Microenvironment and Immunology

Cancer Research

Selective Depletion of CD4$^+$CD25$^+$Foxp3$^+$ Regulatory T Cells by Low-Dose Cyclophosphamide Is Explained by Reduced Intracellular ATP Levels

Jie Zhao[1], Yuchun Cao[2], Zhang Lei[3], Zhuoshun Yang[3], Biao Zhang[3], and Bo Huang[3]

Abstract

CD4$^+$CD25$^+$Foxp3$^+$ regulatory T (Treg) cells have been shown to play important roles in mediating cancer development. Although cyclophosphamide (CY) has shown promise as a drug to selectively target Treg cells with low-dose *in vivo*, the underlying molecular mechanism remains unclear. In this report, we provide evidence that ATP, the energy molecule and signal element, accounts for the selective depletion of Treg cells by low-dose CY. Relative to conventional T cells or other cell types, ATP levels were much lower in Treg cells. This was due to Treg cells that downregulate one microRNA, miR-142-3p, and upregulate ecto-nucleoside triphosphate diphosphohydrolase CD39. The transfection of miR-142-3p or the blockade of CD39 could increase intracellular ATP levels of Treg cells, consequently decreasing the sensitivity of Treg cells to low-dose CY. On the other hand, the transfection of miR-142-3p inhibitor or the addition of soluble CD39 to the cultured CD4$^+$CD25$^-$ T cells resulted in the decrease of intracellular ATP levels and increase of sensitivity of conventional T cells to low-dose CY. Furthermore, we found that the low levels of ATP attenuated the synthesis of glutathione, leading to the decrease of CY detoxification, thus increasing the sensitivity of Treg cells to low-dose CY. Therefore, we here identify a molecular pathway through which low-dose CY selectively ablates Treg cells. Our findings also imply that low levels of ATP are probably related to Treg cell function. *Cancer Res; 70(12); 4850–8. ©2010 AACR.*

Introduction

CD4$^+$CD25$^+$FoxP3$^+$ regulatory T (Treg) cells are of paramount importance to the maintenance of peripheral self-tolerance and avoidance of autoimmunity (1, 2). However, under pathologic conditions, cancers can use Treg cells for immune evasion. The number of functional Treg cells is elevated in cancer patients, leading to the observed immunosuppression (3–7). Therefore, to potentiate elimination of tumors by the immune systems, targeting Treg cells may be beneficial. Cyclophosphamide (CY) has recently shown promise as a drug to target Treg cells (8–10).

CY, an alkylating agent, is widely used in the treatment of malignancies as well as autoimmune disorders (11). However, in low doses, CY has been reported to reduce suppressor function of uncharacterized T cells (12, 13). It is now clear that low-dose CY can selectively ablate CD4$^+$CD25$^+$ Treg cells, leading to the enhancement of immune responses (14, 15). Based on this principle, low-dose CY has been successfully tested in the treatment of various types of tumor (16–18). Moreover, our observations indicated that low-dose CY can effectively prevent the recurrence of condylomata acuminata caused by human papillomavirus (HPV) by selectively depleting Treg cells.[4] These findings together indicate that low-dose CY might be an ideal agent to target Treg cells clinically. However, the molecular mechanism by which low-dose CY selectively targets Treg cells is unclear.

CY is a prodrug subject to a series of biological activation steps necessary for its cytotoxic effect (19). These include the following: (*a*) catalysis of CY to 4-hydroxycyclophosphamide by hepatic cytochrome *P*450 isozymes; (*b*) interconversion of 4-hydroxycyclophosphamide with its tautomer, aldophosphamide; (*c*) diffusion of aldophosphamide out of hepatic cells into the circulation, and subsequent uptake by other cells; and (*d*) spontaneous degradation of aldophosphamide to phosphoramide mustard and acrolein. On the other hand, multiple detoxification routes may be used by cells, including oxidation of aldophosphamide to the inactive carboxyphosphamide by aldehyde dehydrogenases (20). In addition, glutathione, the most abundant intracellular nonprotein thiol, also serves to detoxify CY. Both CY itself, phosphoramide

Authors' Affiliations: Departments of [1]Gynecology and Obstetrics, [2]Dermatology, and [3]Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, The People's Republic of China

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

J. Zhao and Y. Cao contributed equally to this work.

Corresponding Author: Bo Huang, Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, The People's Republic of China. Phone: 86-27-83608655; Fax: 86-27-83650754; E-mail: tjhuangbo@hotmail.com.

doi: 10.1158/0008-5472.CAN-10-0283

[4] Y. Cao and B. Huang, unpublished data.

mustard, and other metabolites can be detoxified by conjugation with glutathione (20, 21).

Glutathione is synthesized from glutamate, cysteine, and glycine by two successive reactions with glutamate cysteine ligase and glutathione synthetase, respectively (22). Notably, these two enzymatic reactions are dependent on ATP. Coincidently, ATP levels in Tregs have been shown to be regulated by the low expression of miR-142-3p, resulting in a high level of adenylyl cyclase 9 (23). The latter converts intracellular ATP to cyclic AMP (cAMP). In line with this, Bopp and colleagues (24) showed that naturally occurring Treg cells harbor high levels of cAMP for their suppressive function. In addition, CD39 and CD73 expressed on Treg cells catalyze extracellular ATP to adenosine (25–27), further implying a possible link between altered ATP and Treg cells. We hypothesize that Treg cells express low concentrations of ATP, leading to the decrease of cytosolic glutathione and the increase of sensitivity to low-dose CY.

Materials and Methods

Donor samples

Peripheral blood samples were taken from healthy volunteers and were approved by the Ethical Committee of the Medical Faculty of Tongji Medical College. Informed consent was obtained from all subjects.

Cell sorting

The splenocytes from naive BALB/c mice were labeled with FITC-conjugated CD3, phycoerythrin-conjugated CD4, and allophycocyanin-conjugated CD25 antibodies (eBioscience). The $CD3^+CD4^+CD25^-$ and $CD3^+CD4^+CD25^+$ cells were sorted according to our previous report (23).

In addition, human peripheral blood mononuclear cells (PBMC) were labeled with FITC-conjugated anti-human CD3, phycoerythrin-conjugated anti-human CD4, phycoerythrin-Cy5–conjugated anti-human CD127, and allophycocyanin-conjugated anti-human CD25 antibodies (eBioscience). The $CD3^+CD4^+CD25^+CD127^-$ Treg cells and $CD3^+CD4^+CD25^-CD127^+$ conventional T cells were sorted.

