ORIGINAL ARTICLE

UDC: 615.015.152
DOI: https://doi.org/10.2298/VSP241004010K

Anti-inflammatory properties of an A3 adenosine receptor agonist, piclidenoson, in a model of human peripheral blood mononuclear cell culture

Anti-inflamacijska svojstva agoniste A3 receptora adenozina, piklidenozona, na modelu kultivisanih humanih mononuklearnih ćelija periferne krvi

Ljiljana Kozić*, Vanja Mališ*, Marija Drakul*, Sara Rakočević*, Dejan Bokonjić*, Dušan Mihajlović*†

***University of East Sarajevo, Faculty of Medicine Foča, Foča, Republic of Srpska, Bosnia and Herzegovina; †Military Medical Academy, Clinic for Dermatovenerology, Belgrade, Serbia**

Abstract

Background/Aim. Piclidenoson (CF101, IB-MECA), a selective agonist of the A3 adenosine receptor (A3AR), is used in clinical trials for the treatment of psoriasis. Emerging data from *in vitro* and *in vivo* studies suggest that piclidenoson possesses anti-inflammatory and immunomodulatory properties, but its action on human peripheral blood mononuclear cells (PBMCs) remains unknown. The aim of this study was to examine the anti-inflammatory effects of piclidenoson in a model of phytohaemagglutinin (PHA)-stimulated human PBMCs culture. **Methods.** Human PBMCs were isolated from the venous blood of healthy donors (n = 4) and treated with different concentrations of piclidenoson. Flow cytometry and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test were used to determine cell viability, while the MTT method and the carboxyfluorescein succinimidyl ester (CFSE) staining method were used to analyze the effect of piclidenoson on cell proliferation. Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, IL-23, IL-36, IL-5, interferon (IFN)-γ, IL-17, and IL-10 were measured using a specific sandwich enzyme-linked immunosorbent assay (ELISA). **Results.** The results of cytotoxicity tests showed that the highest applied concentration of piclidenoson (1,500 nM) reduced the metabolic activity of PBMCs ($p < 0.05$) and increased the percentage of late apoptotic ($p < 0.05$) and necrotic cells ($p < 0.01$). Non-toxic concentrations (250, 500, and 1,000 nM) decreased the proliferation of PBMCs ($p < 0.05$) compared to the control cells. These concentrations also decreased the production of TNF-α ($p < 0.001$). Piclidenoson at concentrations of 250 and 1,000 nM reduced the production of IL-23 ($p < 0.05$) while the concentrations of 500 and 1,000 nM reduced the production of IL-36 ($p < 0.05$). Piclidenoson at 1,000 nM increased IL-1β production, while other concentrations decreased its production ($p < 0.01$). The highest concentration (1,000 nM) inhibited the production of IL-5 ($p < 0.05$) and IFN-γ ($p < 0.01$) while all applied concentrations inhibited the production of IL-17 ($p < 0.001$). Furthermore, piclidenoson increased the production of IL-10 in all applied concentrations ($p < 0.01$). **Conclusion.** At non-toxic concentrations, piclidenoson exerts anti-inflammatory properties associated with the inhibition of proliferation and modulation of cytokine production in PHA-stimulated PBMCs culture.

Key words:
blood; cytokines; flow cytometry; inflammation; in vitro techniques; leukocytes, mononuclear; phytohemagglutinins; receptors, purinergic.

Apstrakt

Uvod/Cilj. Piklidenozon (CF101, IB-MECA), selektivni agonist A3 adenozinskog receptora (A3AR), koristi se u kliničkim ispitivanjima za lečenje psorijaze. Brojni podaci *in vitro* i *in vivo* studija ukazuju da piklidenozon poseduje anti-inflamacijska i imunomodulacijska svojstva, ali njegovo dejstvo na humane mononuklearne ćelije periferne krvi (*peripheral blood mononuclear cells* – PBMCs) nije u potpunosti istraženo. Cilj ovog istaživanja bio je da se ispitaju anti-inflamacijski efekti piklidenozona na modelu kultivisanih, fitohemaglutininom (*phytohaemagglutinin* – PHA)-stimulisanih, humanih PBMCs. **Metode.** Humane PBMCs izolovane su iz

Correspondence to: Dušan Mihajlović, Military Medical Academy, Clinic for Dermatovenerology, Crnotavska 17, 11 000 Belgrade, Serbia. E-mail: dusan.a.mihajlovic@gmail.com

venske krvi zdravih donora (n = 4) i tretirane različitim koncentracijama piklidenozona. Protočna citometrija i *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* – MTT test korišćeni su za određivanje vijabilnosti ćelija, dok su MTT metoda i metoda bojenja *carboxyfluorescein succinimidyl ester* (CFSE) korišćeni za analizu efekta piklidenozona na proliferaciju ćelija. Nivoi faktora nekroze tumora (*tumor necrosis factor* – TNF)-α, interleukina (IL)-6, IL-1β, IL-23, IL-36, IL-5, interferona (IFN)-γ, IL-17 i IL-10 određeni su specifičnim „sendvič" *enzyme-linked immunosorbent assay* – ELISA testom. **Rezultati.** Rezultati testova citotoksičnosti pokazali su da je najveća primenjena koncentracija piklidenozona (1 500 nM) smanjila metaboličku aktivnost PBMCs ($p < 0,05$) i povećala procenat ćelija u kasnoj apoptozi ($p < 0,05$) i nekrotičnih ćelija ($p < 0,01$). Netoksične koncentracije (250, 500, i 1 000 nM) smanjile su proliferaciju PBMCs ($p < 0,05$) u poređenju sa kontrolnim ćelijama. Ove koncentracije su takođe smanjile produkciju TNF-α ($p < 0,001$). Piklidenozon u koncentracijama od 250 i 1 000 nM smanjio je produkciju IL-23 ($p < 0,05$), dok su koncentracije od 500 i 1 000 nM smanjile produkcju IL-36 ($p < 0,05$). Piklidenozon je u koncentraciji od 1 000 nM povećao produkciju IL-1β, dok su ostale koncentracije smanjile njegovu produkciju ($p < 0,01$). Najveća koncentracija (1 000 nM) inhibirala je produkciju IL-5 ($p < 0,05$) i IFN-γ ($p < 0,01$), dok su sve primenjene koncentracije inhibirale produkciju IL-17 ($p < 0,001$). Takođe, piklidenozon je u svim primenjenim koncentracijama povećao produkciju IL-10 ($p < 0,01$). **Zaključak.** U netoksičnim koncentracijama, piklidenozon ispoljava anti-inflamacijske efekte povezane sa inhibicijom proliferacije i modulacijom produkcije citokina u kulturi PHA-stimulisanih PBMC.

