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Research Article

Noncanonical NF-κB signaling in dendritic cells is required for ATP-driven indoleamine 2,3-dioxygenase 1 induction through $P2Y_{11}$ receptor

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Abstract

Extracellular ATP released from dying cells, including tumor cells, is a key mediator of inflammation and tolerance by binding to purinergic receptors on dendritic cells (DCs), resulting in inflammasome activation (via P2X7R), DC maturation (via $P2Y_{11}R$), and indoleamine-2,3-dioxygenase 1 upregulation. However, the regulation of ATP-driven Indoleamine-2,3-dioxygenase 1 expression in human DCs has been poorly investigated. In this work, we aimed to investigate the ATP-driven molecular regulation of indoleamine-2,3-dioxygenase 1 expression via purinergic receptors and to provide an in-depth characterization of ATP-driven T regulatory cells induced by indoleamine-2,3-dioxygenase 1-expressing DCs. We identified $P2Y_{11}R$ as being responsible for ATP-driven indoleamine-2,3-dioxygenase 1 upregulation, and noncanonical NF-kB as a molecular pathway associated with ATP-dependent indoleamine-2,3-dioxygenase 1 induction through $P2Y_{11}R$. Then, we investigated—but did not confirm—an involvement of inflammasome machinery through P2X7R in indoleamine-2,3-dioxygenase 1 upregulation. Finally, we evaluated the role of ATP catabolism via ATP ectonucleotidases, i.e. CD39 and CD73 and its main product adenosine, in regulating the generation of indoleamine-2,3-dioxygenase 1-driven T regulatory cells. We found that ATP-driven indoleamine-2,3-dioxygenase 1 upregulation is associated with CD73 upregulation and adenosine production. Additionally, ATP-treated indoleamine-2,3-dioxygenase 1-positive mature DCs induce PD-1-expressing bona fide suppressive T regulatory cells via adenosine $A_{2A}R$. Collectively, a more in-depth understanding of ATP-driven immune-regulatory mechanisms through indoleamine-2,3-dioxygenase 1 regulation in human DCs leading to the induction of T regulatory cells can have clinical implications for the development of indoleamine-2,3-dioxygenase 1 inhibitors in cancer patients, especially in combination with immunotherapy such as an anti-CD73 or adenosine receptor agonist and immunogenic chemotherapy.

Keywords: ATP, dendritic cell, immune tolerance, indoleamine-2, 3-dioxygenase 1, tumor

1. Background

ATP is a versatile molecule, mostly located in the cellular cytoplasm under physiological conditions, where it acts as the main source of energy for many essential cellular functions.[1] Under pathological conditions such as tissue injury, inflammatory reactions, hyperreactivity, and tumor cell growth, a large amount of ATP is actively released by immune and cancer cells or passively leaked by dying cells into the extracellular compartment, where it functions as an important mediator of extracellular processes by activating purinergic P2 receptors (P2Rs) (extracellular signaling).[2] Extracellular ATP is a key regulator of inflammatory and immune response by modulating the function of many immune cells.[3] Of these, dendritic cells (DCs) are induced to complete maturation and full competence in antigen presentation through the activation of P2X7 and $P2Y_{11}$ receptors (P2X7R and $P2Y_{11}R$).[4–7] P2X7R initiates the process of DC antigen cross-priming and cross-dressing[8] and the activation of inflammasome NLRP3/ASC/caspase-1, which results in the polarization of IFN-γ-producing $CD8^+$ effector T cells through IL-1β secretion.[4] $P2Y_{11}R$ is involved in DC maturation through the upregulation of CD40, CD80, CD86, and CD83.[5,6]

Indoleamine-2,3-dioxygenase 1 (IDO1) is an intracellular rate-limiting enzyme that catalyzes the metabolism of tryptophan along the kynurenine acid pathway. IDO1 is expressed by immune and cancer cells and can modulate the immune system to arrest inflammation and suppress immunity to cancer, thus being responsible for tumor escape.[9] IDO1 expression requires noncanonical NF-κB signaling, which downregulates proinflammatory cytokine production and results in noninflammatory DCs, thus

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suppressing T-cell activation and promoting the development of regulatory T cells (Tregs).[10] Several signals, such as CD40L, are capable of inducing IDO1 upregulation in DCs through noncanonical NF-κB signaling.[10] Also, extracellular ATP has recently been shown to drive IDO1 upregulation in DCs,[11] but the regulation of ATP-driven IDO1 expression in DCs has been poorly investigated.

Along with its well-known proinflammatory activity as a danger signal, extracellular ATP has recently been recognized as having anti-inflammatory and immunotolerance properties. In this, the ATP-driven activation of the IDO1 pathway in DCs is of utmost relevance in the creation of the immune microenvironment for a large variety of pathological conditions.[12,13] In tumors, we previously demonstrated that the release of ATP from chemotherapy-treated dying acute myeloid leukemia cells is correlated with IDO1 upregulation in mature DCs (mDCs), followed by the induction of Tregs.[11] Although these and other data indicate that the ATP-driven IDO1 upregulation in DCs is a major suppressive mechanism leading to Tregs generation,[11–13] the suppressive properties of ATP-driven Tregs induced by IDO1-expressing DCs have not been fully characterized. In particular, little is known about the role of inhibitory checkpoint receptors such as PD-1,[14–16] the expression of which on Tregs could represent a potential therapeutic target that can synergize with the well-established effects on PD-1-expressing effector T cells.[17,18] Moreover, few if any data are available regarding the association of ATP ectonucleotidases, such as CD39 and CD73, which control the enzymatic digestion of ATP and lead to adenosine production[19] and IDO1-driven Treg generation by DCs.

In this work, we aimed to investigate the ATP-driven molecular regulation of IDO1 expression in DCs via purinergic receptors and to provide an in-depth characterization of ATP-driven Tregs induced by IDO1-expressing DCs.

2. Materials and methods

2.1 Cells

Human $CD14^+$ and $CD3^+$ cells were purified by magnetic separation (MiltenyiBiotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions, from mononuclear cells separated from buffy coats of healthy donors by Ficoll–Hypaque centrifugation (Amersham, Buckinghamshire, UK) after they had signed to confirm their informed consent (local Ethics Committee approval code 94/2016/O/TES). The purity of the cell populations was always >90%.

The cells were grown in an RPMI 1640 medium (Lonza, Milan, Italy), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (MP Biomedicals, Milan, Italy) (complete RPMI), and maintained at 37 °C and 5% CO_2.

2.2 DC generation and maturation

Human monocyte-derived DCs were generated by culturing $CD14^+$ cells (1×10^6 cells/mL) for 5 d in complete RPMI and in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF; 50 ng/mL; MiltenyiBiotec) and IL-4 (800 U/mL; MiltenyiBiotec), as previously described.[20] DC maturation (1×10^6 cells/mL) was induced by ATP (1 mM; Sigma Aldrich), CD40L (2 µg/mL; R and D systems, Minneapolis, USA) or LPS (1 µg/mL; Sigma Aldrich) treatment for 48 h in complete RPMI at 37 °C and 5% CO_2. Following this, the immDCs and treated (mDCs) were used for FACS, western blotting, luminometry or quantitative real-time PCR analysis, or for co-culture with autologous $CD3^+$ T cells to induce Tregs.

2.3 DC phenotype and cytokine production

2.3.1 DC-maturation markers and IDO1 expression

2×10^5 (1×10^6 cells/mL) immDCs and DCs treated for 48 h with ATP (1 mM, Sigma Aldrich) in the presence or absence of P2X7R (AZ 10606120 dihydrochloride; Tocris, Bristol, UK; 10 µM; 1 h of pretreatment) or $P2Y_{11}R$ (NF340; Tocris; 10 µM; 1 h of pretreatment) antagonists, or with CD40L (2 µg/mL), were used. Additionally, for experiments on inflammasome involvement, the inflammasome inhibitors Ac-YVAD-cmk Caspase-1 (YVAD; 8 µM; Invivogen, San Diego, USA; 1 h of pretreatment) or BAY 11-7082 (Bay; 10 µM; Invivogen; 1 h of pretreatment) were used.

For experiments on noncanonical NF-κB pathway involvement, the inhibitor of the canonical NF-κB pathway IKK-gamma NEMO (IKK-γ; 15 µg/mL; Novus Biological, Colorado, USA; 2 h of pretreatment) was used.

2.3.2. Intracellular IL-1β staining

2×10^5 (1×10^6 cells/mL) immDCs and DCs treated for 5 h and 15 min with ATP (1 mM, Sigma Aldrich) in the presence or absence of P2X7R (AZ 10606120 dihydrochloride; Tocris; 10 µM; 1 h of pretreatment) or $P2Y_{11}R$ (NF340; Tocris; 10 µM; 1 h of pretreatment) antagonists and with LPS (1 µg/mL; Sigma Aldrich) followed by overnight treatment with Brefeldin A (20 µg/mL; Sigma Aldrich), were used.