The sorted Treg cells used for the experiments were >95% in purity by flow cytometric analysis of intracellular Foxp3 (Supplementary Fig. S1).

Intracellular ATP detection

The isolated Treg cells or conventional T cells were cultured in the presence of 10 ng/mL interleukin 2 (IL-2) for 12 hours. Cells (2×10^4) were used to measure the concentration of intracellular ATP with the ATP Bioluminescent Somatic Cell Assay kit (Sigma) according the manufacturer's instruction. The result was expressed by mole per cell.

Treg cell induction *in vitro*

Transforming growth factor β (TGF-β) was used to induce the conversion of murine $CD4^+CD25^-$ T cells into Treg cells according to the previous report (28). Briefly, $CD4^+CD25^-$ T cells were stimulated with 0.5 μg/mL anti-CD3 in the presence of irradiated T cell–depleted splenocytes as allophycocyanins, 1 ng/mL recombinant murine IL-2, and 5 ng/mL recombinant murine TGF-β1 (PeproTech) for 7 days of culture.

For human Treg induction, purified $CD4^+CD25^-CD45RA^+$ T cells were activated with 5 μg/mL plate-bound anti-CD3, 1 μg/mL soluble anti-CD28, and 1 ng/mL IL-2 (PeproTech) in the presence of 10 nmol/L all-*trans* retinoic acid ATRA (Sigma) and 5 ng/mL recombinant human TGF-β (PeproTech) for 7 days of culture as previously described (29). ATRA was first dissolved in DMSO at 10 mmol/L and further diluted in complete medium.

In both cases, after 7 days of culture, $CD4^+CD25^+$ cells were sorted for another 12-hour culture in the presence of 1 ng/mL of IL-2 for ATP detection.

Northern blot for microRNA

Total RNA was extracted from cells with Trizol reagent (Invitrogen). RNA (30 μg) was separated on a 15% denaturing polyacrylamide gel. The RNA was then transferred to Genescreen Plus membranes (Perkin-Elmer). Biotin-labeled oligo nucleotide complementary to the mature miR-142-3p was synthesized by Signosis, Inc. The membranes were incubated with labeled probe. Prehybridization and hybridization were carried out using hybridization buffer. The most stringent wash was carried out in 2× SSC and 1% SDS at 37°C.

Transfection assay

Stability-enhanced miR-142-3p (Dharmacon), miR-142-3p inhibitor (Ambion), and the corresponding control oligonucleotides were purchased: miR-142-3p and its inhibitor. The transfection assay was performed according to our previous report (23).

Detection of cell viability

The HepG2 cell line was treated with different concentrations of CY (0, 0.2, 2, 10, and 100 μg/mL) for 6 hours. Then, the supernatants were used to culture Treg cells and conventional T cells for 24 hours. The apoptotic cells were analyzed with FITC-conjugated Annexin V (BD Biosciences) by flow cytometry.

Analysis of Foxp3 expression by reverse transcription-PCR and real-time reverse transcription-PCR

Total RNA was extracted from cells with the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The relative quantity of mRNA was determined by reverse transcription-PCR (RT-PCR; 28 cycles, One-step RT-PCR kit, Qiagen). The mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The primer sequences were as follows: mouse GAPDH, sense 5′-GTGGAGATTGTTGCCATCAACG-3′, antisense 5′-CAGTGGATGCAGGGATG ATGTTCTG-3′; mouse FoxP3 5′-CAAGCAGATCATCTCCTGGATGAG-3′, antisense 5′-GAACATGCGAGTAAACCAATGG-3′; human FoxP3, sense 5′-CATG CCTCCTCTTCTTCCTTG-3′, antisense 5′-GCTGGTGCATGAAATGTGG C-3′; and human GAPDH, sense 5′-CCCTTCATTGACCTCAACTAC-3′, antisense 5′-GGT GATGGGATTTCCATTG-3′.

For real-time RT-PCR assays, the cDNA sequences of the murine or the human Foxp3 gene was retrieved from the National Center for Biotechnology Information database. The primers were designed with the Oligo Primer Analysis 4.0 software and the sequences were blasted (http://www.ncbi.nlm.nih.gov/BLAST/). Real-time RT-PCR was done as previously described (23). The mRNA level of the detected gene was expressed as the relative level to that of GAPDH.

Intracellular glutathione detection

Total cellular glutathione levels were determined using the Glutathione Assay kit (Cayman Chemical) according to the manufacturer's instructions. Briefly, cells were lysed by freeze/thawing and protein was precipitated with 5% 5-sulfosalicylic acid solution. Glutathione in the supernatant was quantified by its ability to convert 5,5-dithiobis (2-nitrobenzoic acid) into the yellow product 2-nitrobenzoic acid, which was measured spectrophotometrically.

Statistics

Results were expressed as mean value ± SD and interpreted by ANOVA-repeated measure test. Differences were considered to be statistically significant when $P < 0.05$.

Results

Treg cells express lower levels of ATP than conventional T cells

$CD4^+CD25^+$ Treg cells and conventional $CD4^+CD25^-$ T cells were isolated from the spleens of naïve mice and the intracellular ATP levels were measured. The level of ATP was 8-fold lower than conventional T cells (Fig. 1A). To confirm these data in human Treg cells, we analyzed $CD4^+CD25^+CD127^-$ Treg cells and $CD4^+CD25^-CD127^+$ conventional T cells isolated from eight normal subjects' peripheral blood. Human Treg cells consistently expressed lower levels of ATP relative to conventional T cells (Fig. 1B). We also measured the ATP concentration in various cell lines derived from both mice and humans, and found that all the cell lines had a higher concentration of intracellular ATP than the cultured Treg cells (Fig. 1C). To further confirm the alteration of ATP levels between Treg cells and conventional T cells, the murine $CD4^+CD25^-$ T cells were induced into $CD4^+CD25^+Foxp3^+$ Treg cells with the stimulation of anti-CD3 antibody and TGF-β (28), or human $CD4^+CD25^-CD45RA^+$ T cells were induced into $CD4^+CD25^+Foxp3^+$ Treg cells with the stimulation of anti-CD3 antibody, ATAR and TGF-β (29). Similarly, the induced $CD4^+CD25^+$ Treg cells expressed lower levels of ATP, compared with the originally isolated conventional T cells (Fig. 1D). Thus, Treg cells have intrinsically low levels of ATP.

