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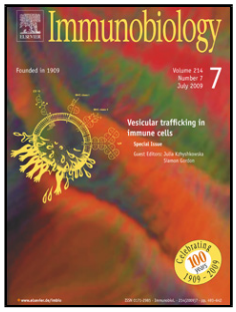

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PII: S0171-2985(15)30037-1
DOI: http://dx.doi.org/doi:10.1016/j.imbio.2015.07.018
Reference: IMBIO 51366

To appear in:

Received date: 20-4-2015
Revised date: 10-7-2015
Accepted date: 23-7-2015

Please cite this article as: Cortés-Garcia, Juan D, López-López, Cintya, Cortez-Espinosa, Nancy, García-Hernández, Mariana H, Guzmán-Flores, Juan M, Layseca-Espinosa, Esther, Portales-Cervantes, Liliana, Portales-Pérez, Diana P, Evaluation of the expression and function of the P2*times*7 Receptor and ART1 in human Regulatory T-Cell subsets.Immunobiology http://dx.doi.org/10.1016/j.imbio.2015.07.018

Evaluation of the expression and function of the P2X7 Receptor and ART1 in human Regulatory T-Cell subsets

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Abbreviations: **ART1=** ADP-ribosyltransferases 1; **ART2=** ADP-ribosyltransferases 2; **ART5=** ADP-ribosyltransferases 5; **ATP=** Adenosine Tri-Phosphate; **CFSE=** Carboxyfluorescein Succinimidyl Ester; **H_2O_2=** Hydrogen Peroxide; **iTreg=** Inducible regulatory T cells; **NAD=** Nicotinamide Adenine Dinucleotide; **NICD=** NAD induced cell death; **Novo=** Noboviocin; **nTreg=** natural regulatory T cells; **P2X7=** Purinergic receptor P2X, ligand-gated ion channel, 7; **PBMC=** Peripheral Blood Mononuclear Cells; **PHA=** Phytohaemagglutinin; **PI=** Propidium Iodide; **PMA=** Phorbol 12-Myristate 13-Acetate;

SEM= Standard Error Mean; **Treg=** Regulatory T cells; **CGS**=CGS-21680; **ZM**= ZM-241385;

Abstract

Regulatory T cells that express CD39 (CD39+ Treg) exhibit specific immunomodulatory properties. Ectonucleotidase CD39 hydrolyses ATP and ADP. ATP is a ligand of the P2X7 receptor and induces the shedding of CD62L and apoptosis. However, the role of ATP in CD39+ Treg cells has not been defined. Furthermore, NAD can activate the P2X7 receptor via ADP-ribosyltransferase (ART) enzymes and cause cell depletion in murine models. We evaluated the expression and function of P2X7 and ART1 in CD39+ Treg and CD39- Treg cells in the presence or absence of ATP and NAD. We isolated peripheral blood mononuclear cells from healthy subjects and purified CD4+ T cells, CD4+CD25+ T cells and CD4+CD25+CD39+ T cells. P2X7 and ART1 expression was assessed by flow cytometry and real-time PCR. Our results showed low P2X7 expression on CD39+ Treg cells and higher levels of ART1 expression in CD4+CD39+ T cells than the other subtypes studied. Neither shedding of CD62L nor cell death of CD39+ Treg or CD39- Treg cells was observed by 1mM ATP or 60μM NAD. In contrast, P2Xs receptor-dependent proliferation with 300 μM ATP, was inhibited by NAD in the different cell types analysed. The NAD proliferation-inhibition was increased with P2Xs and A2a agonist and was reversed with P2Xs and A2a antagonist, therefore NAD inhibits P2Xs-dependent proliferation and A2a activation. In conclusion, our results suggest that the altered function and expression of P2X7 and ART1 in the human CD39+ Treg or CD39- Treg cells could participate in the resistance against cell death induced by ATP or NAD.

Keywords: P2X7 receptor; ART1; ATP; NAD; immune regulation; CD39.

Introduction

The maintenance of homeostasis in the immune system is critical to prevent the development of autoimmune diseases. Therefore, several mechanisms of regulation, such as regulatory T cells (Treg) that act by suppressing the activity of effector T cells, have been developed (Buckner & Ziegler, 2004). Treg lymphocytes express the phenotype CD4+CD25+Foxp3+ and represent a subset of T cells – natural Treg (nTreg) and inducible Treg (iTreg) cells that originate from the thymus and periphery, respectively (Sakaguchi et al., 2013;Buckner, 2010;Buckner & Ziegler, 2004;Ohkura et al., 2013). The control of both levels and function of Treg cells is necessary to achieve an adequate immune response in pathological and physiological processes (Dalla et al., 2011). A subset of nTreg lymphocytes that expresses CD39 and exhibits immunomodulatory properties involved in the regulation of inflammation has been identified (Schuler et al., 2012).

CD39 is an ectoenzyme that hydrolyses ATP and ADP to AMP. It is expressed on human B cells and dendritic cells and constitutively on T-cell subsets. The Treg cells expressing CD39+ have been associated with potent immunosuppressive activity with alterations in CD39 levels have been related to various autoimmune diseases, cancer and viral and bacterial infections (Fletcher et al., 2009;Nikolova et al., 2011;Tang et al., 2012;Zhang et al., 2011;Buckner, 2010;Dwyer et al., 2010). Therefore, these results suggest that the hydrolysis of ATP by CD39 may represent an important regulation mechanism.

Furthermore, extracellular ATP mediates several biological responses in different cell types and tissues. ATP is detected by purinergic receptors, such as P2X: a cation-selective ion channel (Na^+, K^+, Ca^{2+}), and G-protein-coupled P2Y, which participate in different biological processes including differentiation, proliferation, chemotactic signals, and migration (Bulanova et al., 2009;Xu & Liang, 2013a;Jacob et al., 2013). The role of different purinergic receptors in immune cells has been described, such as P2Y11, P2Y14, and P2X1 on neutrophils (Jacob et al., 2013) or P2X4 on γδ+ T cells (Manohar et al., 2012). The P2X7 receptor expressed on T and B lymphocytes, macrophages, and dendritic cells induces apoptosis in macrophages, dendritic cells, and microglia cells (Hong et al., 2009;Rizzo et al., 2009;Caragnano et al., 2012). On the other hand, the nucleotide NAD can also activate the P2X7 receptor through its ribosylation, which is catalysed by ADP-ribosyltransferase enzymes in T lymphocytes and in consequence, inducing the release of CD62L and apoptosis

in a murine model (Hubert et al., 2010). The role of purinergic receptors in Treg cells has been described showing that 1 mM ATP promotes the function of Treg CD4+CD25^{High} (Trabanelli et al., 2012). However, the possible role of the P2X7 receptor on Treg cells that express CD39 has not been defined.

The ADP-ribosyltransferases (ARTs) are a group of proteins anchored to the cell membrane with the ability to transfer a ribosyl group from NAD to different target molecules (Laing et al., 2011;Corda & Di, 2003;Bourgeois et al., 2003). These enzymes are present in a variety of cell types such as epithelial, endothelial, alveolar muscle and immune cells (Hong et al., 2009;Grahnert et al., 2002). In mice, the Treg cells are susceptible to cell death induced by ADP-ribosylation of P2X7 on arginine 125 mediated by ART2.2: a mechanism known as cell death induced by NAD (Hubert et al., 2010;Haag et al., 2007). Five variants of ART (ART1–ART5) have been reported in humans. ART2 is a pseudogene while ART1 and ART5 are detected in lymphocytes. The ART1 variant is associated with the ribosyltransferase activity, since ART5 is only present in the secreted form (Weng et al., 1999;Schilling & Hauschildt, 2012;Paone et al., 2006). Therefore, NAD could activate P2X7 through ART1 at lower concentrations than those required for ATP-based activation (Hubert et al., 2010).