Briefly, 2×10^5 DCs were stained in the dark at room temperature for 15 min using the following antihuman monoclonal antibodies (moAbs): HLA-DR FITC (clone G-46-6; Beckton Dickinson/BD Biosciences, New Jersey, USA), CD14 Pe-Cy7 (clone 63D3; Biolegend, San Diego, USA), CD86 PerCP-Cy5.5 (clone 2331 [FUN-1]; BD Biosciences), CD83 APC (clone HB15; Biolegend), CD80 APC-h7 (clone L307.4; BD Biosciences), CD73 FITC (clone AD2; Biolegend), and IDO1 PE (clone eyedio; Invitrogen, Carlsbad, USA) for IDO1 and DC-maturation markers; and IL-1 β AlexaFluor 647 (AS10; BD Biosciences) for IL-1β production. Before intracellular staining with IDO1 and IL-1β moAbs, the DCs were permeabilized and fixed using BD cytofix/cytoperm fixation and permeabilization solution (BD Biosciences) according to the manufacturer's instructions.

For each sample, isotypic controls were used as a negative fluorescence control. At least 10,000 events of each sample were collected. Analysis was performed on a Cytoflex flow cytometer from Beckman Coulter, and the results were obtained using Kaluza (Beckman Coulter, Indianapolis, USA) analysis software.

2.3.3 Cytokine production

Supernatants were collected from cultures of 1×10^5 (1×10^6 cells/mL) immDCs and DCs treated for 48 h with ATP (1 mM, Sigma Aldrich) in the presence or absence of $P2Y_{11}R$ (NF340; Tocris; 10 µM; 1 h of pretreatment) were assessed by LEGENDplex Human Cytokine Panel 2 (13-plex) (Biolegend). The following human cytokines were analyzed by FACS according to the manufacturer's instructions: TSLP, IL-1α, IL-1β, GM-CSF, IFN-α2, IL-23, IL-12p40, IL-12p70, IL-15, IL-18, IL-11, IL-27, and IL-33.

2.4 DC-induced T-cell proliferation and cytokine production

2.4.1 T-cell proliferation

2×10^4 immDCs and DCs treated for 48 h with ATP (1 mM, Sigma Aldrich) in the presence or absence of $P2Y_{11}R$ (NF340; Tocris; 10 µM; 1 h of pretreatment) were co-cultured with 2×10^5 allogeneic $CD3^+$ T cells stained with CFSE (5 µM) for 5 d. T cells alone

were used as a negative control, and T cells treated with PHA (20 mg/mL; Sigma Aldrich) for 48 h were used as a positive control. Proliferation was analyzed on a FACS Canto II (BD Biosciences) flow cytometer, and the proliferation index was calculated using FCS express 6 software.

2.4.2. T-cell cytokine production

2×10^4 immDCs and DCs treated for 48 h with ATP (1 mM, Sigma Aldrich) in the presence or absence of $P2Y_{11}R$ (NF340; Tocris; 10 µM; 1 h of pretreatment) were co-cultured with 2×10^5 allogeneic $CD3^+$ T cells (1×10^6 cells/mL) for 48 h. T cells alone were used as a negative control. T cells treated with ionomycin (IM, 500 ng/mL; Sigma Aldrich) and phorbol 12-myristate 13-acetate (10 ng/mL; Sigma Aldrich) for 24 h, followed by overnight treatment with brefeldin A (2 µg/mL; BD Biosciences), were used as a positive control. The production of cytokines was assessed using the LEGENDplex HU Th Cytokine Panel (12-plex) (Biolegend). The following human cytokines were analyzed by FACS according to the manufacturer's instructions: IL-5, IL-13, IL-2, IL-6, IL-9, IL-10, IFN-y, TNF-α, IL-17A, IL-17F, IL-4, and IL-22.

2.5 Treg induction by DCs and analysis of suppressive activity

2.5.1 Treg induction

2×10^4 immDCs or DCs matured by ATP (as described in Section 2.2) were co-cultured in the presence or absence of an A_{2A} receptor ($A_{2A}R$) antagonist (SCH 442416; 10 µM; Tocris) for 5 d in complete RPMI with 2×10^5 autologous $CD3^+$ T cells at a ratio of 1:10. $CD3^+$ T cells alone were used as a control. After 5 d of coculturing, the T cells were stained in the dark at room temperature for 15 min using the following antihuman moAbs: CD4 APC-h7 (clone SK3; BD Biosciences), CD25 PeCy7 (clone BC96; Biolegend), CD127 PerCP-Cy5.5 (clone A019D5; Biolegend), CD45RA V500 (clone HI100; Biolegend), PD-1 APC (clone EH12.2H7; Biolegend), OX40 FITC (ACT35; BD Pharmingen), CD73 FITC (clone AD2; Biolegend), CD39 PE (TU66; BD Biosciences), and ICOS PE (G44-26; BD Pharmingen). Intracellular staining of FOXP3 using the Foxp3/transcription factor staining buffer set (eBioscience/Thermo Fisher; Massachusetts, USA) was performed as follows. For each sample, unstained $CD3^+$ T cells were used as a negative fluorescence control. At least 5,000 total Tregs events were collected in each sample. Analysis was performed on a Cytoflex flow cytometer from Beckman Coulter, and the results were obtained using Kaluza (Beckman Coulter) analysis software.

2.5.2. Treg suppression test

Treg suppression was evaluated as the capacity of Tregs to inhibit T-cell proliferation. Briefly, 1.1×10^5 immDCs or DCs matured by ATP in the presence or absence of $P2Y_{11}R$ were co-cultured for 5 d in complete RPMI with 1.1×10^6 allogeneic $CD3^+$ T cells (1×10^6 cells/mL) isolated by the human StraightFrom Buffy Coat REAlease CD3 MicroBead Kit (MiltenyiBiotec). After 5 d of coculturing, $CD4^+$ T cells were isolated by immunomagnetic separation (MiltenyiBiotec), irradiated (5,000 cGy), and co-cultured with autologous $CD3^+$ T cells at a ratio of 1:10 (Treg:T cell) for 5 d. Before coculturing, the $CD3^+$ T cells were activated by human T cell TransAct (MiltenyiBiotec) to induce proliferation and stained with CFSE (5 µM). Proliferation was analyzed on a FACS Canto II (BD Biosciences) flow cytometer, and the proliferation index was calculated using FlowJo_v10.8.1_CL software (BD).

2.6 Quantitative real-time PCR

2.6.1 Expression of IDO1 mRNA in mDCs

5×10^6 immDCs and DCs treated for 48 h with ATP in the presence or absence of P2X7R or $P2Y_{11}R$ antagonists or with CD40L were used.

2.6.2 Expression of IL-1β mRNA in mDCs

5×10^6 immDCs and DCs treated for 5 h and 15 min with LPS in the presence or absence of a BAY inhibitor were used.

2.6.3 Expression of $P2Y_{11}R$ mRNA in mDCs

5×10^6 immDCs and DCs treated for 48 h with ATP in the presence or absence of P2X7R antagonist were used; all the treatments are described in more detail in paragraph 2.3.

The DCs were lysed in an RLT Plus buffer (Qiagen, Düsseldorf, Germany); mRNA was isolated using the RNeasy Mini kit (Qiagen), quantified by spectrophotometry (ND-1000, NanoDrop Technologies, Thermo Fisher), and reverse-transcribed into cDNA using the ImProm-II kit (Promega, Madison, USA). qRT-PCR reactions were performed using a 96-well optical reaction plate. Amplification reactions using human cDNA were carried out using platinum super mix (Thermo Fisher Scientific, Massachusetts, USA). Primer probes used for the target genes were: IDO1, Hs00158027_m1; GAPDH, Hs00266705_g1; IL1B, Hs01555410_m1; and P2RY11, Hs01038858_m1 (all from Applied Biosystems/Thermo Fisher Scientific). Threshold cycle (Ct) values were determined automatically using the 7900HT fast real-time PCR system (Applied Biosystems/Thermo Fisher Scientific). Relative quantification was performed using the ΔCt comparative method.[21] Where indicated, cDNA reverse-transcribed from Universal Human Reference RNA (Agilent technologies, Santa Clara, USA) was used as a control.

2.7 Western blotting

2.7.1 IDO1 cytoplasmic expression and the presence of p52/p100 and Rel-B in nucleus

3×10^6 immDCs and DCs treated for 48 h with ATP in the presence or absence of P2X7R or $P2Y_{11}R$ antagonists or with CD40L, in the presence or absence of IKK-γ (as described in 2.3.), were used for the extraction of cytoplasmic and nuclear proteins.

2.7.2 Cytoplasmic detection of IL-1β

3×10^6 immDCs and DCs treated for 5 h and 15 min with LPS in the presence or absence of IKK-γ were used for the extraction of cytoplasmic proteins.