Both miR-142-3p and CD39 contribute to the low level of ATP in Treg cells

Next, we explored the underlying mechanism for the low levels of ATP in Treg cells. Our previous study showed that very low levels of miR-142-3p in Treg cells leads to a high level of adenylyl cyclase 9 and high production of cAMP (23). Because ATP is the substrate of adenylyl cyclase 9, it was reasonable to speculate that the low expression of

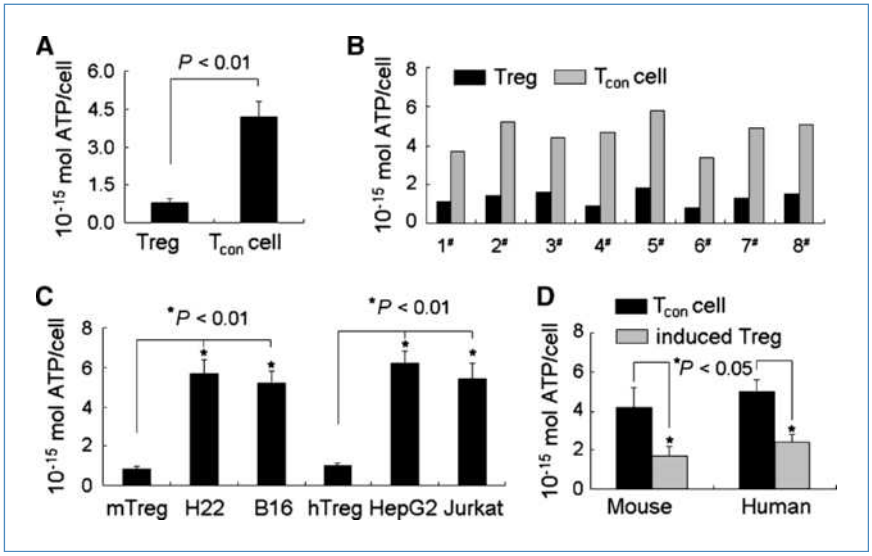

Figure 1. Treg cells express low levels of ATP. A and B, comparison of intracellular ATP concentration between Treg cells and conventional T cells. $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells were isolated from the spleen of naïve mice ($n = 6$), or $CD4^+CD25^+CD127^-$ Treg cells and $CD4^+CD25^-CD127^+$ T cells were isolated from human PBMCs ($n = 8$). After 12 h of culture in the presence of 10 ng/mL of IL-2, the intracellular ATP levels in isolated cells from mice (A) and human (B) were measured with the somatic cell ATP assay kit. C, detection of the intracellular ATP concentration in various cell lines. Murine H22 (hepatocarcinoma) and B16 (melanoma) tumor cell lines, and human HepG2 (hepatocarcinoma) and Jurkat (leukemia) tumor cell lines, were measured. D, the levels of ATP were reduced in induced Treg cells. Naïve BALB/c splenic $CD4^+CD25^-$ T cells or human $CD4^+CD25^-CD45A^+$ T cells were cultured and induced into Treg cells as described in Materials and Methods. The $CD4^+CD25^{high}$ T cells were sorted for 12 h culture in the presence of 10 ng/mL of IL-2. The intracellular ATP levels were measured.

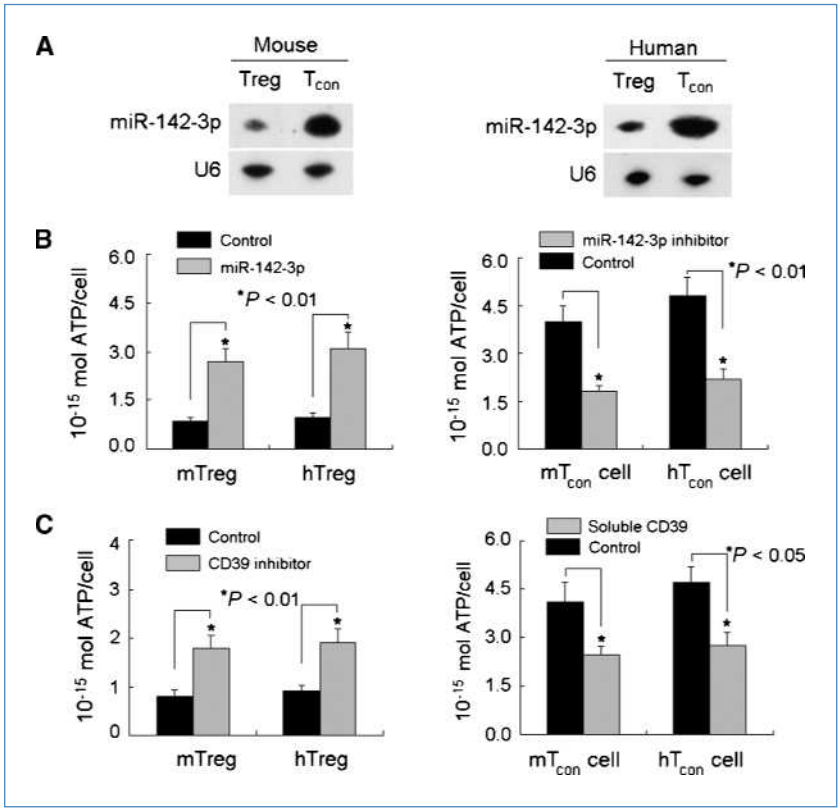

Figure 2. miR-142-3p and CD39 contributes to the low level of ATP in Treg cells. A, Treg cells and conventional T cells were isolated from mice and humans. The expression of miR-142-3p was analyzed by Northern blot. B, the level of miR-142-3p influences the concentration of ATP in Treg cells. The isolated murine or human Treg cells were transfected with miR-142-3p (left) or miR-142-3p inhibitor (right). Twenty-four hours later, the intracellular ATP levels were measured with the somatic cell ATP assay kit. C, the activity of CD39 influences the concentration of ATP in Treg cells. The isolated murine or human Treg cells were treated with CD39 inhibitor ARL67156 (left) or recombinant CD39 (right). Twenty-four hours later, the intracellular ATP levels were measured with the somatic cell ATP assay kit.

