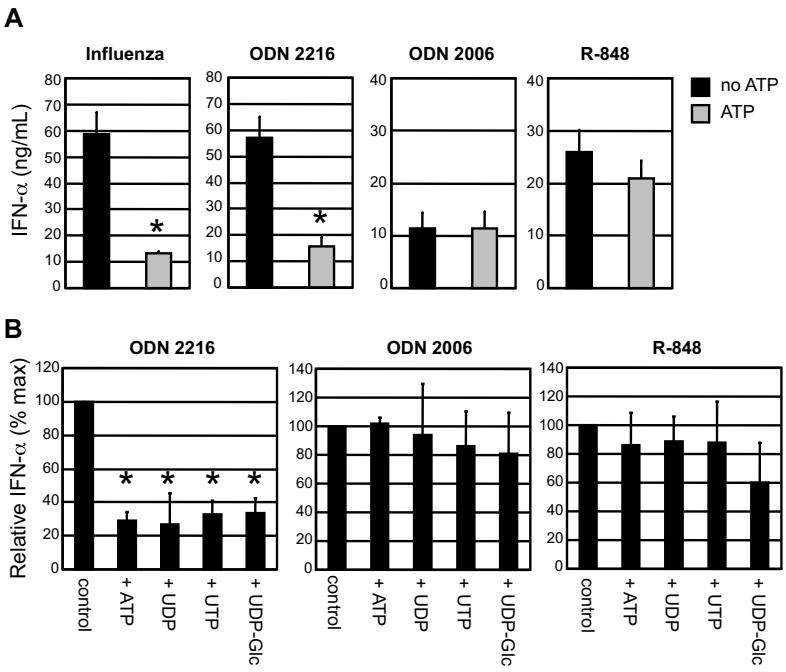

Figure 4. Extracellular nucleotides inhibit IFN-α production in response to influenza virus or CpG ODN 2216. (A) PDCs were activated with either CpG ODN 2216, or CpG ODN 2006, or R-848 or influenza virus in the absence or presence of 100 μM ATP. Supernatants were harvested after 48 hours and concentrations of IFN-α were measured by ELISA. Data are mean values (± SEM) of 5 to 10 different donors. (B) Effect of various nucleotides, each at 100 μM, on IFN-α production in response to TLR ligands. Data represent means of percentage changes (± SEM) of 5 to 10 different donors. Mean values (± SEM) in nanograms/milliliter for CpG ODN 2216, 66.9 (± 7.6); for CpG ODN 2006, 11.3 (± 3.8); and for R-848, 47.0 (± 9.1); *$P < .05$.

(Figure 3). In addition, CD62L, a molecule mediating lymph node homing of PDCs via L-selectin expressed by high endothelial venules, was down-regulated under these conditions. This expression pattern of surface molecules is indicative of a mature PDC phenotype favoring migration to lymph nodes via the lymphatic route.[12]

Extracellular nucleotides inhibit IFN-α production by PDCs

Since a key function of PDCs is sensing and responding to microbial challenge by producing type I IFN, we investigated the effect of P2Y receptor signaling on IFN-α production. None of the nucleotides under investigation induced IFN-α production in PDCs (detection limit of the ELISA: 4.8 pg/mL; data not shown). We next addressed whether nucleotides have an impact on IFN-α production induced by virus or specific TLR ligands. First, we stimulated PDCs with influenza virus (PR-8) in the absence or presence of 100 μM ATP. ATP significantly inhibited IFN-α production by PDCs (Figure 4A). Furthermore, ATP inhibited IFN-α production in response to CpG ODN 2216 (CpG-A), a synthetic TLR9 ligand and potent inducer of type I IFN in PDCs. In contrast, ATP had no significant effect on IFN-α production induced by either CpG ODN 2006 (CpG-B) or by the TLR7/8 ligand R-848.

To assess whether this inhibition is a class effect of P2Y receptor agonists, we studied the effect of other nucleotides on IFN-α production by TLR ligand-activated PDCs. Inhibition of CpG-A–induced IFN-α was seen to a similar degree for ATP, UTP, UDP, and UDP-glucose (each 100 μM) (Figure 4B). Again, no inhibition was seen for PDCs stimulated with CpG ODN 2006 or R-848. Thus, nucleotides inducing Ca^{2+} signaling in PDCs were also effective inhibitors of IFN-α production, indicating a class effect of P2Y receptors.