2.7.3 Validation of IKK-γ functionality

3×10^6 immDCs and DCs treated for 5 h and 15 min with LPS (1 µg/mL; Sigma Aldrich) in the presence or absence of IKK-γ (15 µg/mL; Novus Biological; 2 h of pretreatment) were used for the extraction of nuclear proteins.

The nuclear and cytoplasmic proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) according to the manufacturer's instructions and quantified using the Bradford protein assay. A quantity of 15 to 30 µg of the protein was separated on 10% polyacrylamide gels (Mini-PROTEAN TGX Stain-Free Precast Gels; Bio-Rad, Hercules, USA), transferred to nitrocellulose membranes using the Trans-Blot Turbo system (Bio-Rad), and incubated with a blocking solution (TBS + Tween 0.1% + 5% BSA) for 1 h followed by overnight incubation at 4 °C with the following primary

antibodies: antihuman IDO1 (1:5000; UM500 110CF; OriGene Technologies, Rockville, USA), antihuman IL-1β (1:1000; P420B; Life Technologies/Thermo Fisher Scientific), antihuman p52/p100 (1:500; 4882; Cell Signaling Technology, Massachusetts, USA), antihuman Rel-B (1:1000; 4954; Cell Signaling Technology), c-REL (B-6) (1:200; 6955; Santa Cruz, USA), and p65 (1:1000; 8242; Cell Signaling Technologies). Phospho-Histone H2A.X (Ser139) (1:1500; 9718; Cell Signaling Technology) and anti-β-Actin (C4) (1:5000; 47778; Santa Cruz Biotechnology, Dallas, USA) were used as reference for nuclear and cytoplasmic protein expression respectively. Successively, the membranes were incubated for 1 h with the following respective secondary antibodies: Goat anti-Rabbit IgG (heavy chain), superclonal recombinant secondary antibody, HRP (1:30.000; A27036; Invitrogen), and Goat anti-Mouse IgG Heavy and Light Chain Antibody (1:20.000/30.000; A90-116P; Bethyl, Montgomery, USA). Bound antibodies were revealed by chemiluminescence (Amersham ECL Select, GE Healthcare, Chicago, USA) on ChemiDoc Imaging and Analysis Software (Bio-Rad). The density of the protein bands was analyzed using ImageJ software (a Java-based image processing program developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation at University of Wisconsin, USA).

2.8 Luminometry

2.8.1 Adenosine release

The supernatants from 2×10^5 (1×10^6 cells/mL) immDCs and DCs treated with ATP for 48 h were collected and stained for adenosine using the Adenosine Assay Kit (MET-5090; Cell Biolabs, San Diego, USA) according to the manufacturer's instructions. The measurement of fluorescence correlated with adenosine concentration was performed on a GloMax Discovery Microplate Reader (Promega).

2.8.1 IL-1β release

The supernatants from 2×10^5 (1×10^6 cells/mL) immDCs and DCs treated with LPS for different time intervals (0; 4; 4.25; 4.5; 4.75; 5; 5.25; and 5.5 h) were collected and stained for IL-1β using the Lumit Human IL-1β Immunoassay kit (Promega) according to the manufacturer's instructions. The measurement of luminescence correlated with IL-1β concentration was performed on a Tecan spectrophotometer (Thermo Fisher Scientific).

2.9 Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM) of the values obtained in the experiments. Statistical analyses were performed with GraphPad Prism 10 software (GraphPad Software, Inc., La Jolla, USA), using ANOVA or unpaired *t*-testing. *P*-values of <0.05 were considered to be statistically significant.

3. Results

3.1 ATP-driven IDO1 expression in DCs is modulated differently through P2X7R and $P2Y_{11}R$

Human monocyte-derived immDCs were treated with ATP (1 mM) in the presence or absence of P2X7R or $P2Y_{11}R$ antagonists (α-P2X7R or α-$P2Y_{11}R$, respectively; see 2.3.) and then evaluated for IDO1 expression both at the mRNA and protein levels. As shown in Fig. 1A, IDO1 was significantly upregulated at the mRNA level in ATP-treated DCs compared with immDCs (857.7 ± 102.8, mean fold change ± SEM; $P \leq 0.01$). A significant upregulation was also observed in DCs treated with CD40L, known as strong IDO1 inducers in human monocyte-derived DCs,[10] and then used as a positive control, compared with immDCs (1109.0 ± 191.3; $P \leq 0.01$). In the presence of α-P2X7R and α-$P2Y_{11}R$, the IDO1 expression was modulated differently. In particular, its further upregulation in the presence of α-P2X7R and downregulation in the presence of α-$P2Y_{11}R$ was observed in comparison with ATP-treated DCs (1441.0 ± 96.7 and 291.4 ± 196.7, respectively vs 857.7 ± 102.8; $P \leq 0.05$ for both). The same pattern of IDO1 expression and modulation by P2Rs was observed also at the protein level, as shown Fig. 1B–D. In particular, IDO1 was strongly upregulated in ATP-treated DCs compared with immDCs (33.48 ± 5.09 vs 1.62 ± 0.27; mean ± SEM; $P \leq 0.0001$). In the presence of α-P2X7R and α-$P2Y_{11}R$ antagonists, the IDO1 expression was modulated differently. As with mRNA, the IDO1 protein was further upregulated in the presence of α-P2X7R compared with ATP-treated DCs (75.54 ± 5.43 vs 33.48 ± 5.09; $P \leq 0.0001$), whereas it was significantly downregulated in the presence of α-$P2Y_{11}R$ compared with ATP-treated DCs (4.80 ± 0.75 vs 33.48 ± 5.09; $P \leq 0.001$). The same pattern was also observed for DC-maturation markers such as CD86 and CD83, which were upregulated after ATP treatment and modulated differently in the presence of α-P2Rs antagonists (Fig. 1C and D). Importantly, as shown in Fig. 1E, the IDO1 upregulation was observed exclusively in ATP-treated mDCs expressing CD83 compared with immDCs. In particular, as shown in Supplementary Fig. S1, IDO1 and CD83 were co-expressed exclusively on $CD80^+CD86^+$ DCs after ATP treatment, providing further evidence that ATP upregulates IDO1 expression in mDCs.

To better characterize these $IDO1^+$ DCs, the cytokine profile of ATP-treated DCs in the presence or absence of α-$P2Y_{11}R$, as well as their capacity to induce T-cell proliferation, were analyzed. After ATP treatment, DCs increased the production of all tested pro and anti-inflammatory cytokines (see Section 2.3). However, only IL-27-known also for its ability to maintain immune tolerance through Tregs[22]—and IFN-α2, which plays a key role in modulating inflammatory responses,[23] were significantly upregulated, as shown in Supplementary Fig. S2. Interestingly, a significant upregulation of IL-11 and IL-15 was observed after ATP treatment in the presence of α-$P2Y_{11}R$ compared with the condition with ATP alone. At the functional level, only a slight increase in T-cell proliferation was induced by ATP-treated DCs (Supplementary Fig. S3). The cytokine profile of T cells co-cultured with ATP-treated DCs was consistent with the observed T-cell proliferation results, globally exhibiting only a modest upregulation of both proinflammatory and anti-inflammatory cytokines. Notwithstanding, there was a significant upregulation of IL-4 in T-cell co-cultures with ATP-treated DCs as compared with immDCs. Moreover, the treatment of DCs with ATP and anti-$P2Y_{11}R$ resulted in increased T-cell production of IL-17A as compared with ATP alone (Supplementary Fig. S4).

Taken together, these data indicate differential modulation of ATP-driven IDO1 expression in human monocyte-derived DCs by purinergic receptors P2X7R and $P2Y_{11}R$. Specifically, IDO1 is negatively regulated by P2X7R while positively regulated by $P2Y_{11}R$. ATP-treated DCs, co-expressing IDO1 and maturation markers, such as CD83, CD890, and CD86, exhibit an enhanced production of tolerogenic over inflammatory cytokines, such as IL-27 and, when exposed to anti-$P2Y_{11}R$, have a specific capacity of increasing T cell production of IL-17A.