Ključne reči:
krv; citokini; citometrija, protočna; zapaljenje; in vitro; leukociti, mononuklearni; fitohemaglutinini; receptori, purinergički.

Introduction

Elevated levels of A3 adenosine receptor (A3AR) expression have been identified in mononuclear and tumor cells from patients diagnosed with breast, colon, lung, pancreatic, and melanoma cancers [1–5]. In addition to being a therapeutic target, the A3AR is recognized as a biological marker of disease due to its overexpression in immune cells (neutrophils, monocytes, eosinophils, macrophages, dendritic cells) and tumor cells compared to healthy cells [1, 6–8]. In recent years, various agonists, antagonists, and modulators of the A3AR have been explored for potential therapeutic applications [9–13].

Piclidenoson (IB-MECA, CF101), a selective A3AR agonist, has undergone testing in preclinical models of colitis, uveitis, rheumatoid arthritis (RA), and osteoarthritis [14–16]. This A3AR agonist, demonstrating promising results as an anti-inflammatory and anti-cancer agent, is currently in phase III clinical trials for the treatment of psoriasis [17–20].

Emerging data from *in vivo* and *in vitro* experiments suggest that piclidenoson possesses anti-inflammatory and immunomodulatory properties. In murine models of endotoxemia, administration of piclidenoson (dose range, 0.2–0.5 mg/kg) reduced lipopolysaccharide (LPS)-induced plasma levels of interleukin (IL)-12 and interferon (IFN)-γ [21]. In a collagen-induced RA model, piclidenoson (0.5 mg/kg) significantly decreased tumor necrosis factor (TNF)-α expression levels in the lymph nodes, spleen, and synovial tissue [22]. Furthermore, piclidenoson (dose range, 10–100 μM) demonstrates the ability to inhibit the respiratory burst of human monocytes by inhibiting the activity of nicotinamide adenine dinucleotide phosphate oxidase *in vitro* [23]. Madi et al. [24] showed that the A3AR is overexpressed in peripheral blood mononuclear cells (PBMCs) from patients with RA compared to healthy subjects, and this overexpression is associated with an increase in nuclear factor (NF)-κB protein expression in PBMCs. In the same study, piclidenoson (10 nM) decreased A3AR expression in phytohaemagglutinin (PHA) and LPS-stimulated PBMCs, as well as reduced TNF-α production in LPS-stimulated PBMCs *in vitro*. PBMCs play a significant role in defending the body against infections, cancer, and other external threats.

The aim of this study was to examine the effects of piclidenoson on the proliferation and cytokine production of PBMCs *in vitro*.

Methods

Human PBMCs were collected from a group of healthy volunteers (n = 4) who signed an informed consent. The study was approved by the Ethics Committee of the Faculty of Medicine Foča, University of East Sarajevo, Bosnia and Herzegovina, (No. 01-2-32, from June 5, 2023).

Peripheral blood mononuclear cells

PBMCs were isolated from the blood of healthy volunteers using Lymphoprep density gradient centrifugation, as previously described [25, 26]. After layering on the Histopaque – 1077 gradient (Sigma-Aldrich, Darmstadt, Germany; density 1,077 g/mL), the blood was centrifuged at 2,200 rpm for 20 minutes at room temperature. The PBMC layer was washed three times with 0.02% sodium-ethylenediaminetetraacetic acid (NaEDTA) in phosphate-buffered saline. Cells were resuspended in a complete medium comprising of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol (all from Sigma-Aldrich, Darmstadt, Germany), 2 mM l-glutamine, and antibiotics: 20 μg/ mL of gentamicin, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (all from Galenika, Belgrade, Serbia), for

further use. Cell viability was determined using the trypan blue staining.

Piclidenoson

A3AR agonist with the chemical name 1-deoxy-1-[6-[[(iodophenyl)methyl]amino]-9H-purine-9-yl]-N-methyl-(-D-ribofuranuronamide), piclidenoson (IB-MECA), was produced by Can-Fite BioPharma Ltd., Petah Tikva, Israel. A stock solution of 10 mM was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Darmstadt, Germany).

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as the first method to assess the cytotoxicity of piclidenoson on PBMCs. The PBMCs (3×10^5 cells *per* well) were seeded in 96-well plates (Sarstedt, Nümbrecht, Germany) containing a complete medium. The cells were then treated with increasing concentrations of piclidenoson (250 nM – 1,000 nM) and incubated for 24 hrs at 37 °C with 5% CO_2 and 90% humidity. Cells without piclidenoson were used as a control.

For the proliferation assay, PBMCs were stimulated with 10 μg/mL PHA (Sigma-Aldrich, Darmstadt, Germany) and cultured alone or with piclidenoson (250 nM – 1,500 nM) for 72 hrs. After the incubation period, a solution of MTT (Sigma-Aldrich, Darmstadt, Germany) with a final concentration of 500 μg/mL was added to the wells and incubated for an additional 4 hrs. Subsequently, 10% sodium dodecyl sulfate (Merck KGaA, Darmstadt, Germany) was added to each well, and the results were analyzed the next day using a multimode reader (Synergy HTX, BioTek, Winooski, Vermont, USA) at wavelengths of 670 nm and 570 nm. The values were presented as metabolic activity (in percentages) relative to the analogous negative controls, which were used as 100%.

Flow cytometry

Flow cytometry was the second method for evaluating the cytotoxicity of piclidenoson and its effects on the proliferation of PBMCs.

Apoptosis and necrosis were assessed using an Annexin V/Propidium Iodide (PI) staining kit (BioLegend, San Diego, California), following the manufacturer's protocol. Flow cytometric analysis was conducted using a flow cytometer (Attune, Thermo Fisher Scientific). Data obtained were analyzed offline using the FlowJo X software. Necrotic cells were identified by exclusive PI staining, while cells stained only with Annexin V-fluorescein isothiocyanate were considered to be in the initial apoptotic phase. Cells positive for both markers were recognized as being in the late phase of apoptosis. Results were reported as percentages.

For the analysis of proliferation, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) staining dye (Thermo Fisher Scientific, Dreieich, Germany), treated with piclidenoson (250 nM–1,000 nM) and then stimulated with PHA (10 μg/mL) for the next 72 hrs. After incubation, cells were collected and stained with PI (50 μg/mL, Sigma-Aldrich, Darmstadt, Germany).

Cytokine production

TNF-α, IL-6, IL-23, IL-36, IL-lβ, IL-10, IL-5, IL-17, and IFN-γ production were determined using the enzyme-linked immunosorbent assay (ELISA) method, according to manufactory instructions (all from R&D Systems, Minneapolis, USA). After isolation, PBMCs (2×10^5 *per* sample) were treated with different concentrations of piclidenoson for 1 hr and then stimulated with PHA (30 μg/mL). After 72 hrs of incubation (37 °C, 5% CO_2), cells were centrifuged, and supernatants were collected to analyze cytokine levels. Unstimulated cells were used as a control.