miR-142-3p contributed to the low levels of ATP in Treg cells. Consistently, we found, by Northern blot, much higher miR-142-3p in conventional T cells and much lower miR-142-3p in mouse and human Treg cells (Fig. 2A). We then transfected $CD4^+CD25^+$ Treg cells with miR-142-3p and observed, as predicted, an increase in ATP concentration in Treg cells (Fig. 2B, left). On the other hand, transfecting the inhibitor of miR-142-3p into conventional T cells resulted in the decrease of intracellular ATP (Fig. 2B, right). Similar results were also observed in human T cells (Fig. 2B). In addition to miR-142-3p, ectonucleotidase CD39 can catalyze ATP effectively and has been reported to be mainly expressed on Treg cells rather than conventional T cells (25, 26), which we have also confirmed (Supplementary Fig. S2). To determine if CD39 plays a role in the maintenance of low levels of ATP in Treg cells, we tested the CD39 ecto-ATPase inhibitor ARL67156 (20 μg/mL, Sigma), which resulted in the increase of ATP in murine and human Treg cells (Fig. 2C, left). In parallel, we also cultured $CD4^+CD25^-$ T cells isolated from mouse spleen or human PBMCs in the presence or absence of recombinant mouse or human CD39 (0.5 μg/mL; R & D Systems). Consistently, the addition of CD39 decreased the concentration of ATP in conventional T cells (Fig. 2C, right). Taken together, these data showed that both miR-142-3p and CD39 may contribute to the maintenance of low levels ATP in Treg cells.

Low-dose CY killing Treg cells is triggered by low level of ATP

Next, we asked whether low levels of ATP explained the selective killing of Treg cells by low-dose CY. To address this question, we first explored whether Treg cells could be selectively killed by low-dose CY *in vitro*. We found that CY is not toxic to cultured splenocytes (Supplementary Fig. S3). This may be due to the fact that CY is a prodrug and needs the biotransformation in hepatocytes. Therefore, we treated human hepatocyte cell line HepG2 with different concentrations of CY for 6 hours to generate 4-hydroxycyclophosphamide and added the supernatants to murine Treg cells and conventional T cells, respectively. Treg cells, but not conventional T cells, treated with 2 μg/mL activated CY were killed; higher concentrations killed both Treg cells and conventional T cells (Supplementary Fig. S4; Fig. 3A). Consistently, incubation with 2 μg/mL CY–treated supernatant resulted in the depletion of Treg cells rather than conventional $CD4^+$ T cells in bulk murine splenocytes or human PBMCs, which was confirmed by both Foxp3 expression and analysis of live T cells (Fig. 3B–D). In addition, the analysis of apoptotic T cells showed that the Annexin V^+ cells preferentially were $CD4^+CD25^+$ cells after killing by 2 μg/mL CY–treated supernatant (Supplementary Fig. S5). Interestingly, although conventional T cells were not killed by 2 μg/mL CY–treated supernatant, the transfection of miR-142-3p inhibitor or the addition of soluble CD39 to the culture medium to decrease

intracellular ATP levels or hydrolyze the extracellular ATP could result in the killing of conventional T cells by 2 μg/mL CY–treated supernatant (Supplementary Fig. S6). Taken together, these data indicated that the low-dose active form of CY generated from hepatocytes may selectively target Treg cells and such killing seems to connect to energy molecule ATP.

Next, we tested whether the ATP concentration affected Treg cytotoxicity mediated by 2 μg/mL CY–treated supernatant. We added different concentrations of ATP to the culture system and found that 5 μg/mL ATP rendered Treg cells resistant to the cytotoxicity induced by 2 μg/mL CY–treated supernatant (Fig. 4A and B). We also found that the addition of 5 μg/mL ATP increased the intracellular ATP concentration in cultured Treg cells to that of conventional T cells (Fig. 4C). More significantly, in cells from human subjects, 5 μg/mL ATP enhanced the resistance of Treg cells to the cytotoxicity induced by 2 μg/mL CY–treated supernatant (Fig. 4A and B). To clarify that the influence of 5 μg/mL ATP on Treg cells, we tested the apoptosis of Treg cells and found that apoptosis was not altered by 5 μg/mL ATP (data not shown). In addition, we here also transfected miR-142-3p to Treg cells and found that the resultant increase of intracellular ATP levels conferred Treg cells resistant to the cytotoxicity induced by 2 μg/mL CY–treated supernatant (Fig. 4D). Taken together, these data suggested that low level of ATP guides the selective killing of Treg cells by low-dose CY.

Low levels of ATP decreases the production of glutathione, leading to the sensitivity of Treg cells to low-dose CY

Next, we investigated the mechanism by which low levels of ATP sensitize Treg cells to low-dose CY. The active metabolite of CY can be detoxified by conjugation with glutathione. We hypothesized that low level of ATP in Treg cells diminished the synthesis of glutathione, impairing detoxification and thus increasing the sensitivity of Treg cells to low-dose CY. To test this, we compared the levels of glutathione in Treg cells and conventional T cells, and found that Treg cells had 10-fold less glutathione than conventional T cells (Fig. 5A). To verify whether such low glutathione mediates the killing of Treg cells by low-dose CY, we added glutathione (1 mmol/L) to Treg cell treated with 2 μg/mL CY–treated supernatant. Addition of glutathione attenuated the sensitivity of Treg cells to low-dose CY (Fig. 5B, left). In contrast, addition of buthionine sulfoximine (12.5 μmol/L; Sigma), an inhibitor of γ-glutamylcysteine synthetase, to the media induced the killing of conventional T cells by low-dose CY (Fig. 5B, right). Therefore, the decrease of glutathione may induce the killing of Treg cells by low-dose CY. Glutathione levels were also affected by ATP.