3.2 ATP-driven IDO1 upregulation through P2X7R in DCs does not involve inflammasome

Our data demonstrate that IDO1 is further upregulated in mDCs after ATP treatment in the presence of α-P2X7R as compared

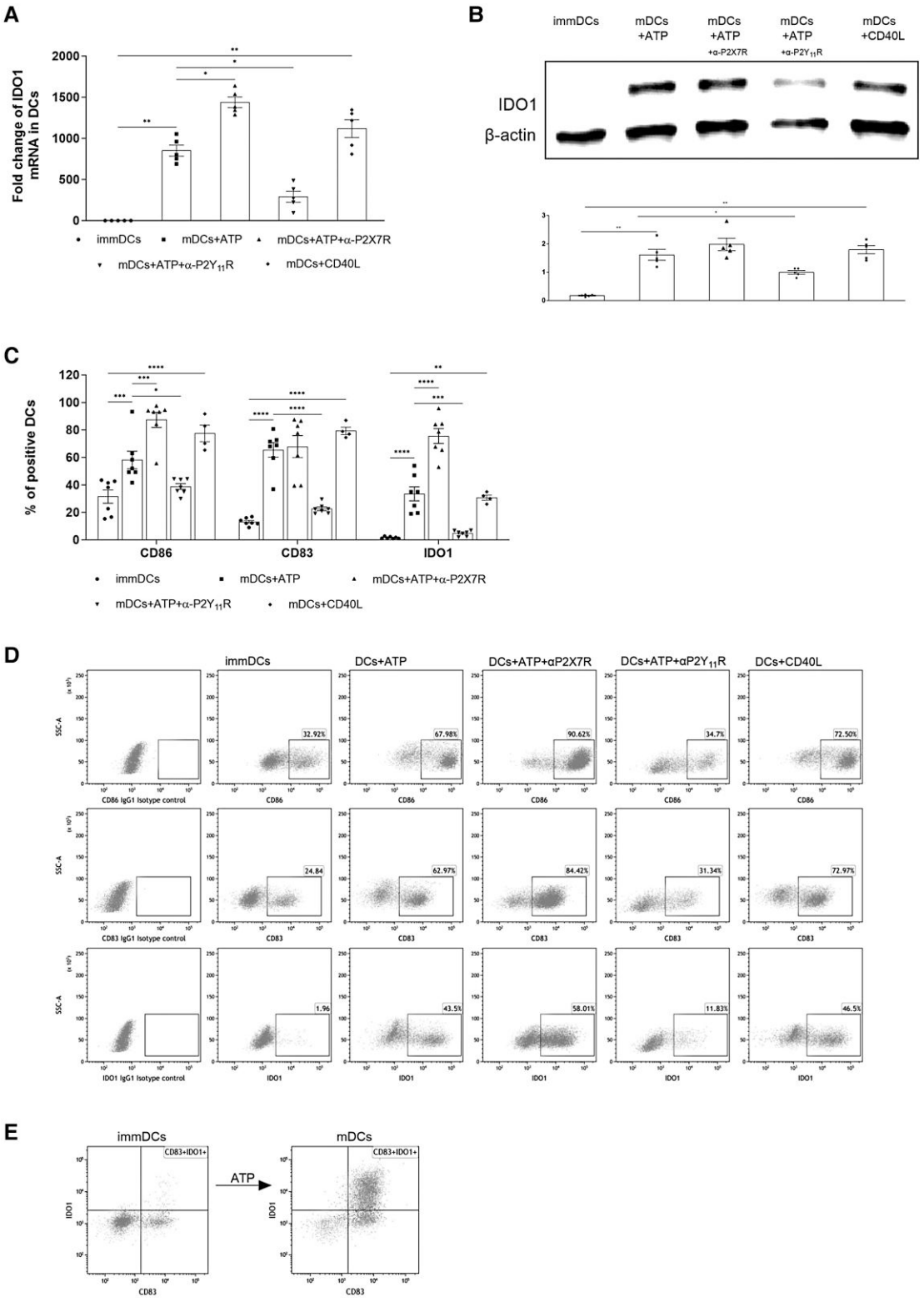

Fig. 1. Different ATP-dependent regulation of IDO1 expression by purinergic receptors P2X7R and P2Y$_{11}$R in human monocyte-derived DCs. A) Quantitative real-time PCR analysis of IDO1 mRNA expression in immDCs and mDCs treated with ATP (1 mM) in the absence or presence of P2X7R or P2Y$_{11}$R antagonists (α-P2X7R or α-P2Y$_{11}$R, respectively; both at c = 10 μM). CD40L (c = 2 μg/mL) was used as a positive control of IDO1 expression. The values are represented as the mean ± SEM of fold change (immDCs = 1) of 5 independent experiments. *$P \leq 0.05$; **$P \leq 0.01$. B) WB analysis of cytoplasmic IDO1 expression in immDCs and mDCs treated with ATP in the absence or presence of α-P2X7R or α-P2Y$_{11}$R. CD40L was used as a positive control of IDO1 expression. Expression of β-actin in cytoplasm was used as a reference, one representative experiment is shown. Densitometry of protein bands of mean ± SEM of 5 independent experiments is shown. **$P \leq 0.01$. C) FACS analysis of IDO1 expression in immDCs and mDCs treated with ATP in the absence or presence of α-P2X7R or α-P2Y$_{11}$R. CD40L was used as a positive control of IDO1 expression. The values are represented as the mean ± SEM of 6 independent experiments. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$. D) FACS analysis of IDO1, CD86, and CD83 expression in immDCs and mDCs treated with ATP in the absence or presence of α-P2X7R or α-P2Y$_{11}$R. CD40L was used as a positive control of IDO1 expression; one representative experiment is shown. E) FACS analysis of IDO1 and CD83 co-expression in immDCs and mDCs treated with ATP; one representative experiment is shown.

with ATP alone. Given the crucial role of inflammasome in mediating P2X7R-derived signal transduction in DCs,[24] we hypothesized an effect of inflammasome machinery on ATP-driven IDO1 expression through P2X7R. To address this point, we analyzed IDO1 expression in ATP-treated DCs in the presence of inflammasome inhibitors Ac-YVAD-cmk (the caspase-1 inhibitor; YVAD) or BAY 11-7082 (the NLRP3 inflammasome inhibitor; BAY). To evaluate the interaction of inflammasome inhibition with the activation of purinergic receptors, this set of experiments was also performed in the presence or absence of α-P2X7R or α-$P2Y_{11}$R. Both inflammasome inhibitors were functionally active as demonstrated by their capacity to abolish LPS-induced IL-1β protein production (YVAD inhibitor) and to prevent the transcription of NLRP3 inflammasome machinery (BAY inhibitor) (Supplementary Fig. S5A–D). As shown in Fig. 2, we did not confirm an involvement of inflammasome, the activation of which results in IL-1β production, in regulating IDO1 expression through P2X7R. Indeed, no upregulation of IDO1 in the presence of neither of the 2 inflammasome inhibitors was observed (Fig. 2A and B). To rule out a possible mutual compensatory mechanism in the expression of P2X7R and $P2Y_{11}$R, we tested whether an increase in $P2Y_{11}$R might be responsible for IDO1 upregulation in ATP-treated mDCs in the presence of α-P2X7R. As shown in Fig. 2, the $P2Y_{11}$R upregulation was observed neither at the mRNA level (Fig. 2C) nor at the protein level (Fig. 2D and E).

Taken together, these data suggest that neither inflammasome nor the compensatory upregulation of $P2Y_{11}$R are involved in ATP-driven IDO1 regulation through P2X7R in DCs.

3.3 ATP-driven IDO1 induction through $P2Y_{11}$R is associated in DCs with the activation of noncanonical NF-κB signaling

We then investigated the ATP-driven intracellular regulation of IDO1 expression through purinergic receptors based on the hypothesis that an involvement of noncanonical NF-κB signaling might be present. We analyzed both the cytoplasmic IDO1 expression of DCs treated with ATP in the presence or absence of α-P2X7R and α-$P2Y_{11}$R and the nuclear expression of noncanonical NF-κB transcriptional factors Rel-B and p52/p100 in the same DCs. The correct separation of the cytoplasmic and nuclear fractions of DCs is shown in Supplementary Fig. S6. To exclude any type of canonical NF-κB involvement, the IKK-gamma (IKK-γ), a specific inhibitor of the canonical NF-κB pathway, was used.

To verify the functionality of the IKK-γ inhibitor, we analyzed the presence of canonical NF-κB transcriptional factors p65 and C-Rel in the nuclei of DCs treated with LPS in the absence or presence of IKK-γ. As shown in Fig. 3, a decrease of both C-Rel and p65 in the presence of IKK-γ was observed in the nuclei of DCs treated with LPS compared with the condition without an inhibitor.

We then tested whether the cytoplasmic expression of IDO1 was associated with the presence of Rel-B and p52 in the nuclei of the same DCs. In particular, as shown in Fig. 4A, the Rel-B and p52 were detected in the nuclei of ATP-treated DCs with positive cytoplasmic IDO1 expression. Importantly, the different IDO1 expression in the presence of α-P2X7R and α-$P2Y_{11}$R was correlated with the presence of Rel-B and p52 in the nucleus (Fig. 4A and Supplementary Fig. S7). Moreover, differences of $IDO1^+CD83^+$ DCs in the presence of IKK-γ were not observed compared with the condition without IKK-γ (Fig. 4B). No changes were observed in the expression of p52 and Rel-B in the presence of IKK-γ (Fig. 4C and Supplementary Fig. S7), thus ruling out a possible involvement of the canonical NF-κB pathway. The results strongly support our hypothesis that the noncanonical NF-κB pathway is responsible for IDO1 induction in DCs through $P2Y_{11}$R.