Statistical analysis

The results were assessed using analysis of variance (ANOVA) test and the Student's *t*-test. Values of $p < 0.05$ were regarded as statistically significant. Mean values from different experiments were compared, and all data is presented as mean ± standard deviation. The data was analyzed using GraphPad Prism software (GraphPad, La Jolla, CA).

Results

Metabolic activity of PBMCs was significantly decreased at a piclidenoson concentration of 1,500 nM ($p < 0.05$) (Figure 1A). As shown in Figure 1B and C, flow cytometry confirmed the results obtained by MTT. Piclidenoson at a concentration of 1,500 nM decreased the viability of the cells and increased the percentage of late apoptotic and necrotic cells ($p < 0.01$, $p < 0.05$, $p < 0.01$, respectively).

As shown in Figure 2A, piclidenoson at concentrations of 250, 500, and 1,000 nM significantly inhibited PBMC proliferation, as measured by the MTT assay, compared to the corresponding control ($p < 0.05$). CFSE labeling assay confirmed these results (Figure 2B and C). No dose-dependent manner was observed in either assay.

Levels of five pro-inflammatory cytokines (TNF-α, IL-6, IL-23, IL-36, and IL-1β) and four key cytokines of T helper (Th) response, IFN-γ, IL-5, IL-17, and IL-10, were determined from PHA-stimulated PBMC supernatants. Cytokine production was undetectable from PHA-unstimulated cells. TNF-α production was downregulated at all piclidenoson concentrations applied ($p < 0.001$).

There is no modulation of IL-6 production. Piclidenoson at concentrations of 250 nM and 1,000 nM reduced IL-23 production, while there was no statistically significant difference at a concentration of 500 nM ($p < 0.05$). Furthermore, piclidenoson at concentrations of 500 nM and 1,000 nM reduced IL-36 production, with no statistically significant difference at a concentration of 250 nM ($p < 0.05$). Interestingly, an A3AR agonist administered at a concentration of 1,000 nM elevated the production of IL-1β, whereas the other two concentrations (250 nM and 500 nM) resulted in a statistically significant decrease of IL-1β production ($p < 0.01$). Th1 cytokine (IFN-γ) and Th2 cytokine (IL-5) were significantly downregulated at the highest concentration of piclidenoson (1,000 nM) ($p < 0.01$, $p < 0.05$, respectively). Moreover, piclidenoson in all applied concentrations decreased Th17 cytokine (IL-17) production ($p < 0.01$) and increased IL-10 production ($p < 0.01$) (Figure 3).

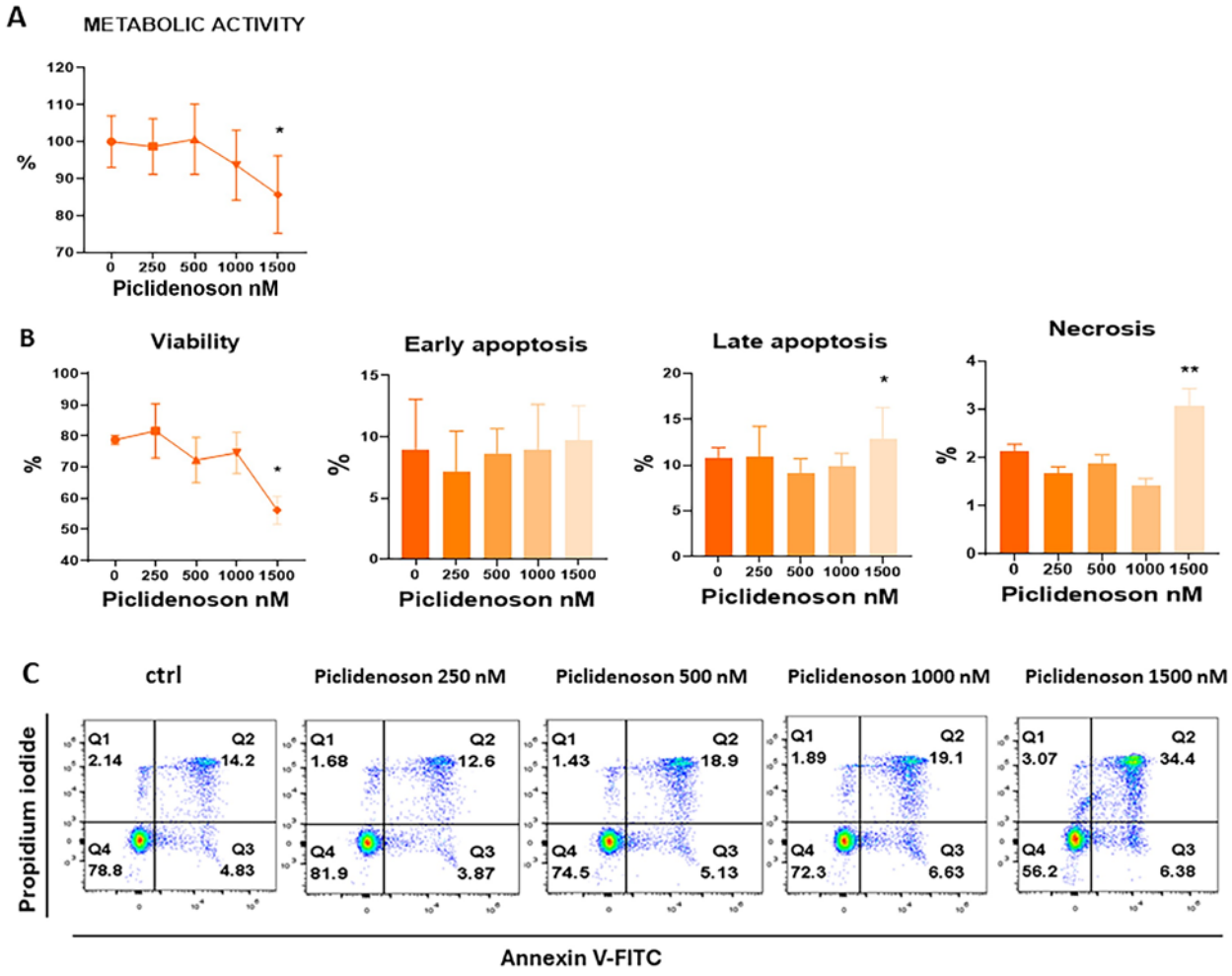

Fig. 1 – Effect of piclidenoson on the metabolic activity, apoptosis, and necrosis of human PBMCs. Cells were treated with increasing concentrations of piclidenoson (250 nM – 1,500 nM) for 24 hrs. Cytotoxicity was determined using the MTT assay and the results are presented as the percent of metabolic activity, relative to the control (100%) (A); the percentage of viable cells, apoptotic cells (Annexin-V$^+$ PI$^-$ for early apoptosis; Annexin-V$^+$ PI$^+$ for late apoptosis), and necrotic cells (Annexin-V$^-$ PI$^+$) is presented, as determined by Annexin-V/PI staining and flow cytometry analysis (B), with a representative example shown in panel C. Values are presented as mean ± standard deviation from 4 independent experiments (A, B).
*** $p < 0.05$, ** $p < 0.01$ compared with corresponding control (piclidenoson-non-treated PBMCs).**
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FITC – fluorescein isothiocyanate; PBMCs – peripheral blood mononuclear cells; Ctrl – control cells.