As previously mentioned the P2X7 receptor is activated by ligand binding to ATP (Jacob et al., 2013) as well as by the specific binding of the ribosyl groups of NAD (Hubert et al., 2010). Therefore, the aim of this study was to assess the effect of ATP or NAD in the CD39+ Treg and CD39- Treg cells through the ART1-P2X7 pathway.

Material and Methods

Peripheral blood mononuclear cells (PBMC)

Peripheral venous blood samples were obtained from twenty healthy subjects with an age of 23±5 years. Blood samples (40 to 60 mL) were drawn into heparinized syringes and were processed on Ficoll-Hypaque gradients (Sigma, St. Louis, MO) to isolate PBMC. All participants signed an informed consent form. The bioethical committee of the Dr Ignacio Morones Prieto Hospital approved this study (No. 10-11).

Cell separation

The CD4+CD25+ T cells were freshly isolated from the PBMC using the regulatory T-cell separation kit (Miltenyi Biotech, Auburn, CA). Briefly, CD4+ T cells were negatively selected from the total PBMC using a biotin-conjugated antibody cocktail specific for the lineage antigens. Afterwards, the CD25+ T cells were isolated from the CD4+ T-cell population using microbeads bearing anti-CD25 antibodies. The CD39- and CD39+ Treg cells were isolated from CD4+CD25+ T cells using cell sorting on a FACSAria II (BD Bioscience, San Jose, CA). The cells were stained with anti-CD4-PercP-Cy5, anti-CD25-PE and anti-CD39-FITC (eBioscience, San Diego, CA); they were resuspended in 10% newborn calf serum (Gibco, New York) in phosphate buffer saline (PBS) and immediately sorted by flow cytometry.

Cell culture

PBMC, CD4+CD25+ T cells, CD39- or CD39+ Treg cells were cultured in RPMI 1640 medium (Gibco) with 10% newborn calf serum (Gibco), 100 U/mL penicillin and 50 μg/mL streptomycin (Sigma) at 37°C in 5% CO_2. The cells were cultured in the presence or absence of NAD, ATP, and the KN-62 inhibitors or Noboviocin (Sigma). We used anti-CD3/CD28 (eBioscience) or PHA (Sigma) as a positive control of cell culture stimulation.

Surface and intracellular staining

Freshly isolated or activated cells were stained to identify surface molecules. Briefly, the cells were incubated with the antibodies anti-CD4-Percp-Cy5, anti-CD25-APC/Cy7, anti-CD39-FITC (eBioscience) or goat anti-human-ART1 (Santa Cruz Biotechnology, Inc., Dallas, TX) for 30 min at 4°C in the dark. For the case of human ART1 staining the cells were additionally incubated with secondary mouse anti-goat-PE antibodies (Santa Cruz Biotechnology) for 30 minutes at 4°C in the dark. In additional assays, the cells were incubated with anti-CD4-Percp-Cy5, anti-CD25-PE, and anti-CD39-FITC (eBioscience) for 30 min at 4°C in the dark. Appropriate isotype controls were included for each sample. For P2X intracellular staining the cells were fixed with 1% (w/v) paraformaldehyde and, after treatment with 0.1% (w/v) saponin (Sigma) for 5 minutes, washed with the same saponin solution. Rabbit anti-human P2X1 (Alomone Lab. LTd, Israel), rabbit anti-human P2X4 (SantaCruz Biotechnology), or rabbit anti-human-P2X7 (Calbiochem, Billerica, MA) antibodies were added for 30 min at 4°C in the dark. Afterwards, a secondary goat anti-rabbit-APC antibody (Santa Cruz Biotechnology, Inc.) was used. Finally, the cells were washed with PBS, fixed with 200 µL of 1% parafolmaldehyde and stored at 4°C until analysis by flow cytometry on a FACSCanto II instrument (BD Bioscience).

Shedding of CD62L

CD4+CD25+ T cells, CD39- or CD39+ Treg cells were incubated in the presence or absence of 50 ng/mL of PMA (as a positive control), 1 mM ATP, or 60 µM NAD at 37˚C for 30 min. The cells were washed with PBS, stained with α-CD62L-FITC (BD Bioscience) for 30 min, and then analysed by flow cytometry as previously reported (Garcia-Hernandez et al., 2011). The results are expressed as percentage of CD62L+ cells.

Apoptosis assay

Apoptotic cells were detected using the Annexin V-FITC/propidium iodide apoptosis detection kit (eBioscience). PBMC, CD4+CD25+ T cells, CD39-, or CD39+ Treg cells were incubated with or without 60 µM NAD or 1 mM ATP (this concentration was selected due to the activity of P2X7 previously assessed in other lymphocyte subpopulations) in complete RPMI 1640 for 30 minutes at 37°C using 96-well flat-bottom tissue culture plates. Ten microliters of 3% hydrogen peroxide (H_2O_2) were used as positive control for the apoptosis assay. We performed apoptosis assays for several concentrations of NAD (15, 30, 60 and 120

µM; and 1 and 2 mM) to determine the NAD concentration required to induce apoptosis. The detection of apoptosis was performed using a flow cytometer (FACSCanto II).

Flow cytometry

Flow cytometry was performed using a FACSCanto II (Beckman Coulter). The lymphocytes were identified based on characteristic properties of the cells in the forward (FSC) and side scatter (SSC). To evaluate P2X7 expression, the gates were restricted to the CD4+CD25^{High} and CD4+CD25- cells; and then analysed for P2X7+, CD39+, CD39+P2X7+, or CD39-P2X7+. In addition, to evaluate ART1 expression, the gates were restricted to the CD4+CD25^{High} and CD4+CD25- cells; and then analysed for ART1+ and CD39+ART1+.

Proliferation assay

PBMC, CD4+ T cells, CD39-, or CD39+ Treg cells were cultured for 3 or 5 days at 37°C in 96-well flat-bottom tissue culture plates (1x10^5 cells/well) either coated with 5 µg/mL anti-CD3/CD28 mAb (5 days) or in the presence of 10 µg/mL PHA (3 days). Previously, the cells were loaded with 2 µM carboxyfluorescein (CFSE) and incubated at 37°C for 10 minutes. The unbound CFSE was quenched using 250 µL of newborn calf serum, and the cells were washed with 4 mL of complete RPMI 1640 medium. Afterwards, the cells were incubated with or without 500 µM NAD or 300 µM ATP (this concentration of ATP is used as a positive control of proliferation by P2X7). In other culture conditions 0.5 mM Novobiocin or 50 mM KN-62 (which inhibit ARTs and P2X receptors, respectively) was added 45 minutes before the NAD or ATP. In an additional assay, to assess the effect of adenosine in cellular proliferation, PBMC were incubated with 70 µM CGS21680 (A2A agonist) (Sigma-Aldrich) or 1 µM ZM241385 (selective A2A antagonist) (Sigma-Aldrich), for 60 min. Then, the cells were washed with PBS, followed by stimulation with anti-CD3/CD28 mAb for 5 days. The percentage of proliferation was determined using a FACSCanto II flow cytometer.

