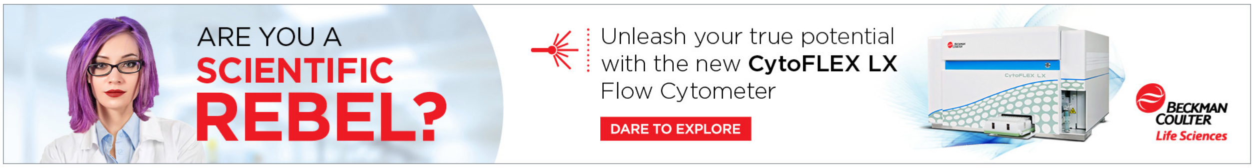

The Journal of Immunology

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Extracellular ATP Exerts Opposite Effects on Activated and Regulatory CD4$^+$ T Cells via Purinergic P2 Receptor Activation

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It has been reported that ATP inhibits or stimulates lymphoid cell proliferation depending on the cellular subset analyzed. In this study, we show that ATP exerts strikingly opposite effects on anti-CD3/CD28–activated and regulatory CD4$^+$ T cells (T$_{regs}$), based on nucleotide concentration. We demonstrate that physiological concentrations of extracellular ATP (1–50 nM) do not affect activated CD4$^+$ T cells and T$_{regs}$. Conversely, higher ATP concentrations have a bimodal effect on activated CD4$^+$ T cells. Whereas 250 nM ATP stimulates proliferation, cytokine release, expression of adhesion molecules, and adhesion, 1 mM ATP induces apoptosis and inhibits activated CD4$^+$ T cell functions. The expression analysis and pharmacological profile of purinergic P2 receptors for extracellular nucleotides suggest that activated CD4$^+$ T cells are induced to apoptosis via the upregulation and engagement of P2X7R and P2X4R. On the contrary, 1 mM ATP enhances proliferation, adhesion, migration, via P2Y2R activation, and immunosuppressive ability of T$_{regs}$. Similar results were obtained when activated CD4$^+$ T cells and T$_{regs}$ were exposed to ATP released by necrotized leukemic cells. Taken together, our results show that different concentrations of extracellular ATP modulate CD4$^+$ T cells according to their activated/regulatory status. Because extracellular ATP concentration highly increases in fast-growing tumors or hyperinflamed tissues, the manipulation of purinergic signaling might represent a new therapeutic target to shift the balance between activated CD4$^+$ T cells and T$_{regs}$. ***The Journal of Immunology*****, 2012, 189: 1303–1310.**

Adenosine triphosphate (ATP) is an important mediator of cell-to-cell communication (1, 2). High concentrations (5–10 mM) of ATP are present in the cellular cytoplasm, whereas low concentrations (1–10 nM) can be found in the extracellular milieu (3). In physiologic conditions, a small amount of ATP is released by regulated exocytosis (4). Under pathologic conditions, a large amount of ATP is released by dying cells upon tissue injury, inflammatory reactions, hyperreactivity, and tumor cell growth (5, 6). Purinergic signaling has been shown to play a pivotal role in allergen-driven lung inflammation (7, 8), in inducing laryngeal AHR (9), in T cell-mediated inflammation in experimental models of type 1 diabetes and inflammatory bowel disease (4), in rheumatoid arthritis and multiple sclerosis (10), and in graft-versus-host disease (11). It has been proposed that extracellular ATP, via activation of purinergic P2 receptors (P2Rs), is an important regulator of inflammatory and immune response (12, 13) by modulating B cells (14), monocytes (MO)/macrophages (15), eosinophils (16), and dendritic cells (DCs) (17, 18). More recently, extracellular nucleotides have been shown capable to modulate mesenchymal as well as normal and leukemic hematopoietic stem cell functions (19–22). However, currently, a coherent picture of ATP-mediated responses of T cells is not available, because of the contrasting results obtained in different studies (23–26). Noteworthy, ATP can be released both from T cells in response to physiologic stimuli and from damaged/stressed or dying tumor cells. Moreover, ATP affects T cell functions via both an autocrine and a paracrine mechanism of action. Therefore, the role of purinergic signaling on T cell activity needs to be further investigated (27).

Two classes of P2Rs have been identified, namely, P2XRs and P2YRs (28). P2XRs are ligand-gated ion channels, whereas P2YRs are seven-membrane–spanning receptors coupled to G proteins (29). Functional P2XRs and P2YRs are expressed in both mouse and human T cells (30, 31).

In this study, we hypothesized that specific CD4$^+$ T cell responses to ATP may depend on two critical factors: nucleotide concentration and CD4$^+$ T cell activated/regulatory status. To this end, we assessed the effects of different exogenous concentrations

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S.T. designed and performed the experiments, analyzed the data, and wrote the paper. D.O., S.G., and V.S. performed cell sorting and cell culture, assessed purinergic P2 receptor expression, and analyzed the data. R.d.P.V. and M.I. analyzed the data and critically reviewed the manuscript. F.D.V. and D.F. performed Ca^{2+} concentration evaluation, analyzed the data, and wrote the paper. R.M.L. and A.C. designed the research, wrote the paper, and gave the final approval for submission of the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BzATP, 2′- and 3′-*O*-(4-benzoylbenzoyl) ATP; [Ca^{2+}]$_i$, intracellular Ca^{2+} concentration; DC, dendritic cell; FN, fibronectin; MO, monocyte; P2R, purinergic P2 receptor; RT, room temperature; T$_{reg}$, regulatory CD4$^+$ T cell.

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of ATP on activated and regulatory CD4$^+$ T cells (T$_{regs}$). In this study, we demonstrate that extracellular ATP is a pivotal player in driving CD4$^+$ T cell activity, thus reinforcing the concept that microenvironment factors modulate the activation of the immune system.

Materials and Methods

Isolation and activation of CD4$^+$ T cells

PBMCs were isolated from buffy coats of healthy donors by Ficoll-Hypaque (Amersham Bioscience, Piscataway, NJ) gradient. CD4$^+$ T cells were purified using MACS$^+$ selection (Miltenyi Biotec, Bergisch Gladbach, Germany). CD4$^+$ T cells were cultured in RPMI 1640 medium (Lonza, Milan, Italy) supplemented with 10% heat-inactivated FBS (Life Technologies-Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (MP Biomedicals, Verona, Italy) at 37°C in 5% CO$_2$. CD4$^+$ T cells were activated for 3 d in flat-bottom 96-well plates precoated with anti-CD3 mAb (2 μg/ml, clone UCHT1; BioLegend, San Diego, CA) in presence of soluble anti-CD28 mAb (1 μg/ml, clone CD28.2; BioLegend).