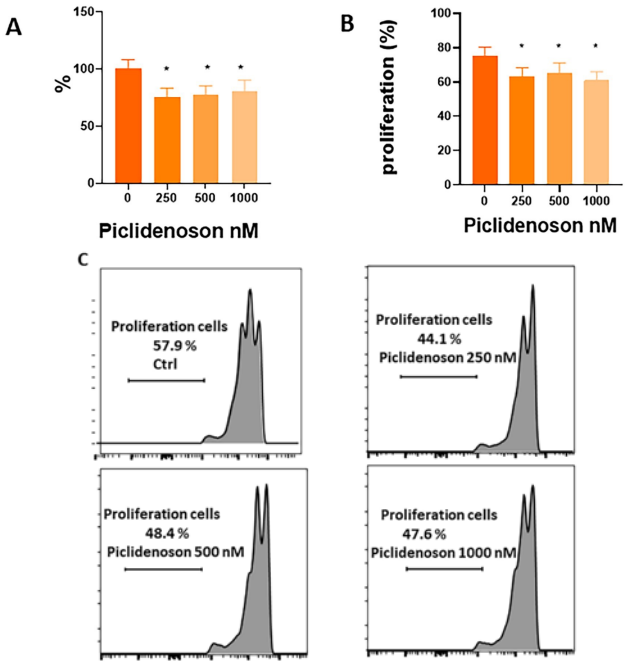

Fig. 2 – The effects of piclidenoson on PHA-stimulated proliferation of PBMCs. Cells were cultivated with PHA alone or PHA with different concentrations of piclidenoson for 3 days. Proliferation was evaluated using MTT assay (the results are presented as the percent relative to the control – 100%) (A) and CFSE staining dye followed by flow cytometry analysis (B, C). Data are expressed as mean ± standard deviation from 4 independent experiments (A, B); the analysis of CFSE dilution is shown on histograms from one representative experiments (C). *$p < 0.05$.
PHA – phytohaemagglutinin; CFSE – carboxyfluorescein succinimidyl ester.
For other abbreviations, see Figure 1.

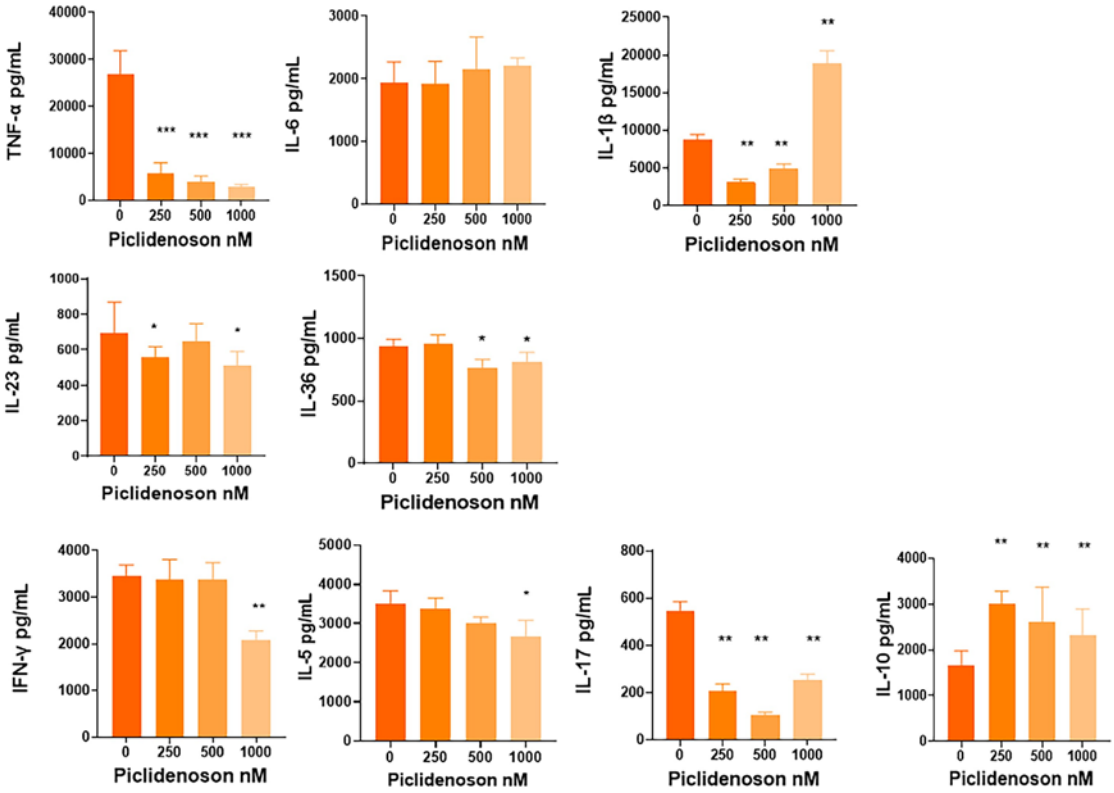

Fig. 3 – Effects of piclidenoson on pro-inflammatory and T-helper cytokine production by PHA-stimulated PBMCs. Cells were cultivated with PHA alone or PHA with different concentrations of piclidenoson for 3 days. Cytokine production was measured in the supernatants of PBMC cultures using ELISA kits. Cytokine levels are expressed as mean ± standard deviation (n = 4). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control.
TNF-α – tumor necrosis factor-alpha; IL – interleukin; n – number; ELISA – Enzyme-Linked Immunosorbent Assay.
For other abbreviations, see Figures 1 and 2.