RT-PCR and real-time PCR

Total RNA was extracted using a phenol-chloroform gradient for each culture condition (7×10^5 cells/well) from each cell type. cDNA was synthesized from 100 ng of total RNA in a volume of 20 µL using the first-strand cDNA synthesis SuperScript II kit (Invitrogen, Grand

Island, NY) with oligo(dT)18 as primers (Invitrogen) according to the manufacturer's recommendations. The cDNA concentration for all samples was adjusted to 100 ng/µL. Real-time PCR was conducted in 10 µL reaction volumes containing 1 µL cDNA, 5 µL PCR buffer SybrGreen Master Mix (Applied Biosystems, San Francisco, CA), and 20 µmol of each primer (IDT Technolgies, CoralVille, IA). The primer sequences were as follows: *Art1*, forward 5'-tgatgtctctgcttcttgtgtct and reverse 5'-gttgagatccgggagagcag-3'; *Art5,* forward 5'-gccaccctcttctctctaacaac-3' and reverse 5'-tgcttctcaaggctgctgga-3'; *18s,* forward 5'-cggctaccacatccaaggaa-3' and reverse 5'-gctggaattaccgcggct-3'. The results were obtained using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001) and different subsets (monocytes, CD4+ T cells and CD4+CD25+ T cells) were compared with PBMC: $2^{-((\Delta Cq \text{ of PBMC})-(\Delta Cq \text{ of any subsets})} = 2^{-((Cq18s\text{-}CqART1 \text{ of PBMC})-(Cq18s\text{-}CqART1 \text{ of any subsets})}$. House-keeping gene (18s) was used for each population when comparative analysis was performed.

Statistical analysis

The data are expressed as the mean ± SEM or median ± interquartile range. The statistical analysis was performed using InStat GraphPad software (InStat GraphPad Inc., San Diego, CA, USA) v. 5.0. The assessment of differences in the expression of the P2X7 receptor and ART1 were determined using both parametric and non-parametric tests. The differences in the function of the P2X7 receptor or ART1 were determined by ANOVA analysis. The differences were considered significant at $p<0.05$.

Results

Low P2X7 expression on CD39+ Treg cells

First, we showed a representative histogram of the P2X7 receptor expression in CD4+ T cells from healthy subject (Fig. 1A). Then, CD4+CD25^{High} as well as CD4+CD25- T cells were identified (Fig. 1B). In Figure 1C a representative dot plot of P2X7 and CD39 expression on gated CD4+CD25^{High} or CD4+CD25- T cells is shown. The percentage of cells expressing P2X7 between the two cell subsets (CD4+CD25^{High} or CD4+CD25- T cells) was not significantly different (Fig. 1D). As previously demonstrated, the CD39 expression on the CD4+CD25^{High} T cell population was significantly higher than in the CD4+CD25- T cells (data not shown). Nonetheless when we analysed the expression levels of the P2X7 molecule on CD39+ Treg cells and effector T cells (CD4+CD25-CD39+), we found that they were significantly lower than on the CD39- Treg cells and CD4+CD25-CD39- cells respectively (Fig. 1E). In addition, the expression of different P2X receptors on gated lymphocytes was evaluated (Fig. 1F) and the levels of P2X1 were increased when compared with the P2X4 and P2X7 receptors (Fig. 1G).

CD4+CD39+ T cells exhibit higher levels of ART1 expression

Next, we evaluated the expression of ART1 in CD39+ Treg and CD39- Treg cells from healthy subjects by flow cytometry (Fig. 2A). In contrast to the observed differences in P2X7 expression, significantly increased ART1 expression in CD4+CD39+ T cells when compared with CD4+ T cells and CD39- Treg cells was detected (Fig. 2B). However, when the ART1 and ART5 mRNA levels were analysed, we observed that the CD4+ T cells exhibited a higher level of ART1 expression when compared with monocytes (Fig. 2C). In contrast, the CD4+CD25+ T cells expressed a higher level of ART5 than monocytes (Fig. 2D).

Neither ATP nor NAD induces shedding of CD62L from CD39+ Treg and CD39- Treg cells

Once we had determined the expression of P2X7 and ART1, we assessed their function in the presence of their natural extracellular ligands. We evaluated the P2X7 receptor-dependent shedding of CD62L in CD4+CD25+ T cells, CD39+ Treg and CD39- Treg cell cultures

exposed to ATP or NAD. Only ATP induced shedding of CD62L from CD4+CD25+ T cells (Fig 3A) or PBMC (data not shown), whereas neither ATP nor NAD induced shedding of CD62L from CD39+ Treg cells (Fig. 3B) or CD39- Treg cells (Fig. 3C).

NAD is unable to induce apoptosis of CD39+ Treg and CD39- Treg cells

We next assessed whether NAD induces apoptosis of Treg subsets by flow cytometry. Using annexin/PI markers we observed that 1 mM ATP, but not NAD, induced apoptosis of CD4+CD25+ T cells (Fig. 4A). In contrast, both ATP and NAD were unable to induce apoptosis of CD39+ Treg or CD39- Treg cells (Fig. 4B). We evaluated whether NAD induces apoptosis over a range of different incubation times (30 min, 90 min, 16 h, and 24 h), but we were unable to induce apoptosis as reported previously in murine models (data not shown). In addition, to rule out the effect of an inadequate concentration of NAD, we explored different NAD concentrations (15, 30, 60 and 120 µM; and 1 and 2 mM), and still no apoptosis in PBMC was observed (Fig. 4C).

NAD induces inhibition of proliferation through P2 purinergic and A2A receptors

To continue evaluating the possible effect of NAD or ATP, we established proliferation assays where cells (CD4+ T cells, CD39- Treg or CD39+ Treg cells) were stimulated with PHA or anti-CD3/CD28; co-cultured with ATP or NAD and in the presence or absence of ATP/NAD inhibitors. We used 300 µM ATP as positive control of the proliferation that is dependent on P2X7 as previously reported (Trabanelli, Ocadlikova, Gulinelli, Curti, Salvestrini, Vieira, Idzko, Di, Ferrari & Lemoli, 2012). In Figure A a representative dot plot of the proliferation assay on PBMC is shown. In contrast to the ATP effect in proliferation, NAD inhibited proliferation in PBMC (Fig. 5A) and in CD4+ T cells (Fig. 5B). Although Treg cell proliferation was detected in low levels when compared with CD4+ T cells, similar effects induced by NAD were observed in CD39- Treg (Fig. 5C) and CD39+ Treg cells (Fig. 5D). In addition, when we cultured cells in the presence of KN-62, an inhibitor of P2X receptors, and NAD; a reversal of the NAD-induced proliferation inhibition was observed (Fig. 5A–B).

On the other hand, when ATP and KN-62 were added to the cell culture, an inhibition of ATP-induced proliferation was detected (Fig. 5A–B); but this phenomenon was not observed in CD39+ Treg and CD39- Treg cells (Fig. 5C-D).

In a preliminary assay to determine if other mechanisms in the inhibition of the proliferation induced by NAD were involved, PBMC were incubated in the presence of 70 µM CGS (A2a receptor agonist) or 1 µM ZM (A2a receptor antagonist). The inhibition of the proliferation induced by NAD was reverted in the presence of ZM (Fig. 5E), suggesting A2A receptor activation.