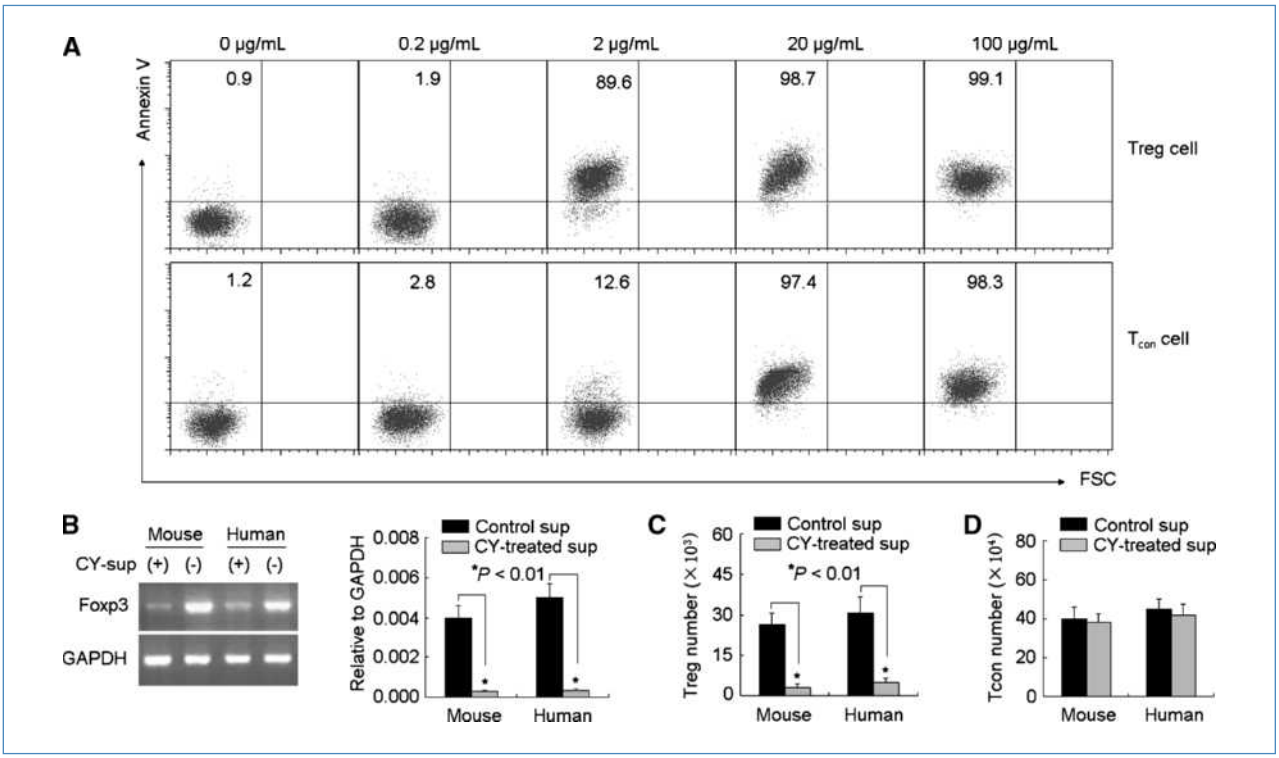

Figure 3. Treg cells could be selectively killed by low-dose CY. A, the selective depletion of Treg cells by low-dose CY. Human hepatocyte cell line HepG2 was treated with different concentrations of CY (0, 0.2, 2, 20, and 100 μg/mL) for 6 h. The supernatants were used to culture the isolated Treg cells and conventional T cells for 48 h. Then, cells were harvested and stained with FITC-Annexin V for flow cytometric analysis. B to D, HepG2 cell line was treated with 2 μg/mL of CY for 6 h. The supernatants were used to culture mouse splenocytes or human PBMCs for 48 h. The expressions of Foxp3 were detected by both RT-PCR (B, left) and real-time RT-PCR (B, right), and the numbers of Treg cells (C) and conventional T cells (D) were counted by flow cytometry.

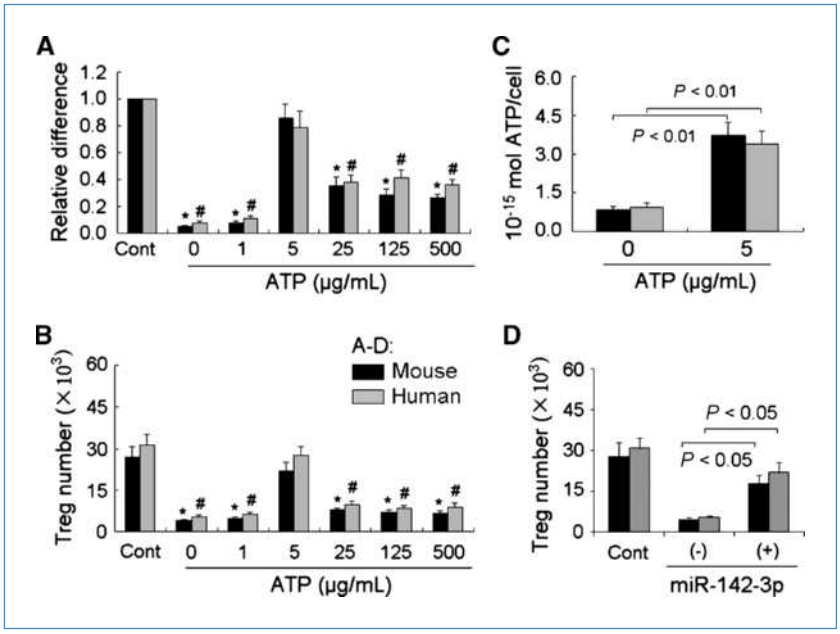

Figure 4. ATP concentration affects the cytotoxicity of low-dose CY to Treg cells. A and B, HepG2 cell line was treated with 2 μg/mL of CY for 6 h. The supernatant was used to culture murine splenocytes or human PBMCs in the presence of 0, 1, 5, 25, 125, or 500 μg/mL ATP for 48 h. The expression of Foxp3 was detected by real-time RT-PCR (A). The data were expressed relative to the cells cultured with normal media, in which the Foxp3 expression was designed as 1. The Treg cell number was counted by flow cytometry (B). * and #, $P < 0.01$, compared with the corresponding control group (neither CY treatment nor ATP addition). C, addition of 5 μg/mL ATP increased the intracellular ATP concentration in cultured Treg cells. HepG2 cell line was treated with 2 μg/mL of CY. Six hours later, the supernatant was used to culture mouse splenocytes or human PBMCs in the presence of 5 μg/mL ATP. Twelve hours later, the intracellular ATP levels were measured with the somatic cell ATP assay kit. D, the isolated murine or human Treg cells were transfected with miR-142-3p. Twenty-four hours later, the transfected cells were cultured with the supernatant of 2 μg/mL CY–treated HepG2 cells for 48 h. The Treg cell number was counted by flow cytometry.