3.4 ATP-driven IDO1 upregulation is associated in DCs with CD73 upregulation and adenosine production

Analyzing the phenotype of DCs treated with ATP, a stably high expression of CD39 ectonucleotidase was observed and a significant increase in CD73 ectonucleotidase, which represents the rate-limiting step of ATP conversion to adenosine,[19] was observed after ATP treatment (Fig. 5A and B). As shown in Fig. 5B, an increase in CD73 was induced after ATP treatment compared with immDCs (10.6% ± 2.6% vs 1.1 ± 0.1, mean ± SEM; $P \leq 0.05$). To test whether a potential association between ATP-driven ectonucleotidase upregulation and IDO1 expression may exist in DCs upon ATP exposure, we specifically analyzed both CD39 and CD73 expression in IDO1-expressing DCs. Of note, CD73 upregulation after ATP treatment was observed only in $IDO1^+$, and not in $IDO1^-$ DCs. More specifically, this increase was predominantly observed in $IDO1^+$ DCs expressing maturation-related markers, such as CD83 (Fig. 5C). Of note, the CD73 upregulation in DCs by ATP was associated with an increase of adenosine concentration in supernatants of treated mDC compared with immDCs (5.8 ± 1.25 μM vs 1.52 ± 0.21 μM; $P \leq 0.01$) (Fig. 5D).

Taken together, these results strongly suggest that ATP-driven IDO1 upregulation is associated in DCs with CD73 upregulation and adenosine production.

3.5 ATP-treated IDO1-expressing mDCs induce PD-1-expressing bona fide suppressive Tregs via adenosine A_{2A}R

To determine the parallel involvement of IDO1 upregulation and adenosine production by ATP-treated DCs for Treg induction and suppression, we used ATP-treated $IDO1^+$ DCs, expressing IDO1 and CD73 and demonstrated to produce adenosine, for in vitro tests of Treg induction. As shown in Fig. 6A and Supplementary Fig. S8, ATP-treated mDCs induced a higher number of bona fide and "total" $CD3^+CD4^+CD127^{-/low}CD25^{+/high}FOXP3^+$ Tregs as compared with immDCs (17.3% ± 2.5% vs 3.9% ± 0.5%, mean ± SEM; $P \leq 0.0001$). Similarly to total Tregs, the percentage of "suppressive" Tregs, characterized as $CD3^+CD4^+CD127^{-/low}CD25^{high}CD45RA^-FOXP3^{+/high}$ cells, were also upregulated in the presence of ATP-treated mDCs compared with immDCs (3.5% ± 1.0% vs 0.5% ± 0.2%; $P \leq 0.01$) (Fig. 6B and Supplementary Fig. S8).

To better characterize the suppressive phenotype of Tregs induced by ATP-treated mDCs expressing IDO1, we evaluated the expression of various "fitness markers" such as OX40, CD73, PD-1, CD39, and ICOS on Tregs after coculturing with DCs. Of all the molecules tested, only PD-1 was significantly and positively modulated on suppressive Tregs (Fig. 6C and Supplementary Fig. S8). In particular, we found an increase in the percentage of PD-1 expression in Tregs induced by ATP-treated mDCs compared with immDCs (12.9 ± 2.8 vs 4.9% ± 1.1%; $P \leq 0.01$).

To evaluate the functional properties of these Tregs, their ability to inhibit T-cell proliferation was assessed. Notably, $CD4^+$ T cells induced by ATP-treated mDCs significantly suppressed the proliferation of autologous $CD3^+$ T cells compared with their $CD4^-$ counterparts (proliferation index 2.7 ± 0.5 vs 1.1 ± 0.1; $P \leq 0.0001$) (Fig. 6E and Supplementary Fig. S9). In contrast, this difference between $CD4^+$ and $CD4^-$ T cells was not observed when they were induced by immDCs.

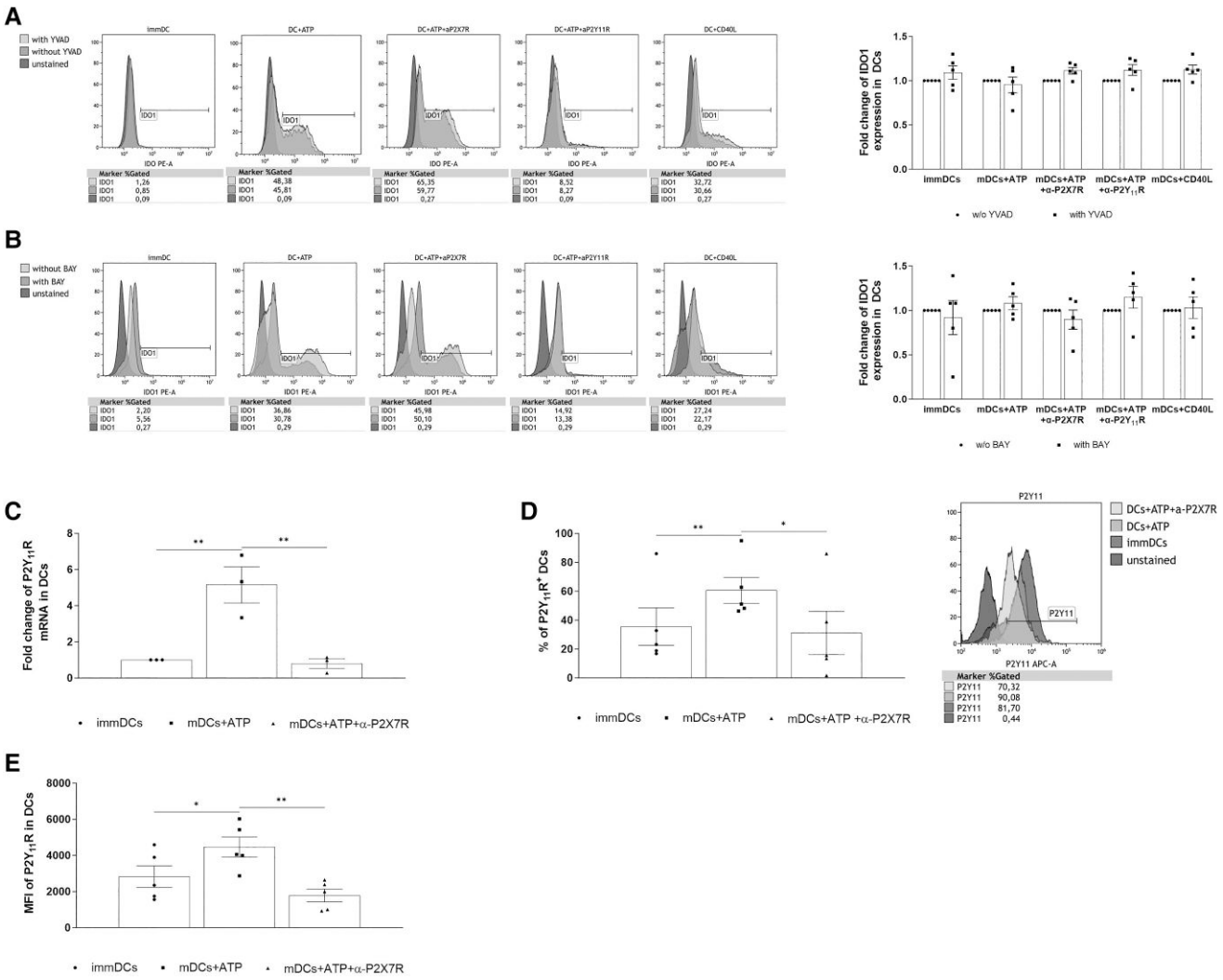

Fig. 2. ATP-driven negative IDO1 regulation through P2X7R in DCs involves neither inflammasome nor compensatory $P2Y_{11}R$ upregulation. A and B) FACS analysis of IDO1 expression in imm DCs and mDCs treated with ATP (c = 1 mM) in the absence or presence of α-P2X7R or α-$P2Y_{11}R$ (both at c = 10 μM), in the absence or presence of inflammasome inhibitor YVAD (c = 8 μM; A) or BAY (c = 10 μM; B) One representative experiment is shown (left panel). The values are represented as the mean ± SEM of fold change (conditions w/o YVAD/BAY = 1) of 5 independent experiments (right panel). C) Quantitative real-time PCR analysis of $P2Y_{11}R$ mRNA expression in immDCs and mDCs treated with ATP in the absence or presence of α-P2X7R. The values are represented as the mean ± SEM of fold change (immDCs = 1) of 5 independent experiments. D) FACS analysis of $P2Y_{11}R$ expression in immDCs and mDCs treated with ATP in the absence or presence of α-P2X7R. The values are represented as the mean ± SEM of 5 independent experiments (left panel). One representative experiment is shown (right panel). E) FACS analysis of $P2Y_{11}R$ MFI in immDCs and mDCs treated with ATP in the absence or presence of α-P2X7R. The values are represented as the mean ± SEM of 5 independent experiments.