Discussion

This study investigated the anti-inflammatory properties of piclidenoson in a model of PHA-stimulated PBMCs. PBMCs are invaluable for *in vitro* immunological research. Predominantly consisting of T lymphocytes, PBMCs offer a practical model for evaluating the effects of various treatments on cell division and cytokine production [27]. PHA interacts with certain carbohydrates on T-cell surfaces and is frequently applied in *in vitro* experiments to stimulate the activation of lymphocytes, transforming them into lymphoblasts that divide, proliferate, and release cytokines [28]. The previous report has shown that PHA stimulation leads to a swift increase in the expression of A3AR in T cells [29].

Piclidenoson is being investigated as a therapeutic option for autoimmune inflammatory disorders such as psoriasis and RA. The results of clinical trials affirm its safety, good tolerability, and significant anti-inflammatory properties [19, 30]. Previous studies have reported increased A3AR expression in the PBMCs of patients with autoimmune inflammatory conditions like Crohn's disease, RA, and psoriasis in contrast to healthy subjects [31]. The precise mechanism of piclidenoson's action remains uncertain. Earlier findings suggest that it involves the modulation of critical signaling proteins like phosphatidylinositol-3 kinase (PI3K), protein kinase A (PKA), protein kinase B (PKB/AKT), and IkappaB kinase. This modulation appears to lead to the disruption of the NF-κB and Wnt/β-catenin pathway and the suppression of inflammatory cytokine production [22]. In this investigation, we found that piclidenoson, applied at non-toxic concentrations, suppressed proliferation and modulated cytokine production of pro-inflammatory cytokines IL-1β, TNF-α, IL-23, and IL-36, as well as Th cytokine profile such as IFN-γ, IL-5, IL-17, and IL-10, in human PBMCs *in vitro*.

Our results demonstrated that piclidenoson, at a concentration of 1,500 nM, increased the number of late apoptotic and necrotic cells and reduced the metabolic activity of PBMCs *in vitro*. Earlier studies showed that A3AR agonists affect cell survival in different ways [32]. At low nanomolar concentrations, they protect human promyelocytic leukemia cell line (HL-60) and pro-monocytic cell line (U937) cells from apoptosis and preserve chick cardiac ventricular myocytes from hypoxic heart damage [33, 34]. However, at high micromolar concentrations (20–40 μM), A3AR agonists induce the death of rat astroglial cells, cerebellar granule neurons, and HL-60 cells [35–37]. According to some studies, A3AR activation leads to apoptotic cell death [33, 36], while other authors report necrotic cell death [37]. Cytotoxicity tests showed that piclidenoson exerts cytotoxic properties at the highest concentration of 1,500 nM, so in further research, we used lower concentrations of 250, 500, and 1,000 nM.

For the first time, our study suggests that piclidenoson inhibits PBMC proliferation in the nanomolar range (250 nM–1,000 nM). According to the results of the MTT test and apoptosis/necrosis assay, inhibited proliferation was not attributed to drug cytotoxicity. Jeffe et al. [38] demonstrated that piclidenoson, at a concentration of 20 μM, can inhibit the proliferation of PHA-stimulated human PBMCs *in vitro*. Similarly, other authors reported that piclidenoson inhibits PHA-stimulated human lymphocyte proliferation at concentrations of 10 μM, 100 μM, and 250 μM [39]. However, they did not employ any cytotoxicity assay method in their research, so the relationship between these effects and cellular apoptosis remains unclear. As reported by Cohen et al. [40], piclidenoson at a nanomolar concentration (10 nM) demonstrated the ability to inhibit the proliferation of immortalized human keratinocyte line cells (HaCaT), which is in accordance with our results.

Based on their cytokine secretion profiles, there are at least four types of Th cells: Th1, Th2, Th17, and T regulatory (T_{reg}). Th1 cells are characterized by the production of IL-2 and IFN-γ, while Th2 cells produce IL-4, IL-5, IL-10, and IL-13. Th17 cells are primarily defined by the production of IL-17A and IL-22 [41]. Lastly, T_{reg} cells, known for their immunosuppressive role, generate IL-10 and transforming growth factor (TGF)-β [42]. Lymphocytes produce pro-inflammatory cytokines, which play crucial roles in both physiological and pathological conditions, modulating immune responses and disease progression. The anti-inflammatory effects of A3AR agonists have been demonstrated in various experimental models and *in vitro* studies. Importantly, our study is the first to examine the effects of piclidenoson on IL-23 and IL-36 production in a model of PHA-stimulated PBMCs. In a model using cultured PBMCs from RA patients, A3AR agonist N^6-(3-iodo-benzyl)-2-chloro-adenosine-5'-N-methyluronamide (CI-IB-MECA) (100 nM) was found to decrease the production of TNF-α, IL-6, and IL-1β, along with reducing NF-κB activation. These effects were observed in PBMCs stimulated with phorbol myristate acetate for 24 hrs [43]. In the model of LPS-stimulated RAW 264.7 mouse macrophage cells, Lee et al. [44] demonstrated that CI-IB-MECA suppressed TNF-α production after 6 hrs of incubation. Additionally, in the same study, CI-IB-MECA reduced IL-1β messenger ribonucleic acid (mRNA) expression after 4 hrs and IL-1β protein expression after 8 hrs. Similarly, in LPS-treated BV-2 microglial cells, adenosine (100 μM) and Cl-IB-MECA (1 μM) suppressed LPS-induced TNF-α protein and mRNA levels by inhibiting PI3K/AKT and NF-κB activation after 4 hrs of incubation [45]. As reported by Cohen et al. [40], piclidenoson (10 nM) inhibits the proliferation of HaCaT cells through the dysregulation of the NF-κB signaling pathway and also suppresses IL-17 and IL-23 expression levels. Our results confirmed that piclidenoson, across all applied concentrations, reduced TNF-α production but did not modify IL-6 production. However, in contrast to previous investigations on A3AR agonists [43, 44, 46], the highest concentration of piclidenoson led to an increase in IL-1β production, while other concentrations decreased its production. The drug may influence the processing and secretion of IL-1β by modulating the activity of inflammasomes or proteases, which are essential for the release of IL-1β from its precursor. The inflammasome nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) activates

Caspase-1, responsible for IL-1β release. Research in a diabetic rat kidney model has shown that A3AR antagonism blocks the increase in Caspase-1 and reduces NF-κB expression in the renal tubular epithelium, leading to a decrease in IL-1β and IL-18 production, which was confirmed by measuring the urinary secretion of these cytokines. The effect of A3AR antagonism with MRS 1220 (0.1 mg/kg) resulted in reduced levels of the profibrotic marker alpha-smooth muscle actin (α-SMA) at the histological level and restoration of proteinuria in diabetic rats, significantly reducing kidney damage. The authors concluded that A3AR antagonism may serve as a potential therapeutic target through selective blockade of the NLRP3 inflammasome [47]. Furthermore, in a mouse macrophage model, the non-selective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (10 μM), acting through A2AR, increased IL-1β secretion by maintaining inflammasome activity *via* the cAMP/PKA/CREB/HIF-1α pathway [48]. A potential explanation for our results may be that piclidenoson, at micromolar concentrations, stimulates IL-1β secretion, maintaining inflammasome activity through A3 or A2 adenosine receptors or another signaling pathway, while at nanomolar concentrations, it inhibits secretion. The molecular mechanisms underlying this process require further investigation.