Purification of T$_{regs}$

After 2 d of activation with anti-CD3 and anti-CD28 mAbs, T$_{regs}$ were purified by using the CD4$^+$CD25$^+$CD127$^{dim/-}$ Regulatory T-Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. T$_{regs}$ were also purified from PBMCs by using the CD4$^+$CD25$^+$ Regulatory T-Cell Isolation Kit (Miltenyi Biotec) and sorted for the presence of CD45RA (clone HI100) or CD45RO (clone UCHL1; BioLegend), by using FACSAria (BD, Franklin Lakes, NJ).

Real-time quantitative PCR

P2R relative quantification was calculated with the ΔCt comparative method, and GAPDH was used as the endogenous reference gene. GAPDH was used as internal control also in activated CD4$^+$ T cells because its mRNA level remains unchanged compared with unstimulated CD4$^+$ T cells (32). CD4$^+$ T cells were used as standard sample. Assay IDs are as follows: P2X$_1$, Hs00175686_m1; P2X$_2$, Hs00247255_m1; P2X$_3$, Hs00175689_m1; P2X$_4$, Hs00602442_m1; P2X$_5$, Hs00531938_m1; P2X$_6$, Hs01003997_m1; P2X$_7$, Hs00175721_m1; P2Y$_1$, Hs00704965_m1; P2Y$_2$, Hs01938383_s1; P2Y$_4$, Hs00267404_s1; P2Y$_6$, Hs00602548_m1; P2Y$_{11}$, Hs01038858_m1; P2Y$_{12}$, Hs00375457_m1; P2Y$_{13}$, Hs00256749_s1; P2Y$_{14}$, Hs00208434_m1; GAPDH (20×) probe dye FAM-MGB 4333764F.

Proliferation assay

Because upon T cell activation T cells change cellular energy metabolism (33), we first compared the proliferation assay based on DNA synthesis ([^{3}H]thymidine; Amersham Pharmacia Biotech, Piscataway, NJ) with the assay based on cellular energy metabolism (Proliferation CellTiter 96 Aqueous One Solution reagent; Promega Italia, Milan, Italy), and we found no differences when activated CD4$^+$ T cells were assayed (Supplemental Fig. 1). Then activated CD4$^+$ T cells or T$_{regs}$ were incubated: 1) for 72 h with increasing concentrations of ATP (1 nM, 50 nM, 250 nM, 1 μM, 50 μM, 250 μM, 1 mM) with or without 1 U/ml functional or heat-denatured (56°C for 30 min) apyrase (Sigma-Aldrich, St. Louis, MO); and 2) for 30 min, 1, 4, 16, or 72 h with 250 nM or 1 mM of ATP. Cells were also incubated with an inhibitor of CD39, ARL67156 (100 μM; Sigma-Aldrich). Cells cultured for <72 h were washed and transferred to complete RPMI 1640 medium for the remaining time to equalize the culture time. Proliferation CellTiter 96 Aqueous One Solution reagent was added for 2 h. OD was measured by an ELISA plate reader (Multiskan Ex; M-Medical, Milan, Italy) at a wavelength of 492 nm. Each variant group was performed in triplicate wells. Data are shown as fold increase compared with basal conditions (cells cultured in RPMI 1640 without ATP).

Cell death assay

Activated CD4$^+$ T cells were cultured for 30 min, 1, 4, 16, or 72 h at 37°C in presence or absence of 1 mM ATP. The culture time was equalized. Cells were also preincubated for 15 min with 45 nM KN-62 (34) or 1.5 μM 5-BDBD (35) (Tocris Bioscience, Ellisville, MO). Cells were suspended in Annexin V binding buffer (Sigma-Aldrich), stained with FITC-conjugated Annexin V and propidium iodide for 10 min at room temperature (RT), and analyzed using BD FACSCantoII equipment (BD). Data are shown as fold increase compared with basal conditions (cells cultured in RPMI 1640 without ATP).

Immunophenotype studies

After 48 h of incubation with ATP, for cell-surface staining, cells were incubated in the dark for 20 min at RT with the following panel of PE-conjugated or FITC-conjugated mAbs: anti-human CD25 (clone BC96), anti-human CD39 (clone eBioA1; eBioscience, San Diego, CA), anti-human CD73 (clone AD2), anti-human CD62L (clone Dreg 56), anti-human CD49d (clone L25), anti-human CD127 (clone hIL-7R–M21; BD), anti-human CD11a (clone HI111), anti-human CD29 (clone TS2/16), and anti-human CD54 (clone HCD54; BioLegend). For Foxp3 intracellular staining, cells were incubated at RT in the dark for 20 min with fix/perm buffer followed by 15 min with perm solution and additional 30 min with anti-human Foxp3 (clone 206D; BioLegend). Cells were analyzed using BD FACSCantoII equipment (BD). A minimum of 10,000 events was collected in list mode on FACSDiva software.

Migration assay

Cell migration was measured using a 5-μm pore polycarbonate filter in 24-well transwell chambers (Corning Costar, Cambridge, MA). In brief, 1 × 10^5 CD4$^+$ T cells were added to the upper chamber. A total of 250 nM or 1 mM ATP was added to the bottom chamber to evaluate their chemotactic activity. In the bottom chamber, 100 nM INS45973 (36) (Inspire Pharmaceutical, Durham, NC) and 5 μM MSR 2768 (Tocris Bioscience) also were used. After an overnight incubation at 37°C, transmigrated cells were recovered and counted. Data are shown as fold increase compared with basal conditions (RPMI 1640 medium without ATP in the bottom).

Cytokine production

CD4$^+$ T cells were incubated with or without ATP (250 nM or 1 mM) for 48 h. The simultaneous measurement of the release of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, TNF-α, and TNF-β was performed by using the FlowCytomix Human Th1/Th2 Sample Kit (Bender MedSystem, Vienna, Austria), according to the manufacturer's instructions. The release of TGF-β1 was measured by the Human TGF-β1 ELISA Kit (DRG Diagnostics, Marburg, Germany), according to the manufacturer's instructions.