Kinetic experiments revealed that inhibition of IFN-α was most efficient when ATP exposure occurred either a few hours before or simultaneously with CpG-A exposure. The inhibitory effect decreased over time and was no longer significant when ATP was added 6 hours after CpG-A activation (Figure 5). ATP did not inhibit R-848–induced IFN-α secretion by PDCs, irrespective of the timing of ATP addition (data not shown).

Ligands of $P2Y_4$, $P2Y_6$, and $P2Y_{14}$ are potent inhibitors of IFN-α production

Targeting specific P2Y receptors may open new treatment options for autoimmune disorders triggered by disregulated type I IFN responses. We therefore analyzed the potency of different P2 receptor agonists in regard to their inhibitory effect on PDC IFN-α production in response to CpG-A. Dose titration experiments revealed striking differences between various nucleotides (Figure 6). IFN-α inhibition was most potent for UDP ($P2Y_6$, EC_{50}: ~ 0.01 μM), followed by UTP ($P2Y_4$, EC_{50}: ~ 1 μM), IDP ($P2Y_6$, EC_{50}: ~ 3 μM), and UDP-glucose ($P2Y_{14}$, EC_{50}: ~ 5 μM). ATP and ADP were effective only in the 100-μM range, whereas AR-C67085 ($P2Y_{11}$) and adenosine (P1 receptors) were ineffective. We previously reported that adenosine can inhibit IFN-α production in mature PDCs via A2a receptors, however, these are not expressed by immature PDCs.[13] Thus, the pyrimidinoreceptors $P2Y_4$ and $P2Y_6$ and the recently cloned $P2Y_{14}$ receptor are potential targets for regulating IFN production in PDCs.

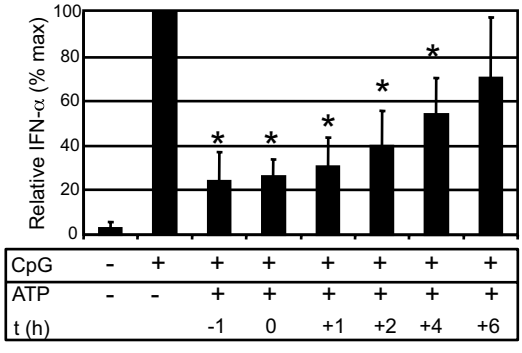

Figure 5. Influence of time point of nucleotide exposure on inhibition of IFN-α production. PDCs were exposed to 100 μM ATP either 1 hour before, simultaneously, or up to 6 hours after activation with CpG ODN 2216. CpG-induced IFN-α production in the absence of nucleotides was normalized to 100%. Data represent means of percent changes (± SEM) of 4 different donors. *$P < .05$.

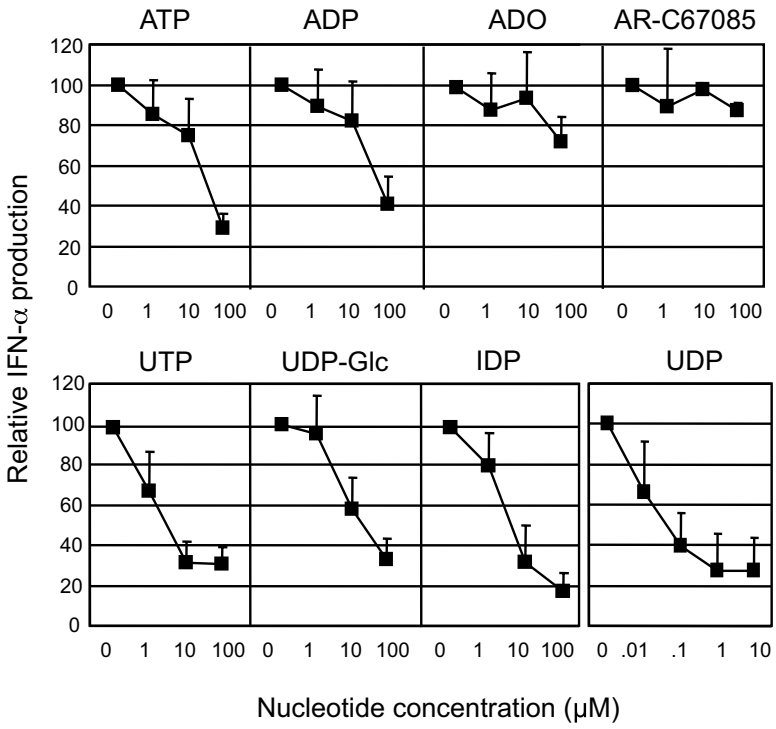

Figure 6. Influence of different P2Y receptor agonists on IFN-α production by PDCs. PDCs were stimulated with CpG ODN 2216 in the absence or presence of ATP, ADP, adenosine (ADO), the $P2Y_{11}$ receptor agonist AR-C67085, UTP, UDP-glucose, IDP, or UDP at the indicated concentrations (note: UDP concentrations differ from other nucleotides). IFN-α production in the absence of nucleotides was normalized to 100%. Data represent mean values (± SEM) of 5 to 10 donors.