In our study, piclidenoson decreased IL-23 production in concentrations of 250 nM and 1,000 nM, but there is no statistically significant difference for concentrations of 500 nM. Furthermore, piclidenoson at concentrations of 500 nM and 1,000 nM has also decreased the production of IL-36 from PHA-stimulated PBMCs. As a pro-inflammatory cytokine, IL-23 is generated by activated monocytes, as well as activated antigen-presenting cells such as dendritic cells (DCs) and macrophages, T cells, B cells, and endothelial cells. It plays a crucial role in the pathogenesis of some diseases by modulating the activities of Th17 cells. These cells, in response to IL-23, produce IL-17 [49]. Elevated IL-17 levels have been observed in various conditions such as psoriasis, multiple sclerosis, Behcet's disease, uveitis, and the synovial fluid of RA patients [50]. The identification of the IL-23/IL-17 pathway has significantly improved the understanding of the pathogenesis of inflammatory diseases and expanded therapeutic possibilities [51, 52]. Within the IL-36 cytokine family, there are three agonists (IL-36α, IL-36β, and IL-36γ) and one antagonist (IL-36 receptor antagonist). Although primarily synthesized by keratinocytes, other cell types, including mononuclear cells, inflammatory macrophages, and DCs, can also produce members of the IL-36 cytokine family [53]. New findings suggest that IL-36 disorder has an important role in some autoimmune disorders, including psoriasis, atopic dermatitis, RA, and allergic contact dermatitis [54]. To our knowledge, there have been no similar studies investigating the impact of A3AR agonist on IL-36 production so far, and it will be very important to conduct similar experiments on other cell lines *in vitro*.

Our findings indicate that piclidenoson, at its highest concentration, reduced the production of IFN-γ and IL-5 and Th1 and Th2 cytokine subsets, respectively. Moreover, all applied concentrations of piclidenoson decreased IL-17 production, a cytokine characteristic of Th17 cells. From these results, we can speculate that piclidenoson at concentrations of 1,000 nM exerts a suppressive effect on the Th1/Th2 response and that all applied concentrations can suppress the Th17 response. Additionally, we observed an increase in the secretion of IL-10 at all tested concentrations of piclidenoson. IL-10, generated by various immune cells, including T and B cells, is very important in regulating excessive inflammation by suppressing the production of pro-inflammatory cytokines [55]. IL-10 can decrease the production of IL-12 by macrophages and DCs, a key cytokine for Th1 differentiation [56]. Likewise, IL-10 can inhibit the proliferation and production of IL-2, IFN-γ, IL-4, IL-5, and TNF-α by CD4 T cells and thus regulate innate and adaptive Th1 and Th2 responses [57]. Our research results on PBMCs and previous *in vitro* studies [58, 59] on LPS-activated RAW 264.7 macrophages and mouse CD4 T lymphocytes indicate that piclidenoson increases the production of IL-10. In this context, we can suggest that lower Th1/Th2 response can be connected with increased IL-10 production. In addition, early study [60] demonstrated that lower IL-17 production is associated with decreased IL-23 production, which is in accordance with our findings. IL-23, released by monocytes/macrophages or DCs, stimulates the generation of IL-17-producing T cells [49, 52].

Conclusion

We showed that piclidenoson exerts anti-inflammatory properties through a model of PHA-stimulated PBMCs. Piclidenoson can modulate PBMC proliferation in nanomolar concentrations and inhibit TNF-α, IL-23, and IL-36 production. Moreover, we showed that the highest concentration of piclidenoson can suppress the production of Th1 and Th2 cytokines, IFN-γ and IL-5. All applied concentrations of piclidenoson can suppress IL-17 production, a cytokine specific to Th17 cells. This knowledge could provide a foundation for further research to support the potential therapeutic applications of piclidenoson. Additional investigations are needed to understand the molecular processes underlying these effects. Furthermore, research on other cell lines will be essential to validate these findings and explore the broader potential of piclidenoson's immunomodulatory effects.

REFERENCES

1. *Varani K, Maniero S, Vincenzi F, Targa M, Stefanelli A, Maniscalco P,* et al. A3 receptors are overexpressed in pleura from patients with mesothelioma and reduce cell growth via Akt/nuclear factor-κB pathway. Am J Respir Crit Care Med 2011; 183(4): 522–30.

2. *Gessi S, Cattabriga E, Avitabile A, Gafa' R, Lanza G, Cavazzini L,* et al. Elevated expression of A3 adenosine receptors in human colorectal cancer is reflected in peripheral blood cells. Clin Cancer Res 2004; 10(17): 5895–901.