Discussion

P2X7 and ART1 receptors play several roles during immunologic conditions. P2X7 has been related to autoimmune diseases (Bartlett et al., 2014;Xu & Liang, 2013b), and has been suggested as pharmacological target in rheumatoid arthritis (Stock et al., 2012;Keystone et al., 2012). ARTs may be used as a pharmacological drug targets in obesity and type 2 diabetes mellitus (Bavec, 2013). It was reported that the activation of P2X7 by ATP inhibits the suppressive potential of Treg cells (Schenk et al., 2011) and the interaction of T lymphocytes with NAD or ATP causes cell death (Haag et al., 2007). Additionally, the sensitivity of cells to ATP correlates with their P2X7 expression levels (Aswad & Dennert, 2006). The results of our study demonstrated, in unstimulated cells, lower levels of P2X7 expression on CD4+CD25^{High}CD39+ Treg cells when compared with CD4+CD25^{High}CD39- Treg cells. Several studies have demonstrated P2X7 receptor expression on different subsets of human immune cells, such as CD4+, CD19+ and CD14+ cells (Garcia-Hernandez et al., 2012). However, no data of the P2X7 expression at the protein level in the subsets of CD4+CD25^{High} T, CD39+ Treg, or CD39- Treg cells have been previously reported.

The elevated levels of P2X7+ cells within the CD4+CD25^{High} T cell subset and diminished frequencies in the CD39+ Treg cell subset might indicate differing sensitivities of these regulatory T-cell subsets to ATP- or NAD-induced cell death (NICD) as reported in CD4+CD25^{High} Treg murine cells (Aswad et al., 2005). However, CD39- Treg cells (Fig. 1E) expressing higher levels of P2X7 than CD39+ Treg cells were ATP-resistant to apoptosis (Fig. 4B). It has been described that the levels of P2X7 receptor expression in CD4+ T cells is increased in the presence of ATP but not in the Treg cells (Trabanelli et al., 2012). We did not observe differences in the P2X7 expression in Treg cells under our culture conditions with ATP or NAD (data not shown).

To identify if another P2X receptor was participating in the ATP effect observed, we evaluated the presence of P2X1 and P2X4 by flow cytometry, and higher P2X1 levels when compared with P2X4 and P2X7 were found (Fig. 1G). P2X1 is a purinergic receptor having an important role in platelets (Clifford et al., 1998), while P2X4 favours TCR activation of γδ T cells (Manohar et l., 2012). Furthermore, both P2X1 and P2X4 receptors regulate T-cell activation at the immune synapses (Woehrle et al., 2010). However, the role in Treg cells needs to be evaluated in further studies.

In a murine model, ART2.2 is described as the enzyme that participates in the transfer of the ribose moiety to different target proteins (Scheuplein et al., 2003;Laing et al., 2011;Weng, et al, 1999;Corda & Di, 2003). However, there is a controversy on which of the four ARTs (ART1, 3, 4, or 5) is expressed on human immune cells (Glowacki et al., 2002;Grahnert et al., 2002;Paone et al., 2006). We evaluated ART1 and ART5 expression on different subsets of human immune cells, since these two enzymes possess ribosyltransferase activity (Grahnert et al., 2002;Del & Balducci, 2008;Krebs et al., 2005). ART1 and ART5 mRNA expression was evaluated in monocytes, CD4+ T cells and CD4+CD25+ T cells. Furthermore, we compared the fold-change in expression relative to PBMC finding that CD4+ T cells expressed higher levels of ART1 mRNA. Nevertheless, CD4+CD25+ T cells expressed more ART5 mRNA. In addition, our results demonstrate that ART1 protein expression is increased in CD4+CD39+ T cells when compared with other subsets of cells in which the expression levels are low; similar to results reported by other authors (Seman et al., 2003;Grahnert et al., 2002). Therefore, these results indicate that ART proteins are constitutively present at different levels on subsets of leukocytes and likely playing a role in immune cell function. In addition, we highlight a possible role of ART1 on CD4+CD39+ T cells, which could act as a reservoir of the CD39+ Treg cells (Schuler et al., 2012). These results are in agreement with those described for cytotoxic T lymphocytes and neutrophils (Wang et al., 1994;Allport et al., 1996).

We assessed the function of the P2X7 receptor in different T-cell subpopulations. Extracellular NAD and ATP trigger the shedding of CD62L and the externalization of phosphatidylserine on murine T cells (Scheuplein et al., 2009) and human monocytes (Garcia-Hernandez et al., 2011). L-Selectin is an important molecule that modulates the migration and homing of the lymphocytes into the lymphoid organs. We did not detect any effect induced by ATP or NAD in shedding of CD62L in the different subpopulations analysed (CD39+ Treg, CD39- Treg or CD4+CD25+ T cells) as reported previously. These results suggest that, the mechanism of P2X7 receptor activation by ART1-mediated ribosylation does not occur in human immune cells as reported in murine models (Aswad & Dennert, 2006;Hong et al., 2009). This conclusion is supported by results observed with patch-clamp assays in which NAD did not activate the P2X7 receptor and behaved like a partial agonist of the P2X7 receptor (Xu et al., 2012). CD39+ Treg cells may be less sensitive to ATP or NAD because they exhibited a lower level of P2X7 and ART1 expression;

however the results with CD39- Treg cells, which expressed higher levels of P2X7 when compared with CD39+ Treg cells, do not support this latter hypothesis.

As previously mentioned 1 mM ATP treatment of monocytes (Bulanova et al., 2009;Rizzo et al., 2009;Garcia-Hernandez et al., 2011) is necessary to induce cell death in a P2X7-activation-dependent manner. Meanwhile, the same concentration of ATP in CD4+CD25+ T cells promoted an increase in proliferation at 72 hours of incubation and, in contrast, exhibited a low percentage of apoptosis within only 30 minutes of ATP exposure (Trabanelli et al., 2012). These data correlate with our results of ATP-induced apoptosis in CD39- Treg and CD39+ Treg cells. On the other hand, when CD4+ T cells were incubated with 250 µM ATP, and increased proliferation was observed (Trabanelli et al., 2012). We found that PBMC and CD4+ T cells, and a small proportion of CD39+ Treg cells, incubated with 300 µM ATP for 5 days showed an increase in proliferation, thus this treatment was used as a positive control of proliferation induced by P2X7 activation.

These results regarding ATP activity in human immune cells are similar to those described for NAD, which was reported to induce cell death via ART2.2-P2X7 activation in a murine model using BALB/c mice (Adriouch et al., 2001;Hubert et al., 2010); nonetheless our results in human immune cell apoptosis are discordant. We were unable to observe apoptosis in the different cell subsets analysed (CD4+CD25+ T, CD39+ Treg or CD39- Treg cells). In addition, NAD did not lead to apoptosis of resting or PHA-stimulated lymphocytes (Bruzzone et al., 2009). Furthermore, as already observed in a murine model, the sensitivity to NAD-induced cell death is related to higher levels of ART2.2 expression. In contrast, both ART2.2-deficient NZW mice and C57BL/6 mice with lower levels of ART2.2 exhibited a lower induction of apoptosis by NAD in T cells and splenocytes, but at the same time exhibited increased inhibition of T-cell proliferation (Adriouch et al., 2001), similar to cytotoxic T lymphocytes and monocytes (Bortell et al., 2001).

Our results in CD4+ T cells, CD39+ Treg cells, and PBMC demonstrated that 500 µM NAD induces inhibition of proliferation and in the presence of KN-62, an inhibitor of P2X7, the inhibition of proliferation is reversed almost to the level reached by the ATP treatment. It is possible that NAD is only able to activate P2X7 to induce proliferation inhibition, not apoptosis. However, another mechanism involved in this process could be that lower levels of ART1 favour the ribosylation of other molecules, such as LFA-1, CD43 (Okamoto et al.,

1998), or P2X receptors since P2X1 showed higher levels than P2X7 in lymphocytes. In addition, NAD induces a rise in [Ca2+]i in activated human monocytes, via the engagement of P2Y1 and P2Y11 receptors (Klein et al., 2009), and inhibits IL-1 expression through a mechanism independent of ERK/JNK in human gingival fibroblasts (Gotoh et al., 2013).