Adhesion experiment

Fibronectin (FN; 5 μg/cm^2; Sigma-Aldrich) or autologous MO (10^5 cells/cm^2) were used to coat 24-well plates. Control plates were coated with PBS-1% BSA. CD4$^+$ T cells (2 × 10^5), pretreated or not with ARL67156 (100 μM), were cultured with or without ATP. After 48-h incubation, cells were washed and plated. After 1 h, nonadherent cells were harvested. Adherent cells were collected by vigorous pipetting after 10 min of incubation with trypsin. Adherent cells were stained with anti-CD4 PE mAb and counted using BD FACSCantoII equipment (BD). A fixed number of 5000 flow-count fluorospheres (Beckman Coulter, Fullerton, CA) was collected in list mode on FACSDiva software. Adhesion on BSA-coated control wells was subtracted from FN or MO-coated wells.

In vitro suppression assay

Purified CD4$^+$CD25$^+$CD127$^-$ cells (T$_{regs}$) were tested in an in vitro suppression assay as previously described (37). T$_{regs}$ were precultured for 24 h with or without 1 mM ATP, irradiated, and added (10^4/well) to cultures consisting of the same donor-derived CFSE-labeled CD3$^+$ T cells (1 × 10^5 /well) and allogeneic irradiated PBMCs (1 × 10^4/well). After 5 d, cultures were analyzed using BD FACSCantoII equipment (BD). For the second set of experiments, T$_{regs}$ were added to the upper chamber of a 0.4-μm pore polycarbonate filter in 24-well transwell chambers (Corning Costar). Data are shown as percentage of inhibition of CD3$^+$ T cell proliferation.

Necrotization of leukemic cells and ATP evaluation

Bone marrow cells were harvested from patients suffering from acute myeloid leukemia at diagnosis. Acute myeloid leukemia cells were resuspended in complete medium at 5 × 10^7 or 1.5 × 10^8 cells/ml. The lysates were obtained by one cycle of freezing/thawing (−80° to +37°C) and passed throughout an insulin syringe. ATP concentration of the supernatants was determined by the ENLITEN rLuciferase/Luciferin Reagent (Promega) according to the manufacturer's instructions.

Data presentation and statistical analysis

Results are expressed as the mean ± SEM of five independent experiments. Statistical significance was assessed by the Student *t* test with $*p < 0.05$ or $**p < 0.01$.

Results

ATP affects T cell proliferation and death

Preliminary experiments demonstrated that activated T cells and T$_{\text{regs}}$ express functional P2Rs, although at a lower level as compared with steady-state unstimulated CD4$^+$ T cells (data not shown). In addition, we found that P2R expression was modulated after exposure to ATP (data not shown). As proof of P2R functionality, intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) changes were measured in response to ATP and UTP. ATP induced a rapid and sustained [Ca^{2+}]$_i$ increase in T$_{\text{regs}}$ and in activated CD4$^+$ T cells (Supplemental Fig. 2Ai). Cell incubation in a Ca^{2+}-free saline solution decreased basal cytosolic Ca^{2+} concentration in both activated CD4$^+$ T cells and T$_{\text{regs}}$, and ATP did not induce any appreciable [Ca^{2+}]$_i$ signal (Supplemental Fig. 2Aii), indicating that the majority of Ca^{2+} ions contributing to the [Ca^{2+}]$_i$ increase derived from influx through the P2X channels and not from Ca^{2+} released from the intracellular stores (P2YR-mediated response). Indeed, P2XRs are ligand-activated Ca^{2+} channels, as their activation by extracellular ATP or 2′- and 3′-*O*-(4-benzoylbenzoyl) ATP (BzATP) induces Ca^{2+} influx through the plasma membrane (5). It has previously been shown that the P2X inhibitor oxidized ATP blocks Ca^{2+} elevation induced by BzATP in circulating T cells (24). Of note, UTP, which does not activate P2XRs, was inactive on both CD4$^+$ T cell populations tested (Supplemental Fig. 2Bi, 2Bii).

We then investigated the biological effects of ATP on CD4$^+$ T cells. As shown in Fig. 1A, dose–response curves for the nucleotide indicated three levels of concentrations at which CD4$^+$ T cell proliferation was differently modulated: 1) low physiological extracellular concentration (between 1 and 50 nM), 2) an intermediate ATP concentration (250 nM), and 3) the high nucleotide concentration (1 mM). At low concentrations, neither activated CD4$^+$ T cells nor T$_{\text{regs}}$ proliferate in response to ATP stimulation. The intermediate concentration (i.e., 250 nM) increased the proliferation of activated CD4$^+$ T cells, whereas 1 mM ATP caused a mean 54% decrease of activated CD4$^+$ T cell proliferation, associated with the induction of apoptosis at 72 h (Fig. 1B). We then tested whether the induction of apoptosis by 1 mM ATP was mediated by P2X7R and/or P2X4R subtypes, which are receptors mediating programmed cell death in other cellular populations (34, 38). Activated CD4$^+$ T cells upregulated P2X4R and P2X7R subtypes when exposed to 1 mM extracellular ATP (Fig. 1C). Therefore, KN-62 and 5-BDBD, two selective antagonists of the P2X7R (34) and P2X4R (35), respectively, were added to cell cultures. As shown in Fig. 1D, the addition of KN-62 and 5-BDBD completely restored the viability of activated CD4$^+$ T cells in the presence of 1 mM ATP, strongly suggesting the involvement of these subtypes in ATP-mediated cell death at high concentration. Because P2X7R is the receptor mainly involved in ATP-mediated apoptosis (34, 38, 39), we confirmed its role in mediating activated CD4$^+$ T cell apoptosis also by using the P2X7-specific siRNA (Supplemental Fig. 3). By contrast, 1 mM extracellular ATP strongly increased T$_{\text{reg}}$ proliferation (Fig. 1A) and did not enhance either T$_{\text{reg}}$ apoptosis (Fig. 1B) or P2X7 and/or P2X4 expression (Fig. 1C).

To assess whether the increase in the proliferation rate of both activated CD4$^+$ T cells and T$_{\text{regs}}$ was due to the presence of the nucleotide in its triphosphate form, we performed the proliferation test by incubating cells with the ATP hydrolyzing enzyme apyrase, which converts ATP to AMP, and with ARL67156, an inhibitor of the ectonucleoside triphosphate diphosphohydrolase 1 CD39, which is strongly expressed on T$_{\text{regs}}$ (40). As shown in Fig. 1E, apyrase counteracted the proliferative activity of ATP, which was restored when apyrase was heat denatured. Moreover, by inhibiting ATP degradation (by inhibiting CD39), T$_{\text{reg}}$ proliferation further increased, thus reinforcing the concept that ATP itself, and not its metabolites, exerts immunomodulatory activity. We also found that 1-h exposure to the appropriate concentrations of ATP (i.e., 250 nM and 1 mM for activated CD4$^+$ T cells and T$_{\text{regs}}$, respectively) was sufficient to increase proliferation of both activated and regulatory T cells (Fig. 1F).