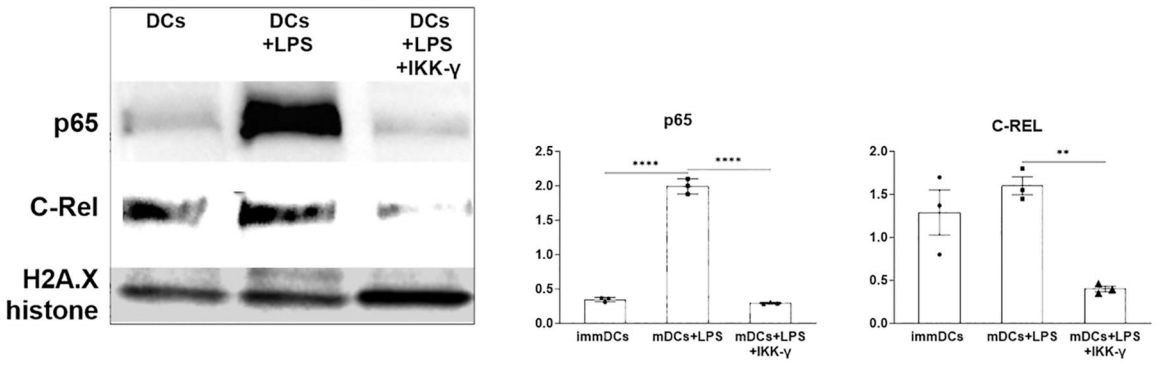

Fig. 3. The inhibitor of canonical NF-κB IKK-γ is functionally active. WB analysis of nuclear expression of p65 and C-Rel in immDCs and DCs treated with LPS (c = 1 μg/mL) in the absence or presence of IKK-γ inhibitor (c = 15 μg/mL). Expression of H2A.X histone was used as a reference. Densitometry of protein bands of mean ± SEM of 3 independent experiments is shown. $^{**}P \leq 0.01$; $^{****}P \leq 0.0001$.

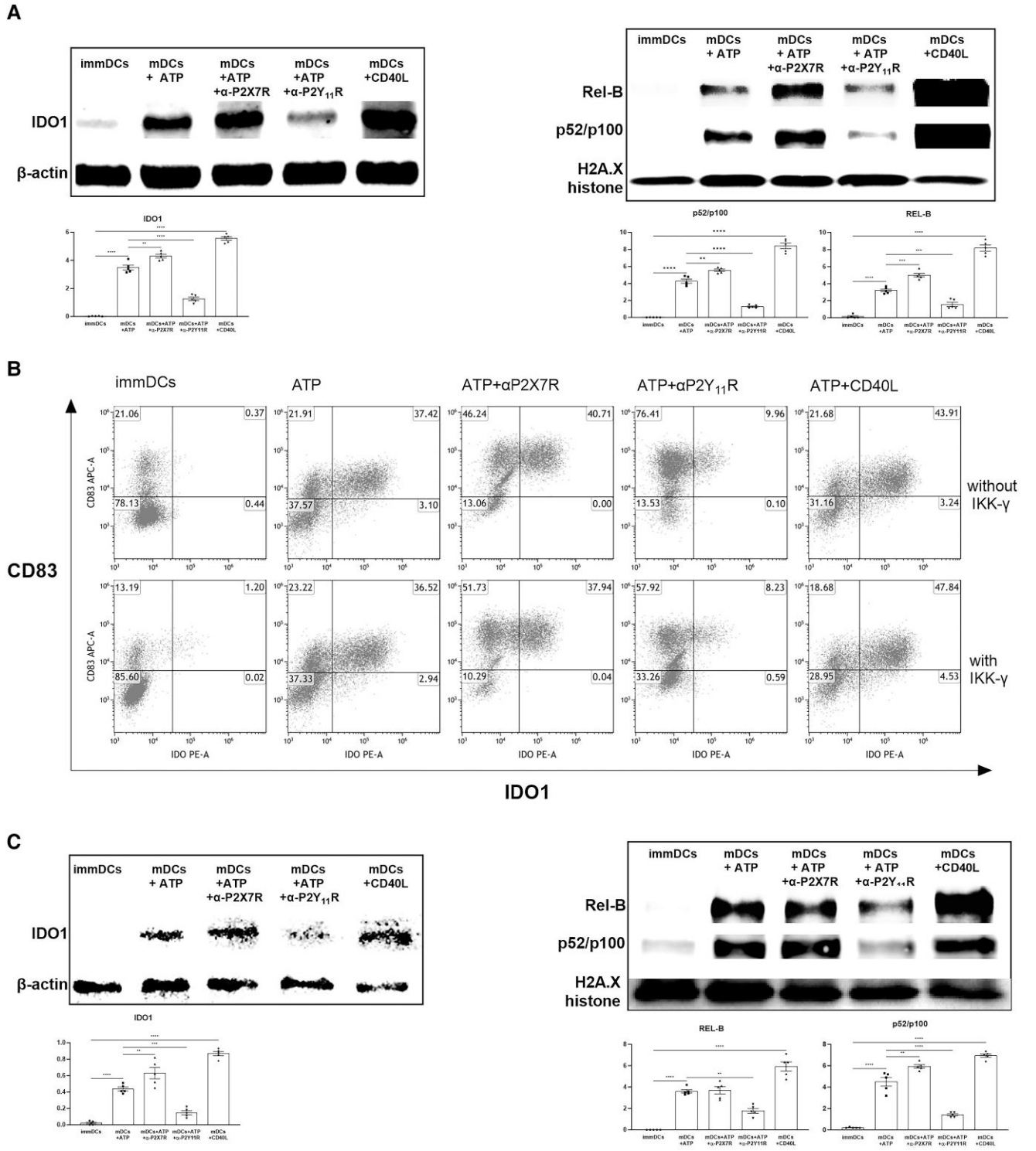

Fig. 4. Noncanonical NF-κB signaling is involved in ATP-dependent regulation of IDO1 expression in DCs by $P2Y_{11}R$. A) WB analysis of cytoplasmic IDO1 (left panel) and nuclear Rel-B and p52/p100 (right panel) expression in immDCs and DCs treated with ATP (c = 1 mM) in the absence or presence of α-P2X7R or α-$P2Y_{11}R$ (both at c = 10 μM). CD40L (c = 2 μg/mL) was used as a positive control of IDO1 expression. Expression of β-actin for cytoplasm and H2A.X histone for nucleus was used as a reference. Densitometry of protein bands of mean ± SEM of 5 independent experiments is shown. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$. B) FACS analysis of $IDO1^{+}CD83^{+}$ expression in immDCs and DCs treated with ATP in the absence or presence of α-P2X7R or α-$P2Y_{11}R$, with or without IKK-γ inhibitor (c = 15 μg/mL). CD40L was used as a positive control of IDO1 expression. The dot plots of one representative experiment are shown. C) WB analysis of cytoplasmic IDO1 (left panel) and nuclear Rel-B and p52/p100 (right panel) expression in immDCs and DCs treated with ATP as described in A) in presence of IKK-γ inhibitor. Densitometry of protein bands of mean ± SEM of 5 independent experiments is shown. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