Furthermore, the ectonucleotidase CD73 produces adenosine from NAD, and this adenosine participates through the A2a receptor to induce proliferation inhibition (Garavaglia et al., 2012). Our preliminary results in PBMC support this notion and suggest an additional mechanism by which NAD modulates the immune function of T cells subpopulations through A2A receptor activation. In addition, it will be necessary to evaluate the effect of NAD in the proliferation of the CD4+CD39+ T cell subset that showed the highest levels of ART1. Finally, although we demonstrated that proliferation inhibition induced by NAD is dependent on P2X7 activation, the complete molecular mechanism whereby NAD acts in subpopulations of immune cells remains to be elucidated.

Conclusions

We showed that CD39+ Treg cells are resistant to cell death induced by ATP or NAD due to diminished expression and function of P2X7 and ART1. Furthermore, NAD inhibits P2Xs dependent proliferation in CD4+ T cells. Therefore, our data suggest that NAD and ATP might modulate the immune function of different subtypes of T cells depending on levels of expression and function of the P2X7 receptor, CD39, and ART proteins combined with the signalling induced by these molecules.

Acknowledgments

This work was supported by grant 169793 (to DP-P) from CONACYT, México. Cortés-García JD was a recipient of a scholarship from CONACYT, México.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Expression of the P2X7 receptor on CD39+ Treg and CD39- Treg cells. PBMC from healthy subjects were stained with anti-CD4-PercP-Cy5, anti-CD25-PE, anti-CD39-FITC, and anti-P2X7 as described in material and methods and analysed by flow cytometry. Representative histogram of P2X7+ cells in the lymphocyte gate (A). Representative dot plot of CD4+CD25+ T or CD4+CD25- T cells (B). Representative dot plots of P2X7 and CD39 expression restricted to the CD4+CD25^{High} and CD4+CD25- cells (C). Comparative levels of P2X7 expression on CD4+CD25^{High} or CD4+CD25- T cells (D). Comparative P2X7 levels on CD39+ or CD39- Treg cells (E). Expression of P2X1, P2X4 and P2X7 receptors on gated lymphocytes based on their forward- and side-scatter characteristics (F). Comparative P2X1, P2X4, and P2X7 levels on gated lymphocytes (G). The results correspond to the mean ± SEM of the percentage of positive cells from seven independent tests. *$p<0.05$. NS= non-significant.

Figure 2. Expression of ADP-ribosyltransferase 1 on CD4+CD39+ T cells. PBMC from healthy subjects were stained with anti-CD4, anti-CD25, anti-CD39, and anti-ART1; and analysed by flow cytometry. Representative histograms of ART1+ cells (A). The percentage of ART1+ cells in the PBMC, CD4+ T, CD4+CD25^{High} T cells, CD4+CD39+ T cells and CD39+ Treg and CD39- Treg populations (B). The results correspond to the median ± range of the percentage of positive cells from four independent tests. *$p<0.05$. The expression of ART1 (C) and ART5 (D) in monocytes, CD4+ T cells, and CD4+CD25+ T cells were analysed by real-time RT-PCR. The results were obtained by the $2^{-\Delta\Delta Cq}$ method, different subsets (monocytes, CD4+ T cells, and CD4+CD25+ T cells) were compared with PBMC: $2^{-((\Delta Cq \text{ of PBMC})-(\Delta Cq \text{ of any subsets})} = 2^{-((Cq18s\text{-}CqART1 \text{ of PBMC})-(Cq18s\text{-}CqART1 \text{ of any subsets})}$, and 18s was employed as house-keeping gene expression. *$p<0.05$.

Figure 3. Effect of ATP or NAD on shedding of CD62L in CD39+ Treg and CD39- Treg cells. The CD4+CD25+ T cells were isolated from the PBMC using the regulatory T-cell separation kit. Then, the CD39- and CD39+ Treg cells were isolated from CD4+CD25+ T cells using cell sorting on a FACSAria II. Next, the cells were cultured with medium (negative control), 50 ng/mL PMA (positive control), 1 mM ATP or 60 µM NAD for 30 minutes, and stained with anti-CD62L as indicated in materials and methods. Representative dot plots of percentage of CD62L+ cells on CD4+CD25^{High} T (A), CD39+ Treg (B) or CD39-

Treg (C) cell populations. The results correspond to representative dot plots of three independent tests.

Figure 4. Effect of ATP or NAD on apoptosis in CD39+ Treg and CD39- Treg cells. The CD4+CD25+ T cells were isolated from the PBMC using the regulatory T-cell separation kit. Then, the CD39- and CD39+ Treg cells were isolated from CD4+CD25+ T cells using cell sorting on a FACSAria II. Next, cells were cultured with RPMI 1640 medium (negative control), 0.3% H_2O_2 (positive control), 1 mM ATP, 60 µM NAD or 50 nM KN-62 for 30 minutes. Then, the cells were stained with an annexin/PI kit and analysed by flow cytometry. Apoptosis levels, as indicated by annexin/PI of CD4+CD25+ T cells (A), CD39+ Treg, and CD39- Treg cells (B). Apoptosis of PBMC after being cultured with increasing concentrations of NAD ranging from 15 µM to 2 mM (C). The results correspond to the mean ± SEM of the percentage of positive cells from three independent tests. *$p<0.05$ when compared with unstimulated cells.

Figure 5. Effect of NAD on the proliferation of CD39+ Treg and CD39- Treg cells. In proliferation assays, the cells were loaded with carboxyfluorescein (CFSE) and cultured with RPMI 1640 medium (negative control), 5 µg/µL anti-CD3/CD28 as a positive control for 5 days as mentioned in materials and methods. Representative plots of proliferation analysis on PBMC (A). Percentage of divided cells on CD4+ T cells (B), CD39- Treg cells (C), and CD39+ Treg cells (D). The results correspond to the mean ± SEM of the percentage of positive cells from three independent tests. *$p<0.05$ with respect to medium alone, **$p<0.05$ when compared with the positive control and ++$p<0.05$ when compared with the ATP treatment. ND= none done. PBMC were incubated with 500 µM NAD in the presence or absence of A2a receptor agonist (70 µM CGS) or A2a receptor antagonist (1 µM ZM) (E). The results correspond to the mean ± SEM of the percentage of positive cells from two independent tests. *$p<0.05$ with respect to medium alone, **$p<0.05$ when compared with the positive control.