Taken together, our data show that 1 h-exposure to specific concentrations of the triphosphate form of adenosine was able to modulate the proliferation of activated CD4$^+$ T cells and T$_{\text{regs}}$, whereas a 72 h-incubation with ATP was required (see Fig. 1B) to induce cell death in activated CD4$^+$ T cells. The process was likely due to the triggering of P2X7R and/or P2X4R subtypes.

ATP concentrations of 250 nM and 1 mM also modulate resting T cell proliferation and death

Resting CD4$^+$ T cells were freshly purified with magnetic beads directly from PBMCs of healthy donors and sorted for CD45RA or CD45RO expression. We found that 1 mM ATP significantly enhanced both resting CD4$^+$ T cell apoptosis and proliferation, whereas 250 nM ATP significantly enhanced T cell apoptosis, but decreased proliferation (Fig. 2A). To understand how the same concentration (1 mM) acted by stimulator of both apoptosis and proliferation, we performed these assays on purified resting CD45RA$^+$ or CD45RO$^+$ CD4$^+$ T cells. We found that 250 nM and 1 mM ATP enhanced only CD4$^+$CD45RO$^+$ T cell apoptosis and had no effect on CD4$^+$CD45RA$^+$ cells, whereas the same nucleotide concentrations enhanced only CD4$^+$CD45RA$^+$ T cell proliferation and had no effect on CD4$^+$CD45RO$^+$ cells (Fig. 2B, 2C).

Therefore, taken together, these data suggest that the effects observed on resting CD4$^+$ T cell population depend on the cell population composition (i.e., the balance between CD45RO$^+$ and CD45RA$^+$ cells), which differs from sample to sample.

Intermediate ATP concentrations modulate activated CD4$^+$ T cell functions

Stimulation of activated CD4$^+$ T cells with the same nucleotide concentration that induced proliferation (250 nM) also increased the secretion of IL-2. No effect was observed on the secretion of IL-1β, IFN-γ, IL-8, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, TNF-β, and TGF-β (data not shown). Of note, when we assessed cytokine production in ATP-treated and control cultures containing the same number of cells (i.e., after adjustment of the cell number according to ATP-induced cell proliferation), we still found a significant increase of secreted IL-2 (Fig. 3A). Thus, the effect of ATP on cytokine secretion seems to be specific and not caused by the mere increase of the number of activated CD4$^+$ T cells.

We then tested whether 250 nM ATP modified the expression of activated CD4$^+$ T cell membrane-associated molecules. As a consequence of ATP treatment, we found a higher expression of Ags involved in cell–matrix and cell–cell adhesion. Specifically, more activated CD4$^+$ T cells expressed CD49d and CD54 (Fig. 3B), whereas, on the contrary, ATP did not modulate CD25, CD127, CD39, CD73, CD11a, CD29, CD62L, and Foxp3 expression (data not shown). Functionally, the enhancement of CD49d and CD54 expression resulted in the increased capacity of CD4$^+$ T cells to adhere to the extracellular matrix (i.e., FN) or to autologous MO (Fig. 3C). Adhesion assay in presence of ARL67156 suggests that the enhancement of adhesion capacity was due to ATP itself. Extracellular ATP did not affect the motility of activated CD4$^+$ T cells (data not shown).

In summary, during CD4$^+$ T cell stimulation by anti-CD3 and anti-CD28 mAbs, the presence of 250 nM ATP contributed to their