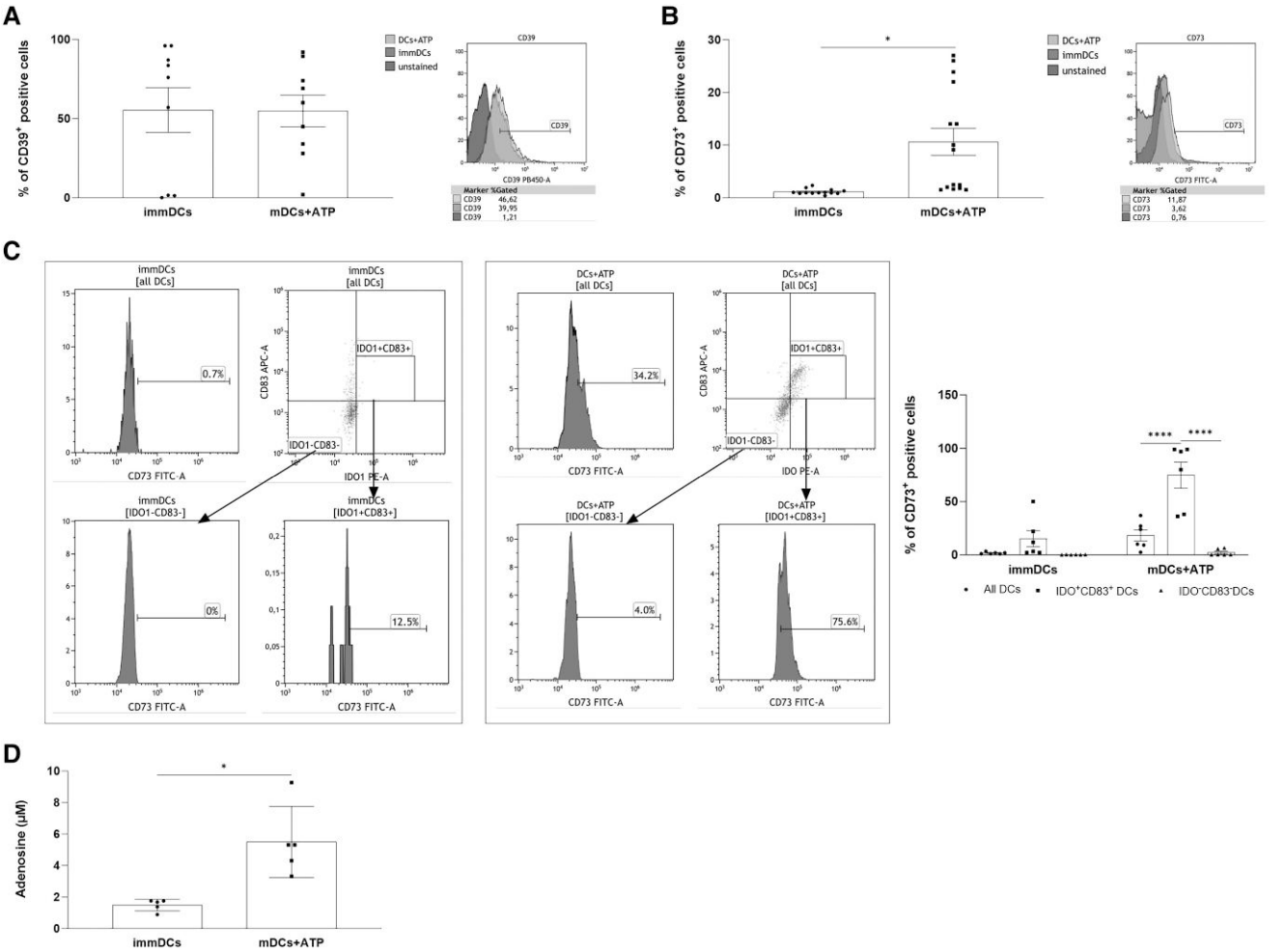

Fig. 5. CD73 upregulation is correlated with adenosine production in ATP-treated IDO1-expressing mDCs. A) FACS analysis of CD39 expression in imm DCs and mDCs treated with ATP (c = 1 mM). The values are represented as the mean ± SEM of 9 independent experiments (left panel). One representative experiment is shown (right panel). B) FACS analysis of CD73 expression in imm DCs and mDCs treated with ATP (c = 1 mM). The values are represented as the mean ± SEM of 15 independent experiments (left panel). One representative experiment is shown (right panel). *$P \leq 0.05$. C) FACS analysis of CD73 expression in all DCs, IDO$^+$CD83$^+$DCs and IDO$^-$CD83$^-$DCs treated with ATP. One representative experiment is shown (2 left panels). The values are represented as the mean ± SEM of 6 independent experiments (right panel) ****$P \leq 0.0001$. D) Luminometric analysis of adenosine production by immDCs and mDCs treated with ATP. The values are represented as the mean ± SEM of 5 independent experiments. *$P \leq 0.05$.

To investigate the potential involvement of adenosine-mediated effects in the induction of this suppressive phenotype of Tregs, we used an $A_{2A}R$ antagonist (α-$A_{2A}R$) for DC-Treg co-cultures. Interestingly, we observed a significant decrease in PD-1 expression on suppressive Tregs in the presence of α-$A_{2A}R$ compared with the condition without an inhibitor (6.2 ± 1.2 vs 14.3% ± 2.4%; $P \leq 0.01$). This decrease was not observed when immDCs were used (5.5 ± 1.6 vs 5.8% ± 1.6%) (Fig. 6D).

These data strongly suggest that ATP-treated IDO1-expressing mDCs induce PD-1 expressing bona fide suppressive Tregs via adenosine A_{2A} receptors.

4. Discussion

In this study, we investigated the ATP-driven immunoregulatory mechanisms that contribute to the capacity of human DCs to induce Tregs. Our data found that the ATP-driven induction of IDO1 expression in DCs is mediated by $P2Y_{11}R$ and requires noncanonical NF-κB signaling. In DCs, moreover, the ATP-driven induction of IDO1 expression is associated with CD73 upregulation and adenosine production, which supports the generation of PD1$^+$ bona fide suppressive Tregs.

Along with its well-known proinflammatory activity as a danger signal, extracellular ATP has recently been recognized as having anti-inflammatory and tolerogenic properties. Extracellular ATP drives IDO1 upregulation in DCs (9, 11), but the regulation of ATP-driven IDO1 expression in DCs has been poorly investigated. Little data are available regarding the ATP-related and receptor-based mechanisms and downstream pathways resulting in IDO1 induction in DCs. Here, we demonstrate for the first time that ATP-dependent IDO1 induction in human DCs is mediated by $P2Y_{11}R$.

Interestingly, our data indicate a different role of P2X7R and $P2Y_{11}R$ in ATP-driven IDO1 modulation in human DCs. Indeed, while IDO1 is positively regulated by $P2Y_{11}R$, a further upregulation of IDO1 expression after ATP treatment was observed in the presence of a P2X7R antagonist compared with ATP treatment alone, suggesting a negative regulation of IDO1 expression via P2X7R. ATP-mediated P2X7R activation leads to inflammasome induction during inflammation,[24] prompting us to hypothesize a