The addition of 5 μg/mL ATP to culture media increased glutathione in Treg cells to roughly the levels found in conventional T cells (Fig. 5C) and, as shown previously, increased resistance to low-dose CY. Moreover, although 5 μg/mL ATP induced the resistance of Treg cells to low-dose CY, this resistance was counteracted by the addition of buthionine sulfoximine (Fig. 5D, left and right). Taken together, these data suggested that low levels of ATP decrease the synthesis of glutathione in Treg cells, thus increasing the sensitivity of Treg cells to low-dose CY.

Discussion

The molecular basis of the selective killing of Treg cells by low-dose CY is unclear. In the present study, we provide evidence that the intrinsic low levels of ATP and consequent low levels of glutathione explain the selective killing of Treg cells by low-dose CY.

Cells normally keep a steady cytosolic concentration of ATP. Here, we report that the intracellular concentration of ATP in Treg cells of either mice or humans, is $\sim 1 \times 10^{-15}$ mol ATP per cell, but that conventional T cells have as much as 4- to 5-fold higher ATP concentration. Compared with conventional $CD4^+$ T cells, Treg cells express much lower levels of miR-142-3p, resulting in elevated synthesis of adenyl cyclase 9, which converts ATP to cAMP (23) and express a much higher level of CD39 to degrade extracellular ATP (26), which probably facilitates the efflux of cytosolic ATP (30). It is known that ATP can be released by various nonexcitatory cells, including T lymphocytes (31, 32). Regardless of that mechanical stimulation and that agonists promote nonexcitatory cells to release ATP, rest nonsecretory cells also physiologically release ATP. Such basal extracellular ATP levels probably reflect a dynamic steady state of cells and the hydrolytic action by ecto-ATPase CD39 may lower extracellular ATP levels. To keep the basal extracellular ATP concentration, more intracellular ATP probably is released, leading to the decrease of cytosolic ATP. On the other hand, the increase of extracellular ATP levels probably prevents the release of intracellular ATP, leading to the increase of intracellular ATP levels. In line with this, our data showed that the addition of ATP increased the intracellular ATP concentration of cultured Treg cells (Fig. 4C). In our present study, we indeed confirm that miR-142-3p and CD39 are involved in the maintenance of low cytosolic ATP levels in Treg cells. However, there may be additional Treg-specific mechanisms that contribute to low ATP levels that we have not yet identified.

One interesting finding in this study is that 5 μg/mL ATP almost totally rendered Treg cells resistant to the cytotoxicity. However, high-dose ATP (≥25 μg/mL) only conferred Treg cells a little resistance. Such inconsistency is probably attributable to that ATP is a signal molecule and may profoundly influence T cells, largely dependent on the quantity

added (33, 34). Yip and colleagues (34) found that although extracellular ATP could stimulate T cells, the decrease of ATP to 40 μmol/L concentration (equal to 20 μg/mL) resulted in the loss of stimulatory effect on T cells, suggesting that extracellular ATP has to reach a certain concentration for signal transduction. Therefore, in our study, the addition of a high concentration of ATP (≥25 μg/mL) probably induces signal transduction in Treg cells, leading to the alteration of intracellular ATP or cell death pathway. For instance, it might be possible that this stimulatory signal promotes intracellular ATP release or increases the apoptosis pathway.

Efflux pump and intracellular metabolism are basically two major detoxification pathways against chemotherapeutic agents. Although $CD4^+$ T cells may express ATP-binding cassette (ABC) transporters such as ABCB1 (35), which requires ATP for function (36), CY is a non–P-glycoprotein substrate drug (37). Therefore, in this study, we did not investigate the role of ABC transporter in CY-mediated Treg cell killing. We show that the decreased glutathione contributes to low-dose CY-mediated Treg cell killing, consistent with previous reports of the antitoxic effect of glutathione in tumor cells against CY (20, 21). The oxidation of aldophosphamide by aldehyde dehydrogenases is another important detoxification pathway. The coenzyme NAD^+ is required for the catalyzing activity of aldehyde dehydrogenases. Interestingly, the cytosolic NAD^+ concentration has been reported to be dependent on the concentration of ATP (38). Therefore, aldehyde dehydrogenase might be involved in the low-dose CY-mediated Treg cell killing, which is currently under study.

Treg cells suppress both antitumor and antivirus immunity in patients and murine models. Several lines of evidence indicate that the interference in Treg biology or depletion of Treg cells is of critical importance in the treatment of cancer and viral infection (39–41). A commonly used chemotherapeutic agent CY can selectively deplete Treg cells when administered more frequenty or "metronomically" at a dose substantially lower than the maximum tolerated dose. Such low-dose CY is not only immunostimulatory but also avoids CY high-dose toxicity (16, 42). In addition, the restoration of Treg numbers and function 10 days after low-dose CY administration implies that a limited period of Treg cell inhibition may avoid potential autoimmunity (15). These features indicate that low-dose CY administration may be an ideal strategy to target Treg cells in cancer- or virus-infected patients. In this regard, our present study, shedding light on the underlying mechanism of low-dose CY selectivity for Treg, has important clinical significance.

In two clinical trials, a single bolus dose of CY combined with vaccines was administrated to treat patients with metastatic breast cancer (9, 43). Slight decreases in the absolute number and the percentage of Treg cells were observed. In contrast, the treatment of nine cancer patients with metronomic low-dose CY showed a selective reduction in Treg cell numbers and preservation of total number of lymphocytes