Inhibition of IFN-α production is downstream of CpG ODN uptake and may involve PKC activation

Since both CpG-A and CpG-B bind to the same receptor (ie, TLR9) but ATP inhibited only CpG-A–induced IFN-α production, we speculated that ATP may influence the uptake of different CpG ODN by PDCs. CpG-A has been shown to self-assemble into higher order tertiary structures via G-tetrad formation of their poly(G) motifs forming nanoparticles in the size range of viruses.[14] The precise uptake mechanism of different CpG ODNs in PDCs is unknown. To test whether ATP has an impact on CpG ODN uptake, we incubated PDCs with fluorochrome-labeled CpG-A or CpG-B in the absence or presence of ATP. As shown in Figure 7A, ATP had no influence on CpG uptake. Thus, inhibition of IFN-α production by ATP likely occurs downstream of CpG internalization.

To confirm that inhibition of IFN-α production is P2Y receptor mediated, we studied the effect of suramin, an inhibitor of various P2Y receptors, including $P2Y_6$,[15] on UDP-mediated IFN-α inhibition in PDCs activated with CpG-A. As shown in Figure 7B, suramin completely abrogated the inhibitory effect of this nucleotide.

Coupling of P2Y receptors to G_q proteins activates intracellular signaling cascades, mainly the phospholipase C_β isoform mobilizing intracellular Ca^{2+} via IP_3. A target for phospholipase C is diacylglycerol (DAG) leading to protein kinase C (PKC) activation. We, therefore, investigated whether enhancing intracellular Ca^{2+} levels by the ionophore, ionomycin, impacts IFN production in PDCs. No significant effect on CpG-A–induced IFN-α was observed (Figure 7C). We next investigated the influence of PMA, a potent activator of most PKC isoforms, on IFN-α production by PDCs. Strikingly, as observed for nucleotides, PMA inhibited both influenza virus- and CpG-A–induced, but not R-848– or CpG-B–induced, IFN-α production (Figure 7D). Thus, PKC activation specifically interferes with influenza virus and CpG-A–induced IFN-α production in PDCs. To further study the role of PKC on IFN-α production, we treated PDCs with various PKC inhibitors (chelerythrine, Ro-31–8220, and U73122). Unexpectedly, PKC inhibition interfered with CpG-A–induced IFN-α production and thus could not be applied in this context (data not shown).

Discussion

In addition to their antiviral properties, type I IFNs are recognized as potent regulators of the innate and adaptive immune response. They enhance the cytotoxicity of natural killer (NK) cells and $CD8^+$ T cells, and, together with IL-12, induce IFN-γ secretion in NK cells, $CD4^+$ T cells, and $CD8^+$ T cells, and, together with IL-6, promote the differentiation of memory B cells into antibody-secreting plasma cells (reviewed in McKenna et al[2]). There is evidence that type I IFNs also play a role in cancer immunosurveillance.[16] These reports highlight the importance of type I IFNs—and PDCs as their main source—in the immune system. Increased numbers of PDCs are found in inflamed lymph nodes,[17] skin lesions associated with systemic lupus erythematosus (SLE),[18,19] psoriasis vulgaris and contact dermatitis,[19] epithelioid cell granulomas,[20] the nasal mucosa during allergic reactions,[21] and tumors,[22-24] likely reflecting their contribution to immune responses during viral infection, autoimmunity, and cancer. A better perceptive of type I IFN regulation will be crucial for understanding the pathogenesis of these diseases and for the development of new therapeutic strategies.