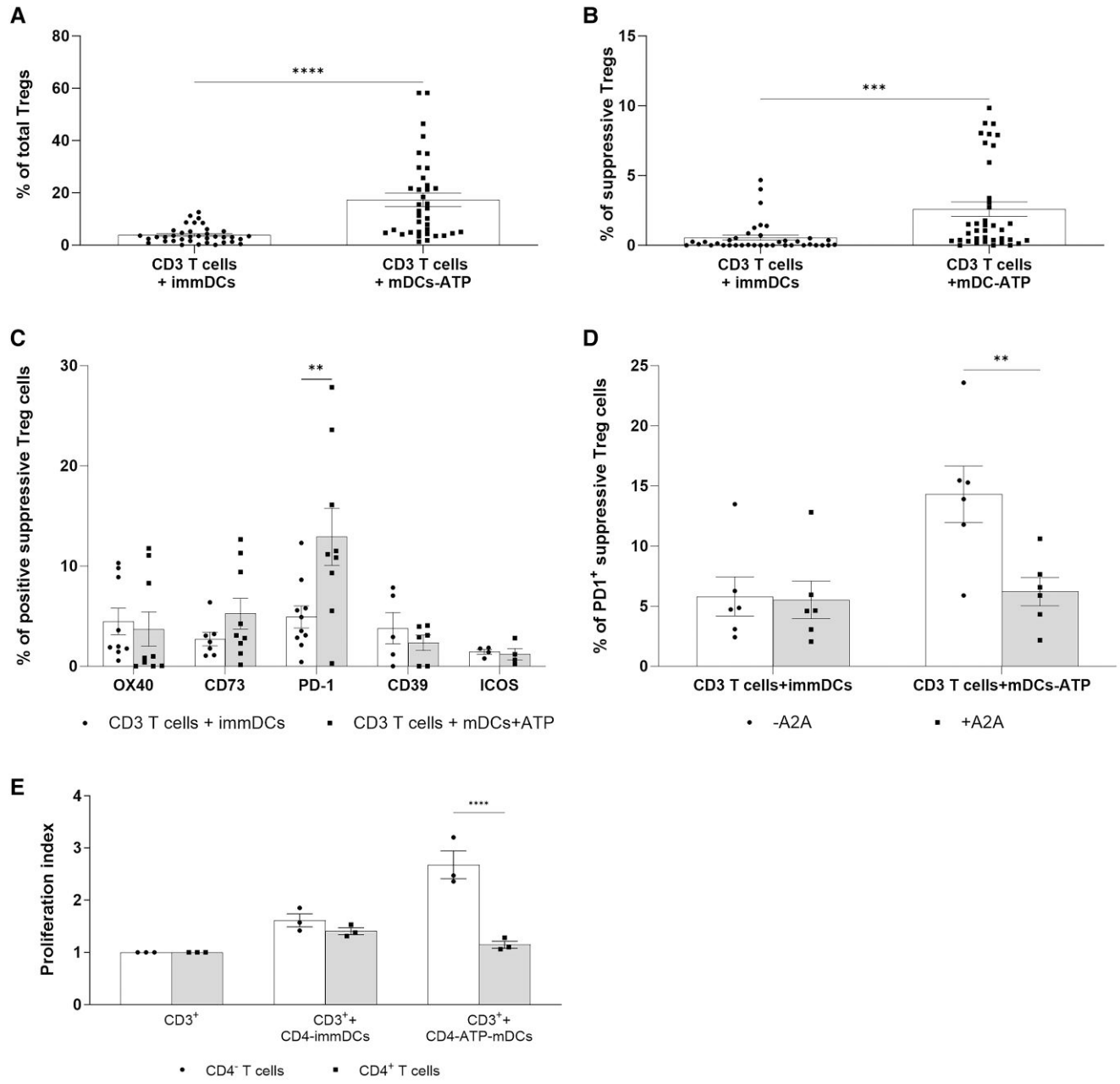

Fig. 6. Phenotypic and functional characterization of Tregs induced by adenosine-producing ATP-treated mDCs expressing IDO1. A) FACS analysis of $CD3^+CD4^+CD127^{-}/^{low}CD25^{+/high}FOXP3^+$ cells (total Tregs) and B) $CD3^+CD4^+CD127^{-/low}CD25^{high}CD45R^-FOXP3^{+/high}$ cells (suppressive Tregs) induced by co-culture of immDCs or ATP-treated mDCs (c = 1 mM) with autologous $CD3^+$ T cells. The values are represented as the mean ± SEM of 36 independent experiments. $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$ compared with immDCs. C) FACS analysis of OX40, CD73, PD-1, CD39 and ICOS expression on suppressive Tregs induced as described in B). The values are represented as the mean ± SEM of 9 independent experiments. $^{**}P \leq 0.01$. D) FACS analysis of PD-1 expression on suppressive Tregs induced as described in B), in presence or absence of α-A_{2A}R (c = 10 μM). The values are represented as the mean ± SEM of 6 independent experiments. $^{**}P \leq 0.01$. E) FACS analysis of proliferation of activated $CFSE^+CD3^+$ T cells after 5 d of co-culture alone ($CD3^+$) or with autologous purificated $CD4^+$ or $CD4^-$ T cells induced by 5-day co-culture of $CD3^+$ T cells with DCs treated with ATP (ATP-mDCs) or immDCs. Proliferation index was evaluated using FlowJo_v10.8.1_CL software (BD). The values are represented as the mean ± SEM of 3 independent experiments. $^{****}P < 0.0001$.

possible inhibiting effect of inflammasome on IDO1 expression. Our data did not support this hypothesis, however, and also ruled out a possible mutual compensatory mechanism in the expression of P2X7R and $P2Y_{11}R$. Accordingly, further studies to delve deeper into this critical aspect of IDO1 regulation in human DCs are strongly needed.

IDO1 is known to possess both enzymatic/catalytic and signaling functions, which are induced by different cytokines and have diverse roles in immune cells.[9,25] The enzymatic activity of IDO1 in DCs is induced by IFN-γ and leads to acute, short-term Tregs induction. This mechanism is important in preventing hyperinflammation during immune response.[26] Conversely, the signaling activity of IDO1 is induced by TGF-β and leads to long-term tolerance in noninflammatory contexts.[25] Both mechanisms play important roles in tumor-associated immune tolerance and can suppress antitumor immunity.[27,28] While IFN-γ-induced IDO1

enzymatic activity is associated with JAK-STAT molecular pathways,[29] the induction of IDO1 expression in response to its signaling activity requires noncanonical NF-κB activation.[30] Indeed, the activation of noncanonical NF-κB is a hallmark of IDO1 signaling in human DCs after CD40L ligation[10] and involves the activation of the IKK kinase and the nuclear translocation of the p52 and Rel-B subunits. In turn, the p52/Rel-B dimer induces the transcriptional expression of Ido1 and Tgfb1 control genes, thus creating a positive feedback loop that represents the molecular basis for long-term immunoregulatory effects in DCs.[25,31] In line with these findings, our data demonstrate that ATP-driven IDO1 induction in human DCs is also associated with noncanonical NF-κB signaling but through $P2Y_{11}R$ activation. However, to exclude a possible involvement of canonical NF-κB, we used a specific inhibitor of this pathway to demonstrate that ATP-driven IDO1 expression through $P2Y_{11}R$ in DCs is induced exclusively through noncanonical NF-κB signaling. It is worth noting that an elevated level of Rel-B in nuclei was also associated with increased DC maturation.[32] Accordingly, our data indicate that the ATP-driven IDO1 induction in DCs is associated with upregulation of DC-maturation markers such as CD83, CD80, and CD86 through $P2Y_{11}R$-initiated noncanonical NF-κB signaling activation.

In cancers, IDO1 expression by tumor cells is a well-recognized and established immunosuppressive mechanism that contributes to immune escape through the generation of Tregs. Based on this strong rationale, IDO1 inhibitors have been developed both in preclinical models and in the clinical setting.[33] Nevertheless, many clinical trials showed disappointing results with no clinical benefit for patients treated with IDO1 inhibitors either in monotherapy or when combined with other agents such as immune checkpoint inhibitors.[34,35] No clear explanation for these results is available, which indicates the need to further investigate resistance mechanisms to IDO1 inhibitors. A better understanding of the regulation of IDO1 induction in DCs as crucial cellular components of the tumor microenvironment,[11,17,27,36–38] and of DC characterization in Tregs induction via IDO1, may help to elucidate the extrinsic mechanisms of tumor cell resistance to IDO1 inhibitors.

After demonstrating the selective induction of IDO1 via noncanonical NF-κB in ATP-treated human DCs, we analyzed Tregs induction through tolerogenic ATP-treated DCs expressing IDO1. Our data confirm both total and suppressive Tregs induction and reveal the upregulation of PD-1 on suppressive Tregs as a novel pattern of response to ATP-stimulated IDO1-expressing DCs. Tumor-infiltrating Tregs can express PD-1, the upregulation of which on suppressive Tregs has been associated with resistance to antitumor therapy and shorter progression-free survival in cancer patients.[39] These data point to PD-1 over-expression by cancer-infiltrating suppressive Tregs via ATP-stimulated IDO1-expressing DCs as a novel path of resistance to immunotherapy strategies, including IDO1 inhibitors, whose capacity to inhibit IDO1-signaling tolerogenic activity especially in DCs is known to be very limited.

In this scenario, the targeting of the most important byproduct of ATP catabolism, i.e. adenosine has the potential to circumvent some of the above-mentioned pitfalls, thus integrating IDO1 inhibition with the blockading of a potent signal associated with Tregs induction. In particular, adenosine has been shown to increase the number of Tregs and to further promote their immunoregulatory activity.[40] In the tumor microenvironment the close balance between ATP release and consumption is completely altered, whereby extracellular ATP can be hydrolyzed by ectonucleotidases expressed on DCs. This 2-step reaction requires both CD39, which catalyzes the hydrolysis of ATP in ADP, and CD73 (the rate-limiting step), which catalyzes the transformation of ADP in adenosine, the main metabolite of ATP.[19,41] In our model, ATP-treated human DCs expressing IDO1 showed a significant upregulation of CD73, which was associated with adenosine production. CD73 upregulation has been observed in many tumor cell types and associated with poor clinical outcomes,[42–44] and anti-CD73 mAbs are currently under clinical investigation in cancer patients with promising early results.[45,46]

After release, adenosine binds the $A_{2A}R$ on Tregs, thus favoring adenosine-dependent suppression signaling and the redirection of the proinflammatory response.[40,43,46] The activation of the $A_{2A}R$ enhances the expression of PD-1 on tumor-infiltrating Tregs,[47] and targeting $A_{2A}R$ alone or in combination with blockading of the PD-1/PD-L1 pathway is under clinical investigation in early clinical trials.[45,48,49] In line with these data, our data demonstrate a significant downregulation of PD-1 on suppressive Tregs in the presence of α-$A_{2A}R$, suggesting a stabilizing adenosine effect on suppressive Tregs and supporting the use of anti-$A_{2A}R$ as a powerful approach to enhancing the antitumor immune response via immunotherapy. Moreover, tumor-bearing P2X7R-null mice were shown to upregulate both CD73 and $A_{2A}R$.[50,51]

In conclusion, our study shed light on the ATP-driven immunoregulatory mechanisms in human DCs. Our data add evidence to the notion that 2 different ATP-dependent mechanisms mediated by DCs may lead to Tregs induction. The first of these is based on IDO1 upregulation, which is induced by ATP through inflammasome-independent noncanonical NF-kB signaling via $P2Y_{11}R$. The second relates to ATP catabolism and involves the production of adenosine, which may lead to the stabilization of IDO1-induced suppressive Tregs through the upregulation of fitness markers like as PD-1. Since IDO1, CD73, $A_{2A}R$ and PD-1 are "drugable" targets under investigation for cancer immunotherapy, our data may have implications in better refining their clinical use and in providing the rationale for a biology-driven combinatorial strategy.

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Author contributions

D.O. design and execution of experiments, data analysis/interpretation, manuscript writing. L.V., S.C.C., and A.P. execution of experiments. E.C. execution of experiments. V.S., M.C., and D.F. data analysis. F.Di.V. and E.A. conceptualization of experiments. M.C. Project Administration/oversight. A.C. study conceptualization, manuscript writing and funding acquisition. All authors edited and approved the final manuscript.

Supplementary material

Supplementary material is available at *Journal of Leukocyte Biology* online.

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Conflict of interest statement. None declared.

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