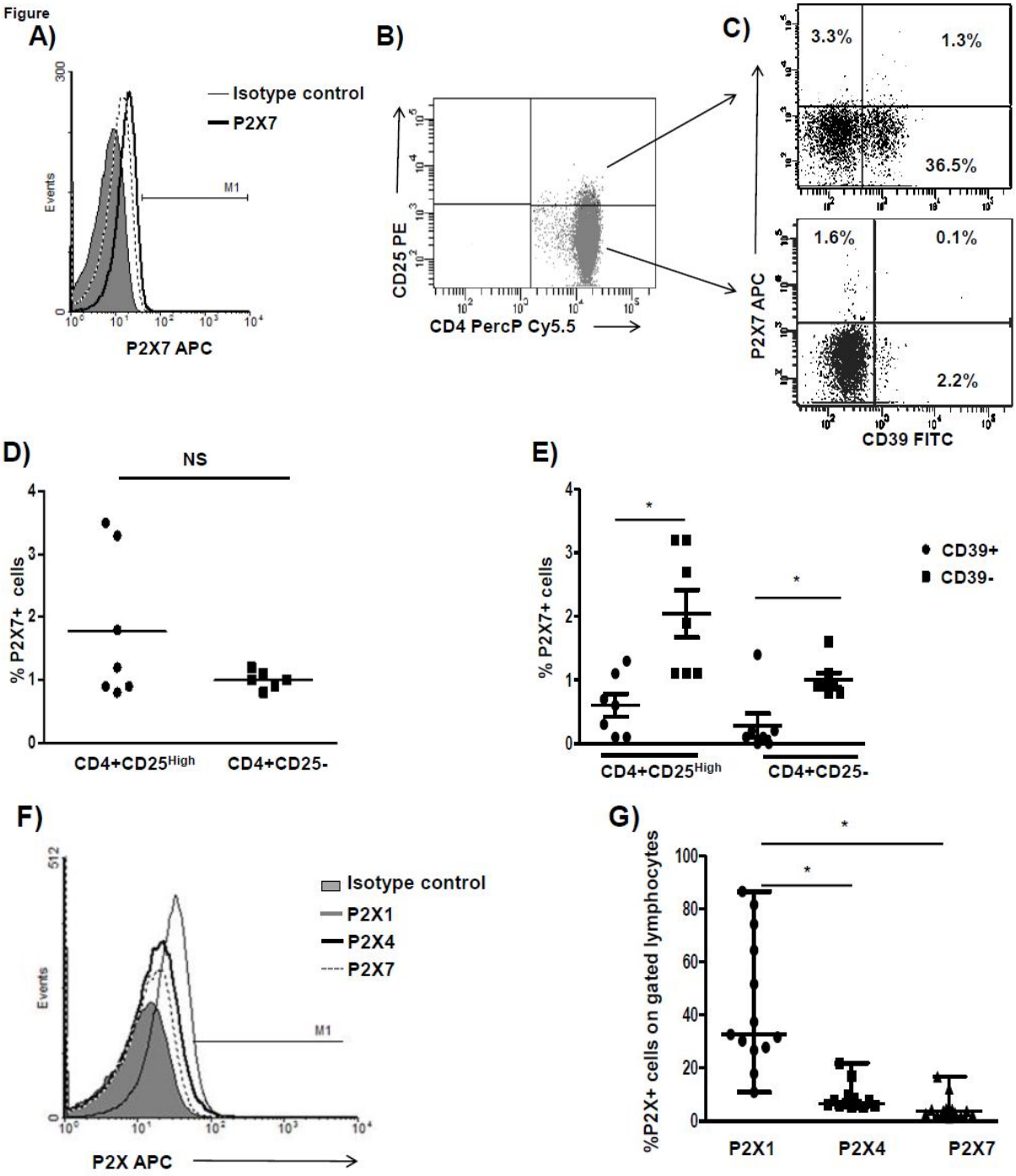


Fig. 1

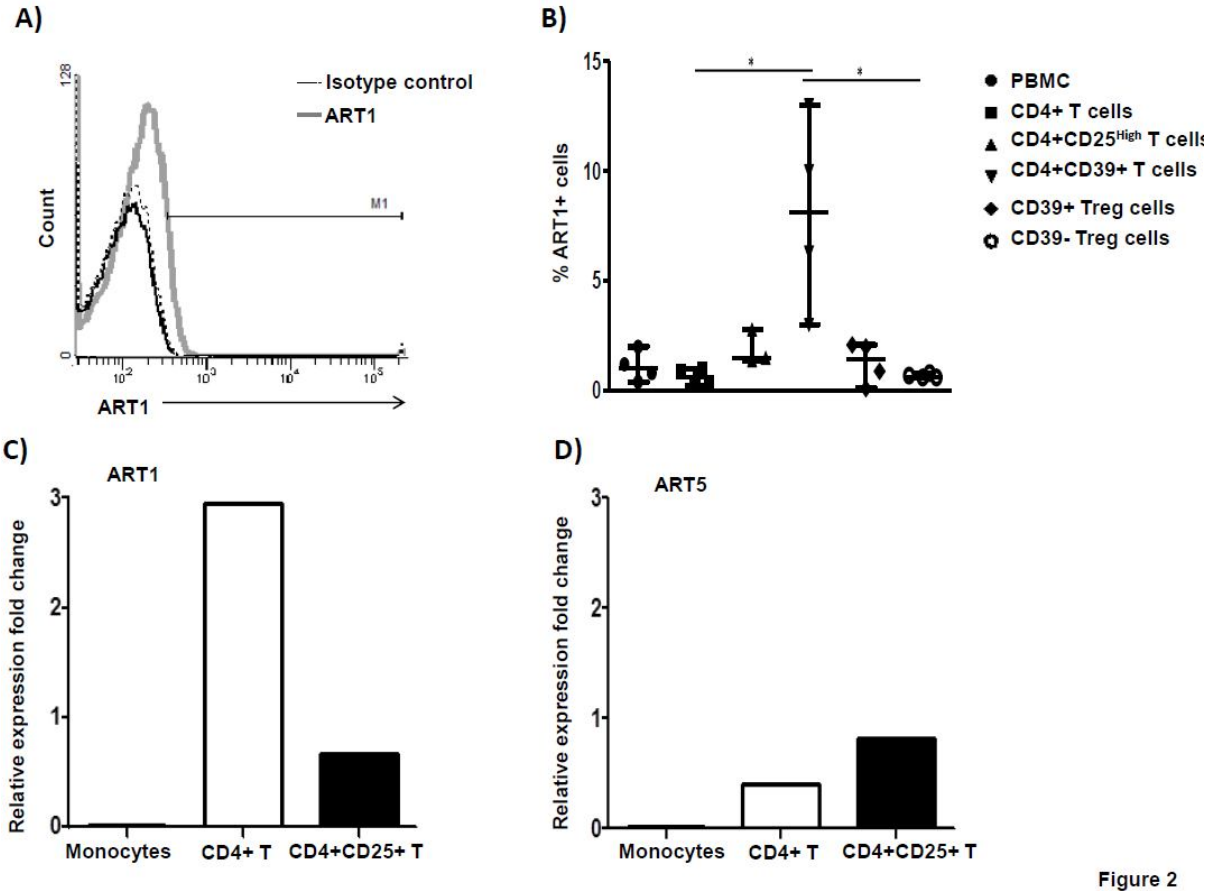


Figure 2

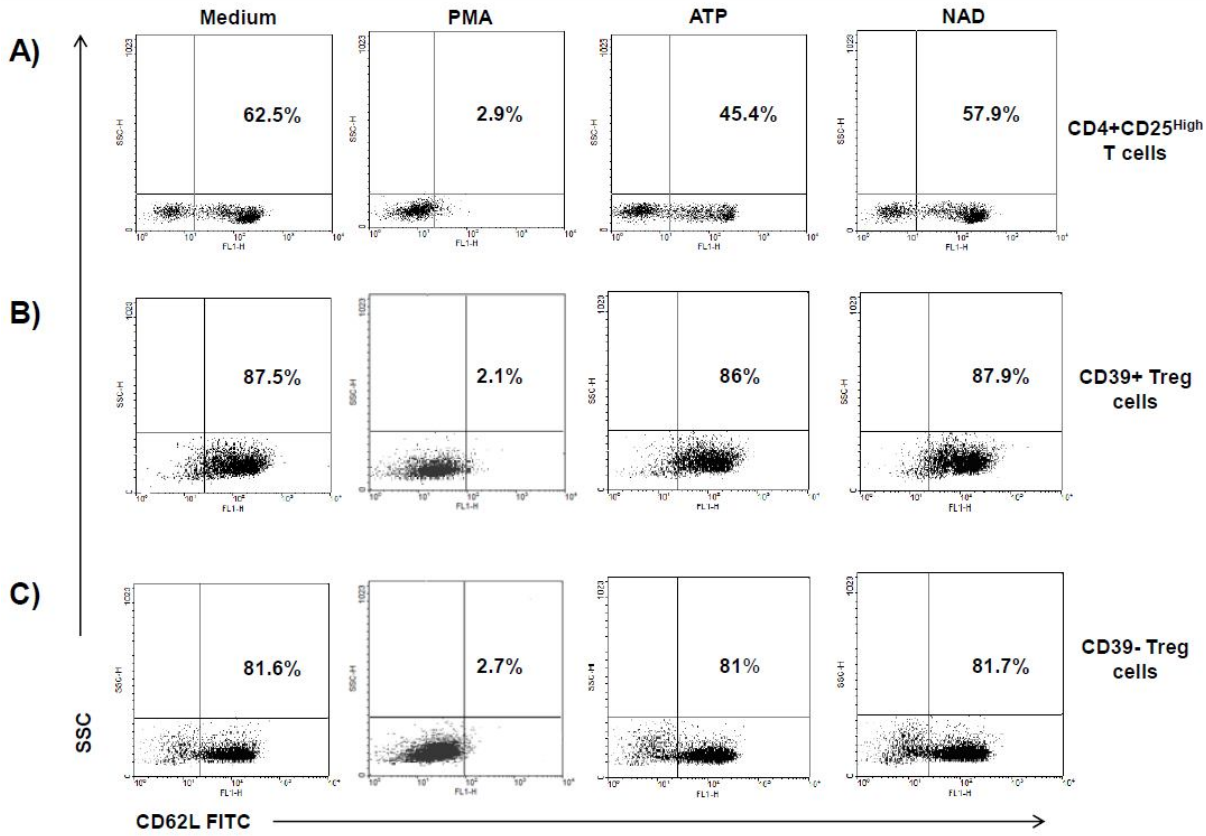