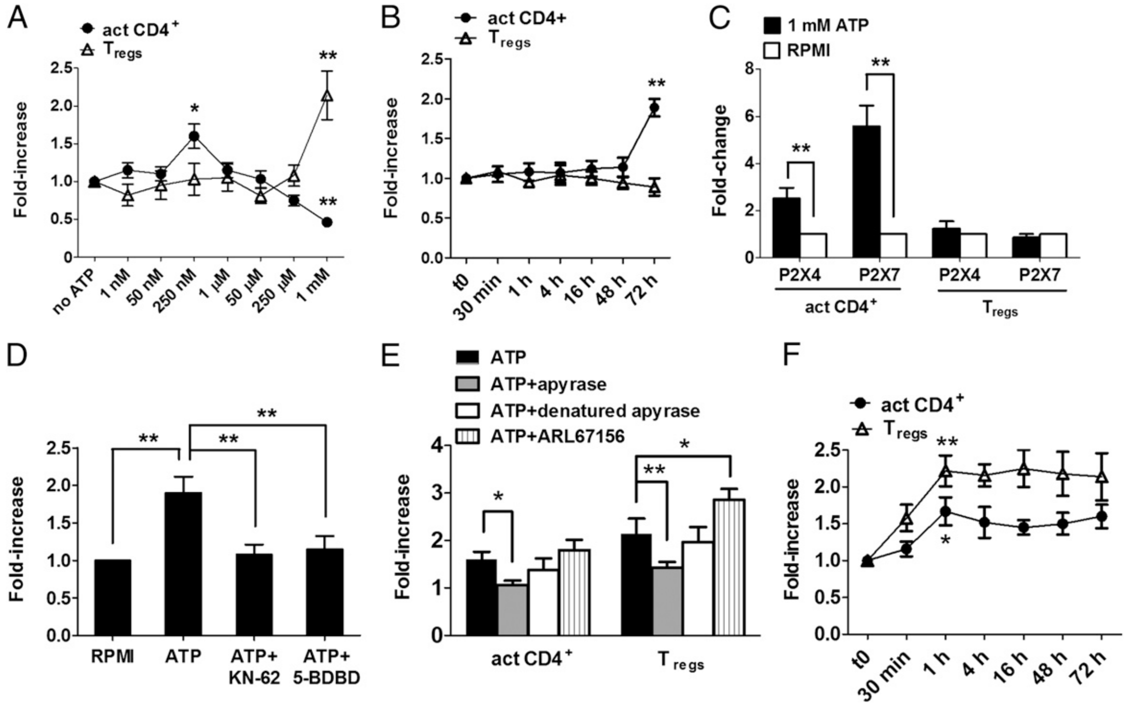


FIGURE 1. Proliferation and apoptosis of activated and T_{regs} exposed to ATP. (**A**) Anti-CD3/CD28 activated CD4$^+$ T cells and T_{regs} were tested for proliferation in response to increasing concentrations of ATP. The results are expressed as fold increase over activated CD4$^+$ T cells and T_{regs} cultured without ATP, used as control samples (CTR; no ATP). Number of CTR cells: activated CD4$^+$ T cells = 798,781 ± 116,250; T_{regs} = 66,783 ± 15,332. (**B**) CD4$^+$ T cells were tested for apoptosis, in response to 1 mM ATP. Apoptosis was evaluated at 30 min, 1, 4, 16, 48, and 72 h (activated CD4$^+$ T cell: t_0 = 14.4 ± 2.4%; T_{regs}: t_0 = 23.7 ± 4.5%). Results are expressed as fold increase between the percentage of apoptotic cells at each time point and the percentage of apoptotic cells cultured without ATP (t_0). (**C**) Comparison of P2X4R and P2X7R mRNA expression in activated CD4$^+$ T cells and T_{regs} cultured for 48 h with or without 1 mM ATP. Results are expressed as fold change comparing ATP-treated samples with CTR. (**D**) Activated CD4$^+$ T cell apoptosis induced by 72 h of 1-mM ATP exposure was tested after T cell pretreatment with KN-62 (45 nM), 5-BDBD (1.5 μM), P2X7 siRNA (200 nM), or CTR siRNA (200 nM). As CTR, cells cultured in RPMI 1640 medium alone were used (13.8 ± 2.6%). (**E** and **F**) Activated CD4$^+$ T cells and T_{regs} were cultured with 250 nM or 1 mM ATP, respectively, and tested for proliferation (E) in presence of apyrase, heat-denatured apyrase (1 U/ml, added to cell culture 30 min before ATP addition), and ARL67156 (100 μM, added to cell culture 30 min before ATP addition), and (F) after an ATP stimulation of 30 min, 1, 4, 16, 48, or 72 h (t_0 = number of activated CD4$^+$ T cells [754,612 ± 98,892] and T_{regs} [74,223 ± 11,672]). Cells cultured for <72 h were washed and transferred to complete RPMI 1640 medium for the remaining time to equalize the culture time. Data are expressed as fold increase comparing ATP-treated samples with CTR. Results represent the mean ± SEM of five independent experiments. $*p < 0.05$, $**p < 0.01$.

activation by inducing responses such as proliferation, secretion of critical cytokines such as IL-2, and adhesion to extracellular matrix and/or MO. T_{reg} functions were not modulated by 250 nM ATP.

High ATP concentrations "turn off" activated CD4$^+$ T cell functions

In contrast with 250 nM ATP, 1 mM ATP inhibited proliferation of activated CD4$^+$ T cells and induced activated CD4$^+$ T cell death (see Fig. 1A, 1B). Moreover, incubation with 1 mM ATP reduced the secretion of some proinflammatory cytokines such as IFN-γ and TNF-α, whereas ATP had no effect on IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-β, TGF-β, IL-2, and IL-8 release (data not shown). Noteworthy, we found a significant decrease of IFN-γ and TNF-α even after adjusting values to the same number of CD4$^+$ T cells in ATP-treated and control cultures (Fig. 4A). Thus, again, we ruled out that changes in cytokines concentration were merely due to the decreased cell number after ATP treatment. In addition, 1 mM ATP modulated the phenotype of activated CD4$^+$ T cells. Indeed, whereas no changes were seen in CD127, CD39, CD73, CD11a, CD29, CD62L, and Foxp3 expression (data not shown), ATP strongly reduced the expression of activation markers and adhesion molecules (Fig. 4B), such as CD25, CD49d, and CD54.

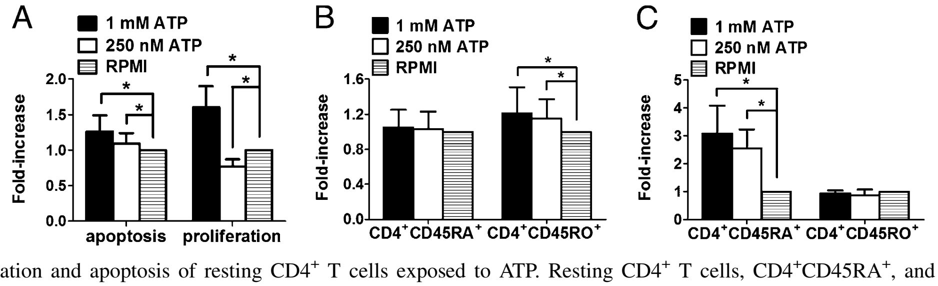


FIGURE 2. Proliferation and apoptosis of resting CD4$^+$ T cells exposed to ATP. Resting CD4$^+$ T cells, CD4$^+$CD45RA$^+$, and CD4$^+$CD45RO$^+$ were cultured in RPMI 1640 medium with or without 1 mM or 250 nM ATP. Results are expressed as fold increase comparing ATP-treated samples with control samples (RPMI). (**A**) Resting CD4$^+$ T cells were tested for apoptosis (RPMI = 24.6 ± 2.4%) and proliferation (RPMI = 174,800 ± 45,342 cells). (**B**) CD4$^+$ CD45RA$^+$ and CD4$^+$CD45RO$^+$ were tested for apoptosis (RPMI = 7.8 ± 3.2% and 14.4 ± 5.2%, respectively) and (**C**) proliferation (RPMI = 198,230 ± 23,640 and 162,300 ± 31,400 cells, respectively). Data are expressed as the mean ± SEM of three independent experiments. $*p < 0.05$.

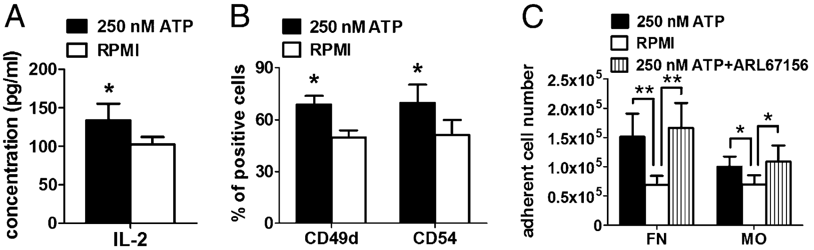


FIGURE 3. Supraphysiological concentrations of ATP affect the functions of activated $CD4^+$ T cells. Activated $CD4^+$ T cells were cultured in RPMI 1640 medium with or without 250 nM ATP. (**A**) IL-2 release, (**B**) expression of CD49d or CD54 on cell surface, and (**C**) adhesion to FN or to autologous MO was tested. Adhesion was performed also in presence of ARL67156 (100 μM). Data are expressed as the mean ± SEM of five independent experiments. $*p < 0.05$, $**p < 0.01$.