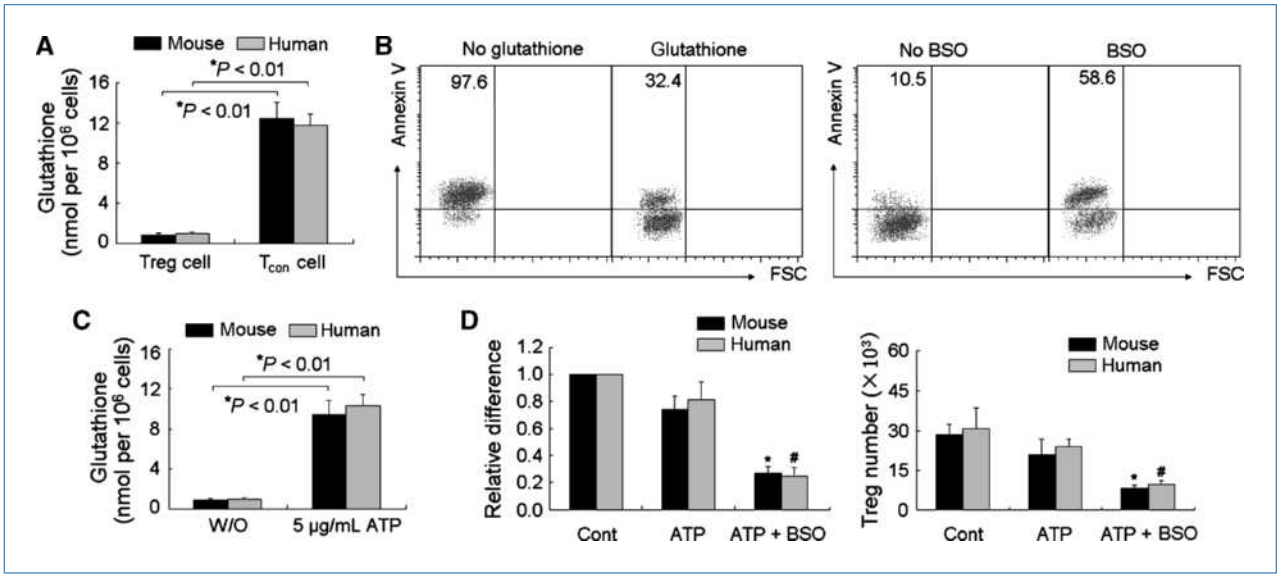

Figure 5. The low level of glutathione results in the killing of Treg cells by low-dose CY. A, Treg cells express low levels of glutathione. The isolated murine or human T cells were used to detect the glutathione level with the glutathione assay kit. Glutathione content was expressed in nmol per 1×10^6 cells. B, glutathione level influences the sensitivity of Treg cells to low-dose CY. HepG2 cell line was treated with 2 μg/mL of CY. Six hours later, the supernatant was used to culture Treg cells in the presence or absence of 1 mmol/L glutathione (left) or conventional T cells in the presence or absence of 12.5 μmol/L buthionine sulfoximine (BSO; right) for 48 h for the flow cytometric analysis of FITC-Annexin V^+ cells. C, ATP affects the level of glutathione. Treg cells were cultured in the presence or absence of 5 μg/mL ATP. The glutathione level was measured with glutathione assay kit. D, inhibition of glutathione synthesis impaired the ATP-induced resistance of Treg cells to low-dose CY. Splenocytes/PBMCs were cultured in the presence or absence of 5 μg/mL ATP and 12.5 μmol/L buthionine sulfoximine. Forty-eight hours later, the expression of Foxp3 was detected by real-time RT-PCR (left). The data were expressed relative to the cells cultured with normal media, in which the Foxp3 expression was designed as 1. The Treg cell number was counted by flow cytometry (right). * and #, $P < 0.01$, compared with the corresponding control group.

and natural killer cells (16). In our unpublished clinical trial against HPV infection–derived genital warts, we also found that by selectively depleting Treg cells, metronomic low-dose CY effectively prevented the recurrence of warts after laser therapy. In that clinical trial, Treg cells and conventional T cells are differentially sensitive to CY. Low-dose CY mostly affects Treg cells rather than conventional T cells; however, high-dose CY affects both Treg cells and conventional T cells. Therefore, the administration of single high-dose CY kills Treg cells as well as other immune cells, but it may be metabolized within a short time, leading to the recovery of Treg cells. In contrast, metronomic low-dose CY treatment may continue to selectively deplete Treg cells. Based on our findings, we suggest that metronomic low-dose CY administration is an ideal approach for Treg cell depletion.

In summary, our data show that a single energy molecule ATP, by virtue of its differential expression levels in $CD4^+CD25^+$ Treg cells and conventional $CD4^+$ T cells, can determine the sensitivity of these cells to low-dose CY. These findings suggest that metronomic low-dose CY administration may be a safe way to deplete Treg cells for the treatment of cancers.

Disclosure of Potential Conflicts of Interest

B. Huang: commercial research grant, Sheng-Qi-An Biotech. The other authors disclosed no potential conflicts of interest.

Acknowledgments

We thank Drs. Lee Leserman (Centre d'Immunologie de Marseille-Luminy) and Jay Unkeless (Mount Sinai School of Medicine) for the helpful discussion and assistance in editing this article.

Grant Support

Scientific Research Foundation of Wuhan City Human Resource for Returned Scholars, the Program for New Century Excellent Talents in University (NCET-08-0219), the Funds for International Cooperation and Exchange of the National Natural Science Foundation of China (30911120482), Special Research Foundation for Universities affiliated with China Ministry of Education (Z2009005), and Sheng-Qi-An Biotech (Wuhan, China).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 01/24/2010; revised 03/24/2010; accepted 04/09/2010; published OnlineFirst 05/25/2010.