Figure 3

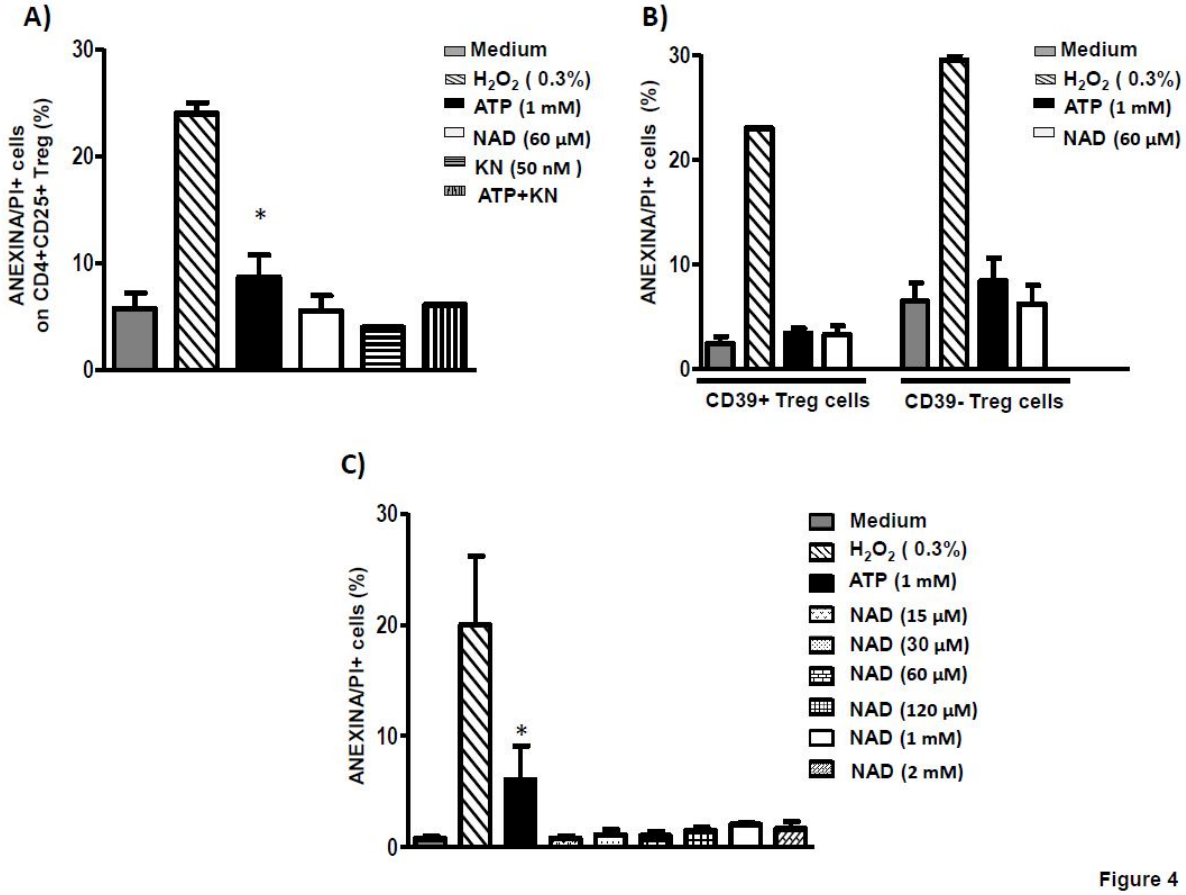
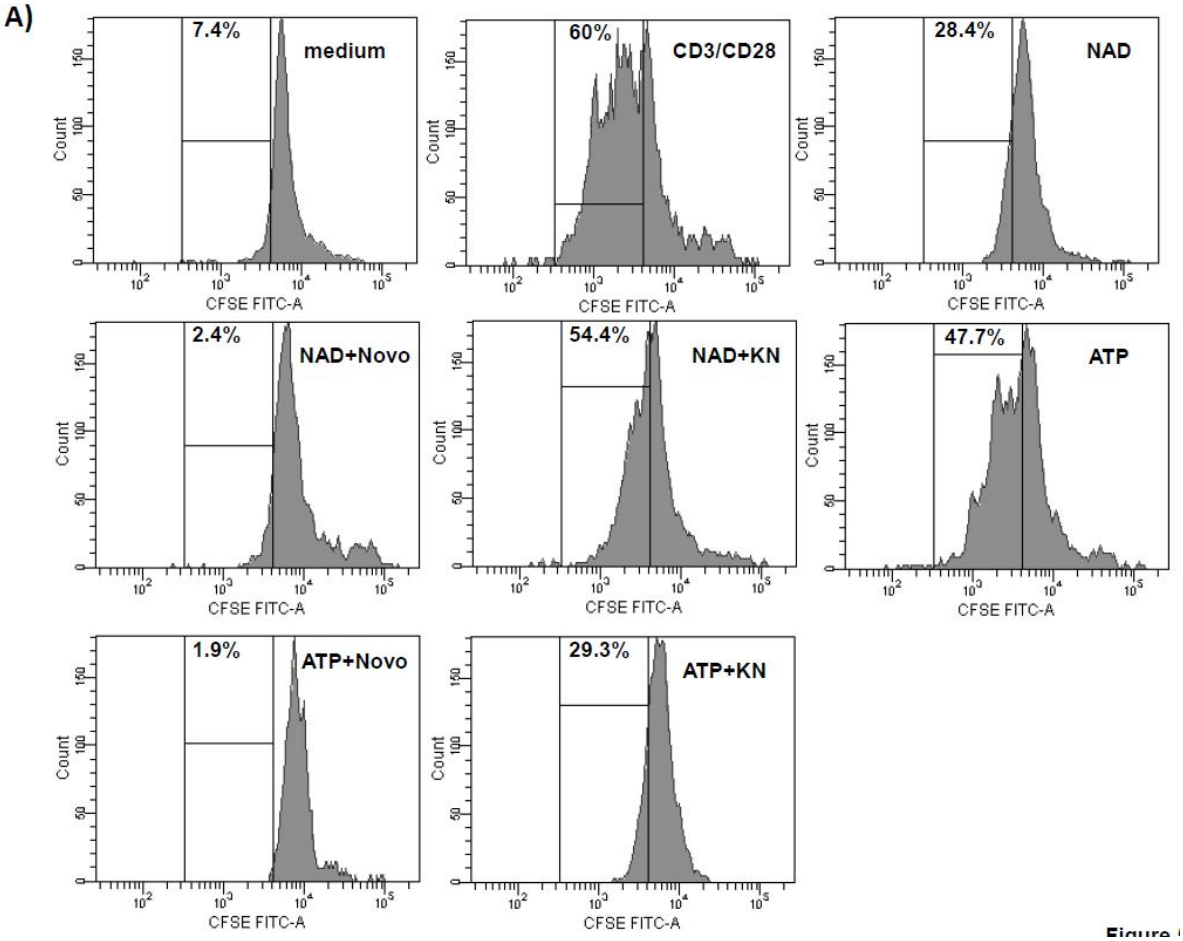
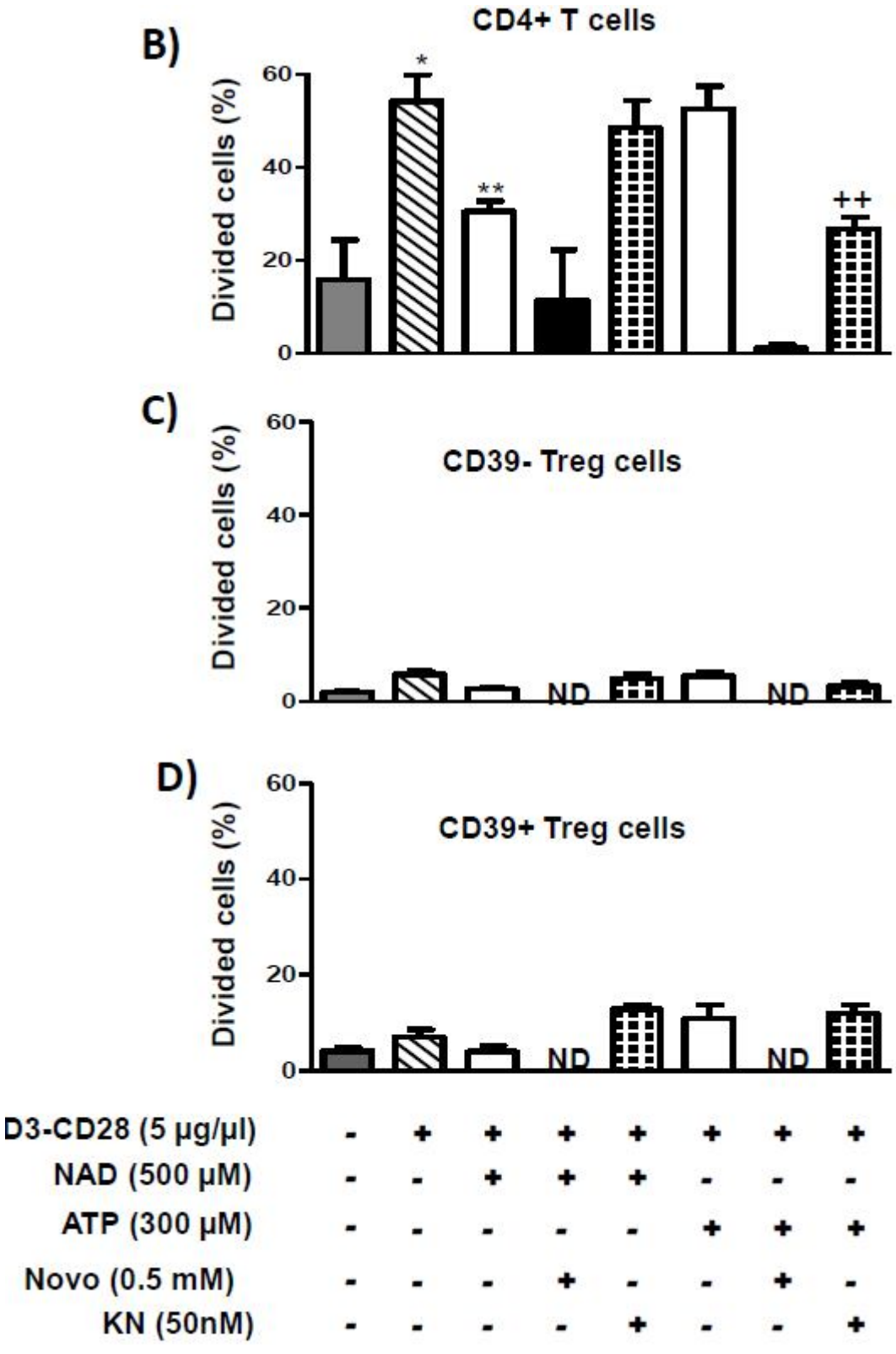