IFN induction in PDCs is triggered through TLR7 and TLR9 ligands, including single-stranded RNA and CpG-rich DNA, respectively, as well as by recognition of virus-specific patterns in the cytosol (reviewed in Asselin-Paturel and Trinchieri[25]). There is an emerging array of soluble and pathogen-derived mediators as well as surface receptors that can negatively regulate IFN-α production. These include cross-linking of PDC surface molecules, such as BDCA-2 and CD123,[26,27] secreted molecules, including histamine,[28] vasoactive intestinal peptide[29] and high mobility group

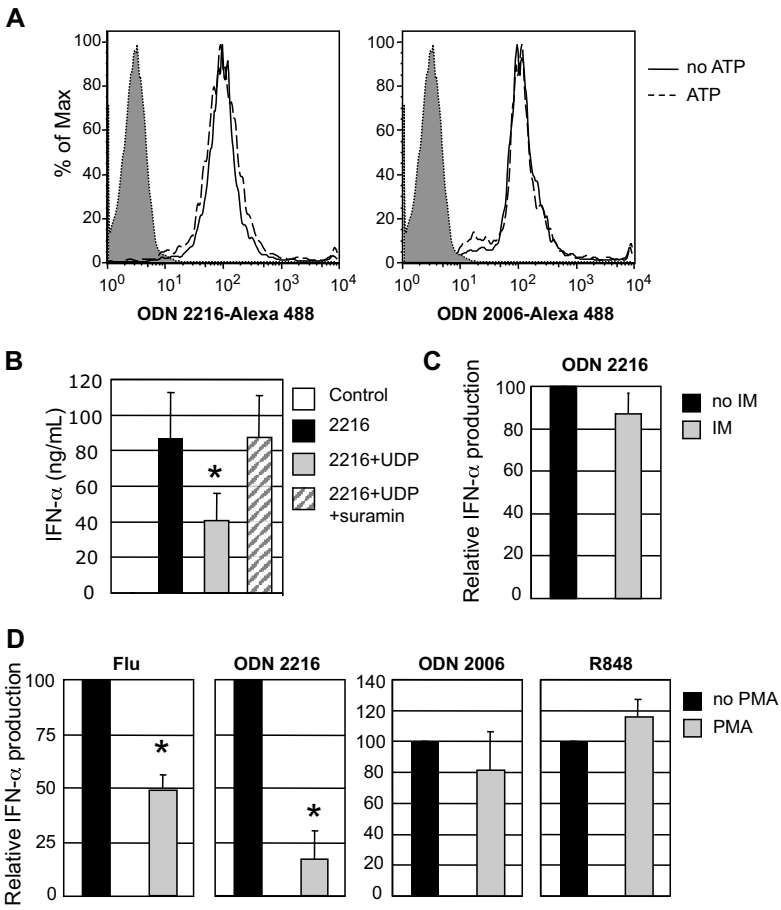

Figure 7. Inhibition of IFN-α production is mediated by P2Y receptors and may involve PKC activation. (A) Influence of nucleotides on CpG ODN uptake by PDCs. PDCs were incubated with Alexa 488–labeled ODN 2216 or ODN 2006 in the absence or presence of 100 μM ATP. ODN uptake was analyzed by fluorescence-activated cell sorting (FACS) analysis after 2 hours. One representative experiment of 2 is shown. (B) Effect of the P2Y receptor antagonist suramin on UDP-mediated inhibition of IFN-α. PDCs were activated with ODN 2216 in the absence or presence of 0.1 μM UDP and suramin. Data represent mean values ± SEM of 3 donors. (C) Effect of ionomycin on IFN-α production. PDCs were activated with CpG ODN 2216 in the absence or presence of 100 ng/mL ionomycin (IM). (D) Effect of PKC activation with PMA on IFN-α production. PDCs were activated with CpG ODN 2216, CpG ODN 2006, influenza virus, or R-848 in the absence or presence of 10 ng/mL PMA. IFN-α levels were measured in the supernatants after 48 hours. Data in panels C,D represent mean values (± SEM) of 4 to 6 donors. *$P < .05$.

B1 protein,[30] as well as nonstimulatory DNA sequences.[31] However, little is known about the pathways by which these act and whether they inhibit through common signaling mechanisms.