3. *Panjehpour M, Hemati S, Forghani MA.* Expression of A1 and A3 adenosine receptors in human breast tumors. Tumori 2012; 98(1): 137–41.
4. *Mazziotta C, Rotondo JC, Lanzillotti C, Campione G, Martini F, Tognon M.* Cancer biology and molecular genetics of A_3 adenosine receptor. Oncogene 2022; 41(3): 301–8.
5. *Mousavi S, Panjehpour M, Izadpanahi MH, Aghaei M.* Expression of adenosine receptor subclasses in malignant and adjacent normal human prostate tissues. Prostate 2015; 75(7): 735–47.
6. *Gessi S, Merighi S, Varani K, Leung E, Mac Lennan S, Borea PA.* The A3 adenosine receptor: an enigmatic player in cell biology. Pharmacol Ther 2008; 117(1): 123–40.
7. *Madi L, Ochaion A, Rath-Wolfson L, Bar-Yehuda S, Erlanger A, Ohana G,* et al. The A3 adenosine receptor is highly expressed in tumor versus normal cells: potential target for tumor growth inhibition. Clin Cancer Res 2004; 10(13): 4472–9.
8. *Bi J, Zheng C, Zheng X.* Increased expression of adenosine A3 receptor in tumor-infiltrating natural killer cells. Cell Mol Immunol 2021; 18(2): 496–7.
9. *Fishman P, Bar-Yehuda S, Liang BT, Jacobson KA.* Pharmacological and therapeutic effects of A3 adenosine receptor agonists. Drug Discov Today 2012; 17(7–8): 359–66.
10. *Stemmer SM, Manojlovic NS, Marinca MV, Petrov P, Cherciu N, Ganea D,* et al. Namodenoson in Advanced Hepatocellular Carcinoma and Child–Pugh B Cirrhosis: Randomized Placebo-Controlled Clinical Trial. Cancers (Basel) 2021; 13(2): 187.
11. *Coppi E, Cherchi F, Venturini M, Lucarini E, Corradetti R, Di Cesare Mannelli L,* et al. Therapeutic Potential of Highly Selective A_3 Adenosine Receptor Ligands in the Central and Peripheral Nervous System. Molecules 2022; 27(6): 1890.
12. *Safadi R, Braun M, Francis A, Milgrom Y, Massarwa M, Hakimian D,* et al. Randomised clinical trial: A phase 2 double-blind study of namodenoson in non-alcoholic fatty liver disease and steatohepatitis. Aliment Pharmacol Ther 2021; 54(11–12): 1405–15.
13. *Itzhak I, Bareket-Samish A, Fishman P.* Namodenoson Inhibits the Growth of Pancreatic Carcinoma via Deregulation of the Wnt/β-catenin, NF-κB, and RAS Signaling Pathways. Biomolecules 2023; 13(11): 1584.
14. *Bar-Yehuda S, Rath-Wolfson L, Del Valle L, Ochaion A, Cohen S, Patoka R,* et al. Induction of an antiinflammatory effect and prevention of cartilage damage in rat knee osteoarthritis by CF101 treatment. Arthritis Rheum 2009; 60(10): 3061–71.
15. *Rath-Wolfson L, Bar-Yehuda S, Madi L, Ochaion A, Cohen S, Zabutti A,* et al. IB-MECA, an A3 adenosine receptor agonist prevents bone resorption in rats with adjuvant induced arthritis. Clin Exp Rheumatol 2006; 24(4): 400–6.
16. *Mabley J, Soriano F, Pacher P, Haskó G, Marton A, Wallace R,* et al. The adenosine A3 receptor agonist, N6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide, is protective in two murine models of colitis. Eur J Pharmacol 2003; 466(3): 323–9.
17. *David M, Akerman L, Ziv M, Kadurina M, Gospodinov D, Pavlotsky F,* et al. Treatment of plaque-type psoriasis with oral CF101: data from an exploratory randomized phase 2 clinical trial. J Eur Acad Dermatol Venereol 2012; 26(3): 361–7.
18. *David M, Gospodinov DK, Gheorghe N, Mateev GS, Rusinova MV, Hristakieva E,* et al. Treatment of plaque-type psoriasis with oral CF101: data from a phase II/III multicenter, randomized, controlled trial. J Drugs Dermatol 2016; 15(8): 931–8.
19. *Fishman P.* Drugs Targeting the A3 Adenosine Receptor: Human Clinical Study Data. Molecules 2022; 27(12): 3680.
20. *Papp KA, Beyska-Rizova S, Gantcheva ML, Slavcheva Simeonova E, Brezoev P, Celic M,* et al. Efficacy and safety of piclidenoson in plaque psoriasis: Results from a randomized phase 3 clinical trial (COMFORT-1). J Eur Acad Dermatol Venereol 2024; 38(6): 1112–20.
21. *Haskó G, Németh ZH, Vizi ES, Salzman AL, Szabó C.* An agonist of adenosine A3 receptors decreases interleukin-12 and interferon-gamma production and prevents lethality in endotoxemic mice. Eur J Pharmacol 1998; 358(3): 261–8.
22. *Fishman P, Bar-Yehuda S, Madi L, Rath-Wolfson L, Ochaion A, Cohen S,* et al. The PI3K-NF-kappaB signal transduction pathway is involved in mediating the anti-inflammatory effect of IB-MECA in adjuvant-induced arthritis. Arthritis Res Ther 2006; 8(1): R33.
23. *Thiele A, Kronstein R, Wetzel A, Gerth A, Nieber K, Hauschildt S.* Regulation of adenosine receptor subtypes during cultivation of human monocytes: role of receptors in preventing lipopolysaccharide-triggered respiratory burst. Infect Immun 2004; 72(3): 1349–57.
24. *Madi L, Cohen S, Ochayin A, Bar-Yehuda S, Barer F, Fishman P.* Overexpression of A3 adenosine receptor in peripheral blood mononuclear cells in rheumatoid arthritis: involvement of nuclear factor-kappaB in mediating receptor level. J Rheumatol 2007; 34(1): 20–6.
25. *Drakul M, Mališ V, Rakočević S, Kozić S, Popović D, Mandić A,* et al. Cytotoxicity and anti-inflammatory properties of a GLP-1 receptor agonist in a model of human peripheral blood mononuclear cell culture. Biomedicinska istraživanja 2024; 15(1): 1–11.
26. *Drakul M, Tomić S, Bekić M, Mihajlović D, Vasiljević M, Rakočević S,* et al. Sitagliptin Induces Tolerogenic Human Dendritic Cells. Int J Mol Sci 2023; 24(23): 16829.
27. *Pourahmad J, Salimi A.* Isolated Human Peripheral Blood Mononuclear Cell (PBMC), a Cost Effective Tool for Predicting Immunosuppressive Effects of Drugs and Xenobiotics. Iran J Pharm Res 2015; 14(4): 979.
28. *Chilson OP, Boylston AW, Crumpton MJ.* Phaseolus vulgaris phytohaemagglutinin (PHA) binds to the human T lymphocyte antigen receptor. EMBO J 1984; 3(13): 3239–45.
29. *Gessi S, Varani K, Merighi S, Cattabriga E, Avitabile A, Gavioli R,* et al. Expression of A3 adenosine receptors in human lymphocytes: up-regulation in T cell activation. Mol Pharmacol 2004; 65(3): 711–9.
30. *Jacobson KA, Merighi S, Varani K, Borea PA, Baraldi S, Aghazadeh Tabrizi M,* et al. A_3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy. Med Res Rev 2018; 38(4): 1031–72.
31. *Ochaion A, Bar-Yehuda S, Cohen S, Barer F, Patoka R, Amital H,* et al. The anti-inflammatory target A(3) adenosine receptor is over-expressed in rheumatoid arthritis, psoriasis and Crohn's disease. Cell Immunol 2009; 258(2): 115−22.
32. *Ohana G, Bar-Yehuda S, Barer F, Fishman P.* Differential effect of adenosine on tumor and normal cell growth: focus on the A3 adenosine receptor. J Cell Physiol 2001; 186(1): 19−23.
33. *Yao Y, Sei Y, Abbracchio MP, Jiang JL, Kim YC, Jacobson KA.* Adenosine A3 receptor agonists protect HL-60 and U-937 cells from apoptosis induced by A3 antagonists. Biochem Biophys Res Commun 1997; 232(2): 317−22.
34. *Stambaugh K, Jacobson KA, Jiang JL, Liang BT.* A novel cardioprotective function of adenosine A1 and A3 receptors during prolonged simulated ischemia. Am J Physiol 1997; 273(1 Pt 2): H501−5.
35. *Abbracchio MP, Brambilla R, Ceruti S, Kim HO, von Lubitz DK, Jacobson KA,* et al. G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. Mol Pharmacol 1995; 48(6): 1038−45.
36. *Kohno Y, Sei Y, Koshiba M, Kim HO, Jacobson KA.* Induction of apoptosis in HL-60 human promyelocytic leukemia cells by adenosine A(3) receptor agonists. Biochem Biophys Res Commun 1996; 219(3): 904−10.
37. *Sei Y, Lubitz DK, Abbracchio MP, Ji XD, Jacobson KA.* Adenosine A3 receptor agonist-induced neurotoxicity in rat cerebellar granule neurons. Drug Dev Res 1997; 40(3): 267−73.