Figure 4



Figure



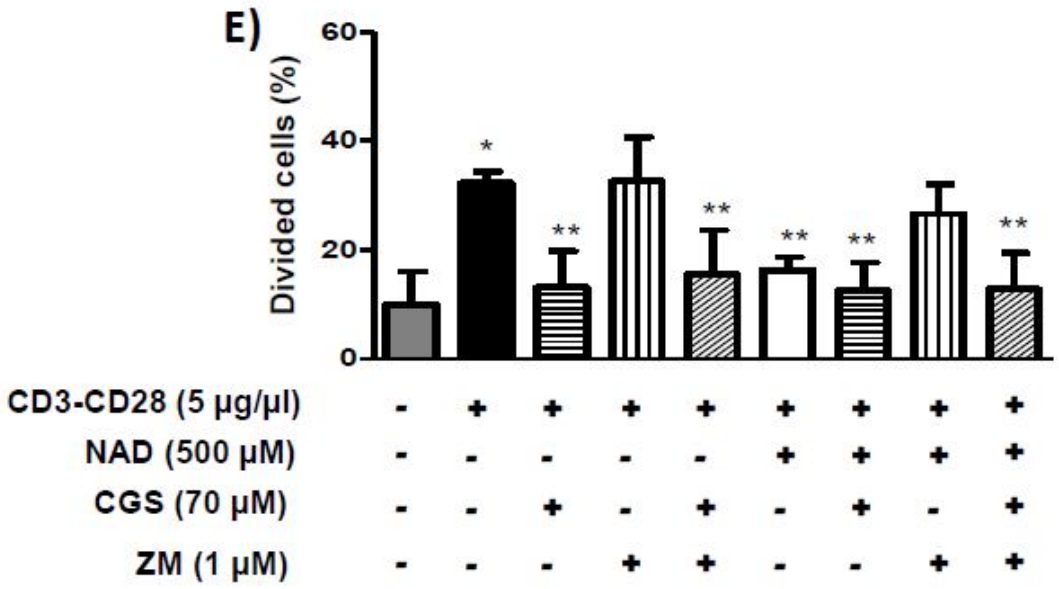


Fig. 5