In the present paper, we provide for the first time evidence of a new class of soluble negative regulators of IFN-α production by PDCs. Extracellular nucleotides, natural ligands of membrane G protein–coupled P2Y receptors, were found to be potent inhibitors of IFN-α production by PDCs. Inhibition was seen for both influenza virus (TLR7)–induced and CpG-A (TLR9)–induced PDC activation. Interestingly, induction of IFN-α in response to the synthetic TLR ligands R-848 (TLR7) and CpG-B (TLR9) was not influenced by nucleotides, indicating that P2Y receptor signaling does not universally shut down IFN production. This finding is surprising considering that both CpG-A and CpG-B signal through TLR9. CpG-A and CpG-B were shown to have distinct physical properties, with CpG-A aggregating spontaneously to form nanoparticles,[14] which might potentially result in different routes of internalization. We could rule out that the mechanism of action of ATP was simply interfering with the uptake of either CpG ODN class by PDCs, implicating a more downstream signaling effect. There is evidence that CpG-A and CpG-B activate PDCs differently, however the precise signaling differences are poorly defined.[32] P2Y receptors are coupled to transductional G proteins. $P2Y_{12,13,14}$ predominantly couple to $G_{i/o}$ proteins inhibiting adenylate cyclase, whereas the uridine nucleotide receptors $P2Y_6$ and $P2Y_4$ use G_q/G_{11} to activate phospholipase C_β/PKC and IP_3 pathways (reviewed in Abbracchio et al[10]). IFN inhibition correlated with strength of calcium signaling and was most potent for ligands of $P2Y_6$ (ie, UDP and IDP) and $P2Y_4$ (UTP), indicating a role for phospholipase C_β/PKC in IFN-α inhibition. Furthermore, the PKC activator PMA exhibited the same functional profile as nucleotides, with potent inhibition of CpG-A– and influenza virus–induced IFN-α production, but not CpG-B– or R-848–induced IFN-α production by PDCs. Unfortunately, the role of PKC on nucleotide-induced IFN-α regulation could not be studied directly, as PKC inhibitors directly interfered with IFN-α production by PDCs.

This leads to the question, at which level does PKC interfere with the regulation of type I IFN production in PDCs? PKC has been shown to play a role in the formation of mature lysosomes.[33] Thus, PKC signaling may result in a more efficient trafficking of endocytosed material, such as CpG ODN, from endosomes into lysosomes in PDCs. Honda et al reported that spatiotemporal regulation of MyD88-IRF-7 signaling is critical for the induction of high levels of IFN in response to TLR9 activation.[34] They also demonstrated that CpG-A, but not CpG-B, is retained for long periods in PDC endosomal compartments thereby activating MyD88-IRF-7, which results in the induction of high levels of IFN-α. A reduced retention time of CpG-A in endosomes via accelerated lysosome formation could explain why nucleotides/PMA have such a potent inhibitory effect on CpG-A–, but not CpG-B–induced IFN in PDCs. Identifying the precise mechanism of IFN inhibition by nucleotides is the subject of further investigations by our laboratory.

Pharmacological studies using nucleotides with distinct affinities for P2Y receptors revealed that uridine nucleotides have the highest inhibitory potency on IFN-α production. UDP ($P2Y_6$) was effective in the nanomolar range, followed by UTP ($P2Y_4$), IDP ($P2Y_6$), and UDP-glucose ($P2Y_{14}$) in the low micromolar range, whereas the adenine nucleotides ATP and ADP were inhibitory only

in the high micromolar range. This finding correlated with functional P2Y receptor analysis measuring Ca^{2+} transients in PDCs and RT-PCR expression profiling. In contrast, PDCs were only weakly sensitive to ATP and insensitive to a specific $P2Y_{11}$ agonist, correlating with the lack of mRNA expression for $P2Y_2$ and $P2Y_{11}$ receptors. In this respect, PDCs differ strikingly from myeloid DCs, in which $P2Y_{11}$ signaling regulates critical functions, such as maturation and induction of migration and cytokine production.[8,9,35] In fact, lack of specific ATP receptors and low sensitivity to ATP in calcium signaling assays may indicate that the biologic effects of ATP on PDCs, such as maturation induction and type I IFN regulation, are rather mediated by the breakdown product ADP generated by ectonucleotidases leading to signaling via $P2Y_{12}$ or $P2Y_{13}$. Ecto-ATPase expression has been described for DCs in lymph nodes[36] and monocyte-derived DCs.[37] As data for PDCs are currently lacking, we assessed CD39 expression by flow cytometry, but no expression was found (data not shown). This finding, however, does not rule out expression of other ectonucleotidases. Since CD39 is ubiquitously expressed in tissues, degradation of extracellular ATP to ADP is likely to play a role in nucleotide-induced signaling in PDCs in vivo.