References

1. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531–62.
2. Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. Nat Rev Immunol 2007;7:305–10.
3. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 2006;6:295–307.
4. Bonertz A, Weitz J, Pietsch DH, et al. Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. J Clin Invest 2009;119:3311–21.
5. Zhou J, Ding T, Pan W, Zhu LY, Li L, Zheng L. Increased intratumoral regulatory T cells are related to intratumoral macrophages and poor prognosis in hepatocellular carcinoma patients. Int J Cancer 2009;125:1640–8.
6. Strauss L, Bergmann C, Gooding W, Johnson JT, Whiteside TL. The frequency and suppressor function of $CD4^+CD25^{high}Foxp3^+$ T cells in the circulation of patients with squamous cell carcinoma of the head and neck. Clin Cancer Res 2007;13:6301–11.
7. Miller AM, Lundberg K, Ozenci V, et al. CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol 2006;177:7398–405.
8. Brode S, Cooke A. Immune-potentiating effects of the chemotherapeutic drug cyclophosphamide. Crit Rev Immunol 2008;28:109–26.
9. Audia S, Nicolas A, Cathelin D, et al. Increase of $CD4^+CD25^+$ regulatory T cells in the peripheral blood of patients with metastatic carcinoma: a phase I clinical trial using cyclophosphamide and immunotherapy to eliminate $CD4^+CD25^+$ T lymphocytes. Clin Exp Immunol 2007;150:523–30.
10. Ghiringhelli F, Menard C, Puig PE, et al. Metronomic cyclophosphamide regimen selectively depletes CD4+CD25+ regulatory T cells and restores T and NK effector functions in end stage cancer patients. Cancer Immunol Immunother 2007;56:641–8.
11. Martin-Suarez I, D'Cruz D, Mansoor M, Fernandes AP, Khamashta MA, Hughes GR. Immunosuppressive treatment in severe connective tissue diseases: effects of low dose intravenous cyclophosphamide. Ann Rheum Dis 1997;56:481–7.
12. Awwad M, North RJ. Cyclophosphamide-induced immunologically mediated regression of a cyclophosphamide-resistant murine tumor: a consequence of eliminating precursor $L3T4^+$ suppressor T-cells. Cancer Res 1989;49:1649–54.
13. Berd D, Mastrangelo MJ. Effect of low dose cyclophosphamide on the immune system of cancer patients: reduction of T-suppressor function without depletion of the CD8+ subset. Cancer Res 1987;47:3317–21.
14. Ghiringhelli F, Larmonier N, Schmitt E, et al. $CD4^+CD25^+$ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004;34:336–44.
15. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of $CD4^+25^+$ T regulatory cell function implicated in enhanced immune response by low dose cyclophosphamide. Blood 2005;105:2862–8.
16. Wada S, Yoshimura K, Hipkiss EL, et al. Cyclophosphamide augments antitumor immunity: studies in an autochthonous prostate cancer model. Cancer Res 2009;69:4309–18.
17. Castano AP, Mroz P, Wu MX, Hamblin MR. Photodynamic therapy plus low-dose cyclophosphamide generates antitumor immunity in a mouse model. Proc Natl Acad Sci U S A 2008;105:5495–500.
18. Daenen LG, Shaked Y, Man S, et al. Low-dose metronomic cyclophosphamide combined with vascular disrupting therapy induces potent antitumor activity in preclinical human tumor xenograft models. Mol Cancer Ther 2009;8:2872–81.
19. Rooney PH, Telfer C, McFadyen MC, Melvin WT, Murray GI. The role of cytochrome *P*450 in cytotoxic bioactivation: future therapeutic directions. Curr Cancer Drug Targets 2004;4:257–65.
20. Gamcsik MP, Dolan ME, Andersson BS, Murray D. Mechanisms of resistance to the toxicity of cyclophosphamide. Curr Pharm Des 1999;5:587–605.
21. Dirven HA, van Ommen B, van Bladeren PJ. Involvement of human glutathione *S*-transferase isoenzymes in the conjugation of cyclophosphamide metabolites with glutathione. Cancer Res 1994;54:6215–20.
22. Anderson ME. Glutathione and glutathione delivery compounds. Adv Pharmacol 1997;38:65–78.

23. Huang B, Zhao J, Lei Z, et al. miR-142-3p restricts cAMP production in CD4+CD25- T cells and CD4+CD25+ TREG cells by targeting AC9 mRNA. EMBO Rep 2009;10:180–5.
24. Bopp T, Becker C, Klein M, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. J Exp Med 2007;204:1303–10.
25. Borsellino G, Kleinewietfeld M, Di Mitri D, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007;110:1225–32.
26. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 2007;204:1257–65.
27. Alam MS, Kurtz CC, Rowlett RM, et al. CD73 is expressed by human regulatory T helper cells and suppresses proinflammatory cytokine production and Helicobacter felis-induced gastritis in mice. J Infect Dis 2009;199:494–504.
28. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3. J Exp Med 2003;198:1875–86.
29. Wang J, Huizinga TW, Toes RE. *De novo* generation and enhanced suppression of human $CD4^+CD25^+$ regulatory T cells by retinoic acid. J Immunol 2009;183:4119–26.
30. Lazarowski ER, Boucher RC, Harden TK. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. Mol Pharmacol 2003;64:785–95.
31. Corriden R, Insel PA, Junger WG. A novel method using fluorescence microscopy for real-time assessment of ATP release from individual cells. Am J Physiol Cell Physiol 2007;293:C1420–5.
32. Schenk U, Westendorf AM, Radaelli E, et al. Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. Sci Signal 2008;1:ra6.
33. Budagian V, Bulanova E, Brovko L, et al. Signaling through P2X7 receptor in human T cells involves p56lck, MAP kinases, and transcription factors AP-1 and NF-κB. J Biol Chem 2003;278:1549–60.
34. Yip L, Woehrle T, Corriden R, et al. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. FASEB J 2009;23:1685–93.
35. Aggarwal S, Tsuruo T, Gupta S. Altered expression and function of P-glycoprotein (170 kDa), encoded by the MDR 1 gene, in T cell subsets from aging humans. J Clin Immunol 1997;17:448–54.
36. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002;2:48–58.
37. Matheny CJ, Lamb MW, Brouwer KR, Pollack GM. Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. Pharmacotherapy 2001;21:778–96.
38. Devin A, Guérin B, Rigoulet M. Cytosolic NAD+ content strictly depends on ATP concentration in isolated liver cells. FEBS Lett 1997;410:329–32.
39. Morse MA, Hobeika AC, Osada T, et al. Depletion of human regulatory T cells specifically enhances antigen specific immune responses to cancer vaccines. Blood 2008;112:610–8.
40. Powell DJ, Jr., Attia P, Ghetie V, Schindler J, Vitetta ES, Rosenberg SA. Partial reduction of human FoxP3+ CD4 T cells *in vivo* after CD25-directed recombinant immunotoxin administration. J Immunother 2008;31:189–98.
41. Allan SE, Broady R, Gregori S, et al. CD4+ T-regulatory cells: toward therapy for human diseases. Immunol Rev 2008;223:391–421.
42. Nizar S, Copier J, Meyer B, et al. T-regulatory cell modulation: the future of cancer immunotherapy? Br J Cancer 2009;100:1697–703.
43. Rech AJ, Vonderheide RH. Clinical use of anti-CD25 antibody daclizumab to enhance immune responses to tumor antigen vaccination by targeting regulatory T cells. Ann N Y Acad Sci 2009;1174:99–106.