Accordingly, at the functional level, the reduction in CD49d and CD54 expression correlated with a reduced capacity of cells to adhere to FN and to autologous MO (Fig. 4C). Adhesion assay in presence of ARL67156 suggested again that the decrease of adhesion capacity of activated $CD4^+$ T cells was due to ATP itself. High ATP concentrations did not affect the motility of activated $CD4^+$ T cells (data not shown).

In summary, during $CD4^+$ T cell stimulation by anti-CD3 and anti-CD28 mAbs, high ATP concentrations inhibited their activation by enhancing apoptosis and diminishing proliferation, cell adhesion, and release of critical proinflammatory cytokines.

High ATP concentrations "turn on" T_{regs}

After demonstrating that 1 mM ATP enhanced T_{regs} proliferation (see Fig. 1A), we investigated the effects of the nucleotide on naive T_{regs}. To this end, peripheral blood circulating $CD4^+CD25^+$ T cells were immunomagnetically purified first, then labeled with CD25, CD45RA, and CD45RO, and sorted to obtain $CD4^+CD25^{high}CD45RA^+$ and $CD4^+CD25^{high}CD45RO^+$ T cells. Selected cell populations were then incubated without or with extracellular ATP (1 nM, 1 μM, 1 mM) and tested for proliferation. As shown in Fig. 5A, both $CD4^+CD25^{high}CD45RA^+$ and $CD4^+CD25^{high}CD45RO^+$ T cells proliferate in presence of 1 mM ATP. On the contrary, when we assessed whether the increase of T_{regs} number was also due to the conversion of $CD4^+CD25^-Foxp3^-$ into $CD4^+CD25^+Foxp3^+$ T cells, we did not find any effect of ATP on this mechanism (data not shown). In addition, 1 mM ATP had no effect on membrane or intracellular marker expression (CD25, CD127, CD39, CD73, CD11a, CD29, CD62L, CD49d, CD54, and Foxp3, data not shown). Conversely, 1 mM ATP itself significantly modulated the adhesion capacity of T_{regs}, as they show a decreased adherence to FN and an increased adherence to autologous MO, also in presence of ARL67156 (Fig. 5B).

We then investigated whether ATP modulates the immunosuppressive ability of T_{regs}. ATP-treated T_{regs} were more effective in reducing T cell proliferation, in comparison with RPMI 1640-treated T_{regs} (Fig. 5C). Because 1 mM ATP had no effect on cytokine secretion (IL-2, IL-1β, IFN-γ, IL-8, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, TNF-β, and TGF-β, data not shown), but modulated T_{regs} adherence to other cells, we hypothesized that the enhancement in T_{regs} suppressive capacity may be related to a cell-to-cell contact-dependent mechanism. To test this hypothesis, we performed the same suppression assay by separating cells with a 0.4-μm pore transwell chamber. As shown in Fig. 5C, when added in the upper chamber of the transwell, T_{regs} precultured with 1 mM ATP had the same efficacy in inhibiting T cell proliferation as compared with T_{regs} precultured with RPMI 1640. Therefore, ATP enhanced the regulatory capacity of T_{regs} by modulating mechanism(s) involved in cell-to-cell contact-dependent suppression.

Finally, using all required controls to differentiate chemotaxis from chemokinesis, we found that 1 mM ATP exerted a chemotactic effect on T_{regs} in a transwell assay, because T_{reg} motility was enhanced only when RPMI 1640 was in the top chamber and ATP in the bottom (Fig. 5D). Because P2Y2R mediates chemotaxis in DCs, eosinophils (41), and neutrophils (42), we investigated its potential involvement in T_{reg} chemotaxis. Noteworthy, T_{regs} upregulated P2Y2R when incubated with 1 mM ATP (Fig. 5E). We performed the migration assay also using the P2Y2R and P2Y4R agonist INS45973 (36) and the P2Y2R agonist MSR 2768. As shown in Fig. 5F, the engagement of P2Y2R, with or without the P2Y4R, mimicked the full activity of ATP on T_{reg} migration.

In summary, 1 mM ATP enhanced T_{reg} proliferation, adhesion capacity to different cell types, P2YR-mediated migration, as well as their suppressive capacity by a cell-to-cell contact-dependent mechanism.

ATP released by necrotic leukemic cells stimulates T_{reg} proliferation

To test whether ATP released by dying tumor cells had the same immunomodulatory properties as extracellular ATP pharmacologically added to $CD4^+$ T cell cultures, we necrotized leukemic blasts by one cycle of freezing/thawing, and used collected supernatants to measure ATP concentration and to stimulate $CD4^+$ T cell proliferation. Fig. 6A shows that ATP concentration was lower when viable leukemic cells were tested and already increased when a small number of cells (5×10^7) died. ATP concentration further increased when a higher number of cells (1.5×10^8) was necrotized. Noteworthy, 1 mM ATP was the most effective nucleotide concentration in modulating activated $CD4^+$ T cells and T_{reg} activity. As expected, when apyrase was added,

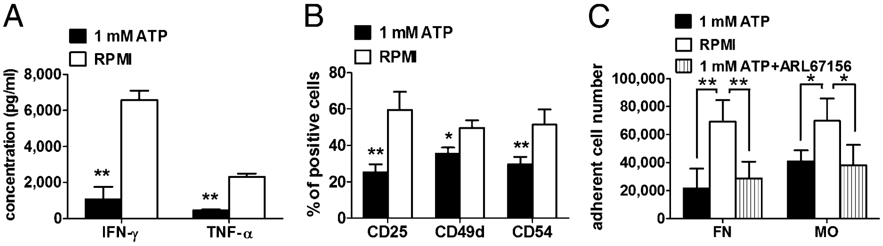


FIGURE 4. High ATP concentrations inhibit activated $CD4^+$ T cells. Activated $CD4^+$ T cells were cultured in RPMI 1640 medium with or without 1 mM ATP. (**A**) IFN-γ and TNF-α release; (**B**) expression of CD25, CD49d, and CD54 on cell surface; and (**C**) adhesion to FN or to autologous MO were tested. Adhesion was performed also in presence of ARL67156 (100 μM). Data are expressed as the mean ± SEM of five independent experiments. $*p < 0.05$, $**p < 0.01$.