It has been proposed that nucleotides induce a distorted maturation of myeloid DCs by inhibiting IL-12p70 but enhancing the production of the anti-inflammatory cytokine IL-10 in response to TLR ligands or CD40L.[5,38] Interestingly, in one study ATP enhanced IL-10 production via the $P2Y_{11}$ receptor, whereas in another study ADP was inhibitory.[37] In contrast to myeloid DCs, PDCs are poor producers of IL-10.[39] In line with this report, IL-10 levels were near the detection limit of the ELISA for all stimulation conditions in our study (data not shown).

What is the physiological consequence of P2Y receptor signaling in PDCs? Nucleotides are released and accumulate at sites of inflammation, tissue damage, or cell stress. PDCs located in the vicinity are likely to be exposed to nucleotide concentrations sufficient to activate P2Y receptors. As a consequence, PDCs up-regulate the chemokine receptor CCR7 and undergo phenotypic maturation, enabling them to migrate from peripheral sites to draining lymph nodes via the lymphatic route and to interact efficiently with T cells. On the other hand, PDCs exposed to IFN-inducing stimuli, such as DNA or virus, will be primed toward a reduced IFN-α response. Inhibition of IFN production by nucleotides may play an important negative regulatory mechanism for avoiding excessive tissue damage or the induction of autoimmunity. As the kinetic experiments demonstrated, nucleotides may (a) condition PDCs to low type I IFN-producing capacity (eg, in situations when dying cells release nucleotides before PDCs are exposed to TLR ligands, such as dsDNA-antibody complexes found in SLE) or (b) inhibit IFN production even several hours after TLR9 activation (release of nucleotides in draining lymph nodes, eg, from activated T cells, may still be sufficient to limit IFN-α production). The distinct P2Y receptor repertoire expressed by PDCs (such as $P2Y_6$, $P2Y_4$, and the recently cloned $P2Y_{14}$ receptor) highlights the possibility for designing new drugs to target this specific P2Y receptor repertoire for inhibiting type I IFN responses in vivo.

Another consequence of nucleotide exposure of PDCs could be tolerance induction to tumors. Tumor cells release nucleotides via lytic (necrosis) as well as nonlytic mechanisms.[40] Tolerogenic PDCs have been isolated from lymph node–draining tumors.[41] These suppressive PDCs were found to express indoleamine 2,3-dioxygenase (IDO). Interestingly, ATP can up-regulate IDO in DCs.[42] Nucleotide release by tumor cells could thus be an immune escape mechanism by (a) inducing IDO in lymph node resident DCs and (b) inhibiting Th1 immune responses by limiting type I IFN production. This hypothesis deserves further evaluation in tumor models.

In conclusion, this is the first report to demonstrate that human PDCs express a characteristic P2Y receptor repertoire rendering them highly sensitive to uridine nucleotides. After nucleotide exposure, PDCs acquire a mature phenotype, which is synergistically enhanced by CD40L. In addition, nucleotides are potent negative regulators of IFN-α production. Since IFN-α is a key cytokine with effects on both innate and adoptive immunity, manipulating its production has potential in the development of novel therapeutic strategies against infectious disease, autoimmune disorders, and cancer. In particular, drugs acting on $P2Y_4$, $P2Y_6$, or $P2Y_{14}$ receptors may prove useful for the treatment of IFN-mediated autoimmune disorders (such as SLE), where overproduction of IFN-α contributes to disease progression.

Acknowledgments

The authors thank Dr. W. Chen for virus preparation and Tristan McAlpine and Marina Zupper for technical assistance.

This work was supported by the Deutsche Krebshilfe (Max Eder Research Grant to M. Schnurr), the Deutsche Forschungsgemeinschaft (GK1202) to M. Schnurr, a Program Grant from the Australian National Health and Medical Research Council (NH&MRC) to E.M., and the Ludwig Institute for Cancer Research. E.M. is an employee of CSL limited and an Honorary Senior Research Fellow of the Ludwig Institute for Cancer Research.

Authorship

Contribution: A.S. designed and performed research and collected and analyzed data; T.T., S.R., N.R., J.V., and M.D. performed research and collected and analyzed data; M. Stuplich performed research and collected data; S.E. and J.C. wrote the paper; E.M. analyzed data and wrote the paper; and M. Schnurr designed and performed research, collected and analyzed data, and wrote the paper.

Conflict-of-interest disclosure: E.M. is employed by CSL Limited, but none of its potential products was studied in the present work. The other authors declare no competing financial interests.

Correspondence: Max Schnurr, Medizinische Klinik der Universität München, Campus Innenstadt, Ziemssenstr. 1, D-80336 München, Germany; e-mail: Max.Schnurr@med.uni-muenchen.de.

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