38. *Jeffe F, Stegmann KA, Broelsch F, Manns MP, Cornberg M, Wedemeyer H.* Adenosine and IFN-{alpha} synergistically increase IFN-gamma production of human NK cells. J Leukoc Biol 2009; 85(3): 452−61.
39. *Schiedel AC, Lacher SK, Linnemann C, Knolle PA, Müller CE.* Antiproliferative effects of selective adenosine receptor agonists and antagonists on human lymphocytes: evidence for receptor-independent mechanisms. Purinergic Signal 2013; 9(3): 351−65.
40. *Cohen S, Barer F, Itzhak I, Silverman MH, Fishman P.* Inhibition of IL-17 and IL-23 in Human Keratinocytes by the A_3 Adenosine Receptor Agonist Piclidenoson. J Immunol Res 2018; 2018: 2310970.
41. *Zhu X, Zhu J.* CD4 T Helper Cell Subsets and Related Human Immunological Disorders. Int J Mol Sci 2020; 21(21): 8011.
42. *Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA.* Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. Immunology 2006; 117(4): 433−42.
43. *Varani K, Padovan M, Vincenzi F, Targa M, Trotta F, Govoni M,* et al. A2A and A3 adenosine receptor expression in rheumatoid arthritis: upregulation, inverse correlation with disease activity score and suppression of inflammatory cytokine and metalloproteinase release. Arthritis Res Ther 2011; 13(6): R197.
44. *Lee* HS, Chung HJ, Lee HW, Jeong LS, Lee SK. Suppression of inflammation response by a novel A_3 adenosine receptor agonist thio-Cl-IB-MECA through inhibition of Akt and NF-κB signaling. Immunobiology 2011; 216(9): 997−1003.
45. *Lee JY, Jhun BS, Oh YT, Lee JH, Choe W, Baik HH,* et al. Activation of adenosine A3 receptor suppresses lipopolysaccharide-induced TNF-alpha production through inhibition of PI 3-kinase/Akt and NF-kappaB activation in murine BV2 microglial cells. Neurosci Lett 2006; 396(1): 1−6. Erratum in: Neurosci Lett 2019; 712: 134486.
46. *Sipka S, Kovács I, Szántó S, Szegedi G, Brugós L, Bruckner G,* et al. Adenosine inhibits the release of interleukin-1beta in activated human peripheral mononuclear cells. Cytokine 2005; 31(4): 258−63.
47. *Garrido W, Jara C, Torres A, Suarez R, Cappelli C, Oyarzún C,* et al. Blockade of the Adenosine A_3 Receptor Attenuates Caspase 1 Activation in Renal Tubule Epithelial Cells and Decreases Interleukins IL-1β and IL-18 in Diabetic Rats. Int J Mol Sci 2019; 20(18): 4531.
48. *Ouyang X, Ghani A, Malik A, Wilder T, Colegio OR, Flavell RA,* et al. Adenosine is required for sustained inflammasome activation via the A_2A receptor and the HIF-1α pathway. Nat Commun 2013; 4: 2909.
49. *Bianchi E, Vecellio M, Rogge L.* Editorial: Role of the IL-23/IL-17 Pathway in Chronic Immune-Mediated Inflammatory Diseases: Mechanisms and Targeted Therapies. Front Immunol 2021; 12: 770275.
50. *Ruiz de Morales JMG, Puig L, Daudén E, Cañete JD, Pablos JL, Martín AO,* et al. Critical role of interleukin (IL)-17 in inflammatory and immune disorders: An updated review of the evidence focusing in controversies. Autoimmun Rev 2020; 19(1): 102429.
51. *Bernardini N, Skroza N, Tolino E, Mambrin A, Anzalone A, Balduzzi V,* et al. IL-17 and its role in inflammatory, autoimmune, and oncological skin diseases: state of art. Int J Dermatol 2020; 59(4): 406−11.
52. *Chan TC, Hawkes JE, Krueger JG.* Interleukin 23 in the skin: role in psoriasis pathogenesis and selective interleukin 23 blockade as treatment. Ther Adv Chronic Dis 2018; 9(5): 111−9.
53. *Gresnigt MS, van de Veerdonk FL.* Biology of IL-36 cytokines and their role in disease. Semin Immunol 2013; 25(6): 458−65.
54. *Sachen KL, Arnold Greving CN, Towne JE.* Role of IL-36 cytokines in psoriasis and other inflammatory skin conditions. Cytokine 2022; 156: 155897.
55. *Iyer SS, Cheng G.* Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol 2012; 32(1): 23−63.
56. *Ma X, Yan W, Zheng H, Du Q, Zhang L, Ban Y,* et al. Regulation of IL-10 and IL-12 production and function in macrophages and dendritic cells. F1000Res 2015; 4: F1000 Faculty Rev − 1465.
57. *Couper KN, Blount DG, Riley EM.* IL-10: the master regulator of immunity to infection. J Immunol 2008; 180(9): 5771−7.
58. *Durante M, Squillace S, Lauro F, Giancotti LA, Coppi E, Cherchi F,* et al. Adenosine A3 agonists reverse neuropathic pain via T cell-mediated production of IL-10. J Clin Invest 2021; 131(7): e139299.
59. *Németh ZH, Lutz CS, Csóka B, Deitch EA, Leibovich SJ, Gause WC,* et al. Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism. J Immunol 2005; 175(12): 8260−70.
60. *Lyakh L, Trinchieri G, Provezza L, Carra G, Gerosa F.* Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. Immunol Rev 2008; 226: 112−31.

Received on October 4, 2024
Revised on November 12, 2024
Revised on December 20, 2024
Accepted on January 15, 2025
Online First March 2025