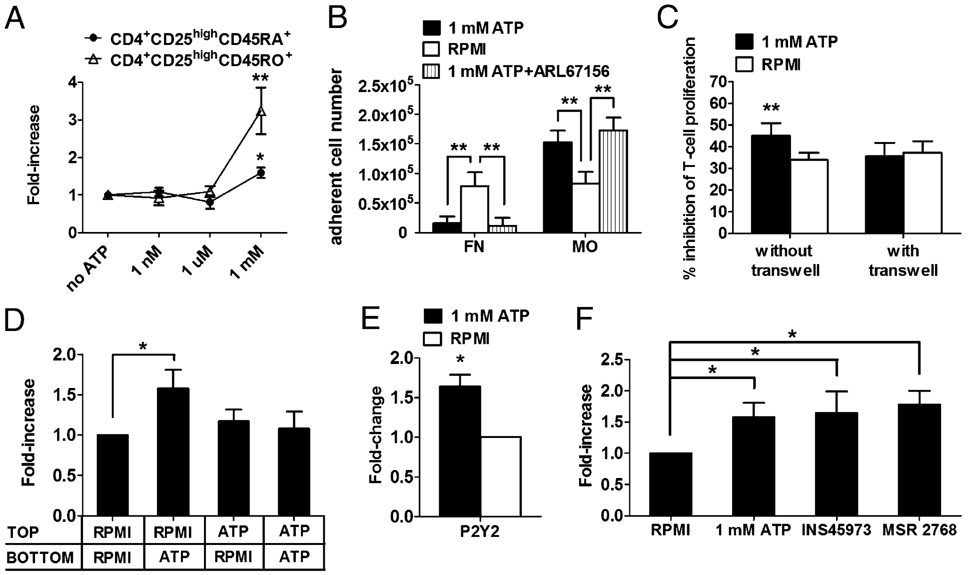


FIGURE 5. High ATP concentrations stimulate T_{regs} functions. (**A**) Circulating $CD4^+CD25^{high}CD45RA^+$ and $CD4^+CD25^{high}CD45RO^+$ were tested for proliferation in response to increasing concentrations of ATP. The results are expressed as fold increase over $CD4^+CD25^{high}CD45RA^+$ and $CD4^+CD25^{high}CD45RO^+$ cells cultured without ATP, used as control samples (CTR). Number of CTR cells: $CD4^+CD25^{high}CD45RA^+$ = 66,150 ± 12,625; $CD4^+CD25^{high}CD45RO^+$ = 60,753 ± 14,382. (**B**) T_{regs} were tested for the adhesion to FN or to autologous MO after incubation with 1 mM ATP, with or without ARL67156 (100 μM). (**C**) T_{regs} were precultured with or without 1 mM ATP and tested for the inhibition of allogeneic T cell proliferation. The test was performed with or without transwell chamber. (**D**) Cells were cultured overnight in transwell chambers with 1 mM ATP either in the bottom or the top chamber, or in both, to test the chemotaxis of T_{regs}. (**E**) Comparison of P2Y2R mRNA expression in T_{regs} cultured for 48 h with or without 1 mM ATP. (**F**) Chemotaxis of T_{regs} was evaluated using transwell assay. ATP, INS45973 (100 nM), or MSR 2768 (5 μM) was added in the bottom chamber. (D and F) As CTR, RPMI 1640 medium alone was used in the bottom chamber, and data are expressed as fold increase over RPMI 1640 (number of migrated cells: 30,195 ± 13,204). Data are expressed as the mean ± SEM of five independent experiments. $*p < 0.05$, $**p < 0.01$.

ATP concentration was strongly reduced. As shown in Fig. 6B, when activated $CD4^+$ T cells and T_{regs} were incubated with supernatants from cell cultures with a low necrotic content, proliferation of activated $CD4^+$ T cells was enhanced, whereas T_{regs} were not affected. In these cultures, the addition of apyrase abolished the stimulation of activated $CD4^+$ T cell proliferation, although it had no effects on T_{regs} proliferation. In contrast, when activated $CD4^+$ T cells and T_{regs} were incubated with supernatants from cultures with massive necrosis of leukemic cells and consequent high ATP release, activated $CD4^+$ T cells proliferation was inhibited, whereas T_{regs} proliferation was strongly enhanced. The presence of apyrase in the supernatants reduced ATP concentration to a level that stimulated proliferation of activated $CD4^+$ T cell and abrogated proliferation of T_{regs}.

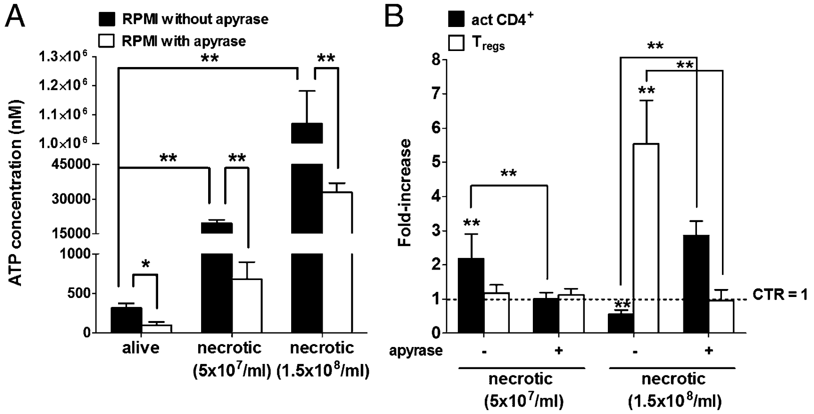
Taken together, these data indicate that necrotic tumor cells release ATP that differently modulates T cell responses, depending on nucleotide concentration and T cell activated/regulatory status.

Discussion

In this study, we investigated the effects of the "danger-signal" extracellular ATP (43) in the modulation of $CD4^+$ T cell functions. Three main findings were observed: 1) low physiological concentrations of ATP did not modulate either proliferation or cell death of activated $CD4^+$ T cells and T_{regs}, 2) intermediate concentrations of ATP contributed to the activation of $CD4^+$ T cells, and 3) high concentrations of ATP "turned off" activated $CD4^+$ T cells, whereas they "turned on" T_{regs}.

Activation of purinergic signaling by 250 nM ATP induced, in activated $CD4^+$ T cells, an enhancement in IL-2 secretion and in CD49d and CD54 expression. It is well-known that IL-2 supports the survival and proliferation of T lymphocytes (44). Therefore, the increase of IL-2 may trigger the enhancement of activated $CD4^+$ T cell proliferation. CD54, also known as ICAM-1, is normally present at low level on the membrane of leukocytes and endothelial cells. When activated, leukocytes bind to endothelial

FIGURE 6. ATP release by necrotic leukemic cells. (**A**) Evaluation of extracellular ATP concentration in alive or necrotic leukemic cells medium. Necrotic leukemic cells were cultured overnight with or without apyrase (1 U/ml). (**B**) Activated $CD4^+$ T cells and T_{regs} were cultured with the medium obtained from necrotic leukemic cells and tested for proliferation. Activated $CD4^+$ T cells and T_{regs} cultured with the medium of alive leukemic cells, with or without apyrase, were used as control sample (CTR). Number of CTR activated $CD4^+$ T cells was 39,565 ± 16,252 without apyrase, and 26,222 ± 19,351 with apyrase. Number of CTR T_{regs} was 28,889 ± 10,357 without apyrase, and 27,332 ± 11,678 with apyrase. Data are expressed as the mean ± SEM of two independent experiments. $*p < 0.05$, $**p < 0.01$.



cells via ICAM-1/LFA-1 and then transmigrate into tissues (45, 46). Our data demonstrate that the expression of CD54, sensitive to cytokine stimulation, is also modulated by specific extracellular ATP concentrations. Moreover, recent evidence shows that DCs are able to use active LFA-1 and can thereby control the contact duration with naive T cells (47). Leukocyte/endothelial interactions are mediated by adhesion molecules, chemokines, and their respective receptors. CD49d, also known as α4-integrin, mediates the G protein-independent capture and subsequent G protein-dependent adhesion of T cells to the VCAM-1. By this mechanism, under inflammatory conditions, circulating lymphocytes and MO/macrophages readily cross the endothelium and reach the inflammation site (48, 49). Because release of ATP by cells occurs as a consequence of bacterial products, viral infection, or inflammation (43, 50), it is not surprising that the nucleotide was able to modulate membrane expression of CD54 and CD49d in $CD4^+$ T cells, thus facilitating transendothelial migration of $CD4^+$ T cells into the inflamed tissue. Moreover, by enhancing the stability of $CD4^+$ T cell/DC contact during their priming under inflammatory conditions, it may favor the success of an Ag-dependent response in situ.

Conversely, stimulation with 1 mM ATP of $CD4^+$ T cells during activation caused the decrease in CD54, CD49d, and CD25, suggesting an ATP-dependent inhibition of $CD4^+$ T cell activation. In fact, under this condition, $CD4^+$ T cell priming may fail because of the instability of CD54/LFA-1 contact. Moreover, the downregulation of CD25 affects the IL-2–dependent T cell survival and proliferation. However, the survival of activated $CD4^+$ T cell is also directly influenced by ATP. High ATP concentration causes, indeed, T cell apoptosis, by upregulating and engaging the P2X4R and P2X7R subtypes. This result is supported by the finding that several cell lines undergo apoptosis through P2X7 engagement when exposed to ATP (34, 38). P2X7 drives the formation of nonselective membrane pores, causing cell death (39). Furthermore, 1 mM ATP also affects the capacity of activated $CD4^+$ T cells to sustain a proinflammatory response, by downregulating the secretion of proinflammatory cytokines, such as IFN-γ and TNF-α. These results are consistent with those published by Duhant and colleagues (51), showing that 100 μM adenosine 5′-*O*-(3-thiotriphosphate) and BzATP diminished activated $CD4^+$ T cell proliferation; secretion of IL-2, IFN-γ, IL-5, and IL-10; and expression of CD25.

Finally, T_{reg} functions are modulated by 250 nM and 1 mM extracellular ATP in an almost opposite manner in comparison with activated $CD4^+$ T cells. In fact, 250 nM extracellular ATP does not exert any modulation of T_{reg} functions, whereas 1 mM stimulates their proliferation, chemotaxis via P2Y2R activation, adhesion to MO, and immunosuppressive ability in a cell-to-cell contact-dependent manner. Thus, the number of T_{regs} is increased by 1 mM of extracellular ATP both by enhancing proliferation and by recruiting T_{regs} on site, but not by converting non-T_{regs} into T_{regs}.

Because a subset of human T_{regs} expresses CD39 and CD73 (40, 52), enzymes that catalyze the hydrolysis of ATP to AMP and of AMP to adenosine and inorganic phosphate, respectively, we investigated whether the triphosphate form of the nucleotide was responsible for the biological effects on T_{regs}, as supposed to adenosine, which is known for its immunoregulatory properties (40). Our experiments, performed in presence of exogenous apyrase, which catalyzes the hydrolysis of ATP to AMP and inorganic phosphate, or in presence of an inhibitor of CD39, demonstrate that ATP in its triphosphate form shows immunomodulatory activity by itself.

Of note, these findings were confirmed when T cell proliferation was assayed with the supernatant of necrotic leukemic cells. Thus, we propose a possible model to correlate our in vitro data with the in vivo scenario. Within a range of ATP concentrations compatible with physiological conditions (1–50 nM), $CD4^+$ T cell proliferation is unaffected. When the concentration of extracellular ATP increases after cellular/tissue necrosis (3, 5, 6), $CD4^+$ T cells receive a “danger signal.” Under this condition, $CD4^+$ T cells are activated and are ready to exert an inflammatory response. At this stage, T_{reg} intervention may not be required. As ATP concentration further increases because of increased cell/tissue necrosis, a feedback loop may occur to avoid hyperinflammation. Under this condition, activated $CD4^+$ T cells are killed and inhibited in their function by the high ATP concentration and by T_{regs}, which are recruited on site and induced to proliferate. In contrast, ATP is released by tumor cells as shown in vivo (6) and in vitro (this article), and ATP stimulatory (T_{regs})/inhibitory (activated $CD4^+$ cells) activity may represent a tumor-escape mechanism from the immune system. This scenario is consistent with the finding of high numbers of T_{regs} in the cellular milieu of advanced tumors (53, 54). The complex interplay between the inflammatory microenvironment, tumor cells, and effector cells of the immune system is currently investigated in our laboratory, in vivo, in animal models.

In summary, our study shows a dose-dependent ATP regulation of $CD4^+$ T cells according to their activated/regulatory status. This mechanism may have an important role in inflammation and tumor cell growth.

Disclosures

The authors have no financial conflicts of interest.

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