Adenosine and the adenosine A_{2A} receptor agonist, CGS21680, upregulate CD39 and CD73 expression through E2F-1 and CREB in regulatory T cells isolated from septic mice

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Abstract. The number of regulatory T cells (Treg cells) and the expression of ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1; also known as CD39) and 5'-ectonucleotidase (NT5E; also known as CD73) on the Treg cell surface are increased during sepsis. In this study, to determine the factors leading to the high expression of CD39 and CD73, and the regulation of the CD39/CD73/adenosine pathway in Treg cells under septic conditions, we constructed a mouse model of sepsis and separated the Treg cells using a flow cytometer. The Treg cells isolated from the peritoneal lavage and splenocytes of the mice were treated with adenosine or the specific adenosine A_{2A} receptor agonist, CGS21680, and were transfected with specific siRNA targeting E2F transcription factor 1 (E2F-1) or cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), which are predicted transcription regulatory factors of CD39 or CD73. The regulatory relationships among these factors were then determined by western blot analysis and dual-luciferase reporter assay. In addition, changes in adenosine metabolism were measured in the treated cells. The results revealed that adenosine and CGS21680 significantly upregulated CD39 and CD73 expression ($P<0.01$). E2F-1 and CREB induced CD39 and CD73 expression, and were upregulated by adenosine and CGS21680. Adenosine triphosphate (ATP) hydrolysis and adenosine generation were inhibited by the knockdown of E2F-1 or CREB, and were accelerated in the presence of CGS21680. Based on these results, it can be inferred that adenosine, the adenosine A_{2A} receptor agonist, E2F-1 and CREB are the possible factors contributing to the high expression of CD39 and CD73 on the Treg cell surface during sepsis. Adenosine and its A_{2A} receptor agonist served as the signal transducer factors of the CD39/CD73/adenosine pathway, accelerating adenosine generation. Our study may benefit further research on adenosine metabolism for the treatment of sepsis.

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Introduction

Sepsis is defined as a systemic inflammatory response to infections caused by bacteria, fungi, viruses or parasites. It can be divided into three severities with increasing severity, namely, sepsis, severe sepsis and septic shock (1). A recent report indicated that the incidence of sepsis is more frequent than previously reported and that over 700 in 100,000 patients admitted to a medical emergency department are diagnosed with sepsis of any severity (2). Sepsis is assoicated with many other disorders, thus making its diagnosis difficult. Many efforts have been made to improve the diagnostic methods and increase the survival rates, such as measuring the lactate level immediately and obtaining blood cultures prior to the administration of antibodies (3). Existing management methods include antibiotics (4), blood cleansing (5), vasopressor agents (3), and so forth. Although the pathophysiology of sepsis has been a research hotspot, further detailed studies are still urgently required.

An increased percentage of regulatory T cells (Treg cells) is one of the main characteristics of sepsis (6). Treg cells are specialized for immune suppression and they are indispensable to the proper control of the adaptive immune response (7). Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1; also known as CD39) and 5'-ectonucleotidase (NT5E; also known as CD73) are primarily expressed in Treg cells as surface markers (8,9), both catalyzing adenosine generation (10). Particularly, CD39 converts adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine

monophosphate (AMP), and CD73 dephosphorylates AMP to adenosine (8). Since studies have demonstrated the pro-inflammatory functions of ATP (11), and the anti-inflammatory functions of adenosine and adenosine A_{2A} receptor (12,13), CD39 and CD73 can play synergistic roles in inhibiting inflammation. Besides, both CD39 and CD73 reduce the mortality assoicated with sepsis (14,15), implying their close association with sepsis. However, the factors leading to the high expression of CD39 and CD73 on the Treg cell surface during sepsis remain unclear.

This study aimed to elucidate the molecular mechanisms responsible for the high expression of CD39 and CD73 on the Treg cell surface, and to investigate the regulatory factors of the CD39/CD73/adenosine pathway. For this purpose, a mouse model of sepsis was constructed using the cecal ligation and puncture (CLP) method. Treg cells were isolated and cultured for drug treatment or siRNA transfection. We analyzed the regulatory effects of adenosine and the specific adenosine A_{2A} receptor agonist, 2-p-(2-carboxyethyl)-phenylethylamino-5'-N-ethylcarboxamidoadenosine (CGS21680). The functions of E2F transcription factor 1 (E2F-1) and cyclic adenosine monophosphate (cAMP) responsive element-binding protein (CREB) as regards the regulation of CD39 or CD73 were verified. Subsequently, we detected changes in the adenosine cycle caused by these factors. Our results shed insight as to why CD39 and CD73 are enriched on the Treg cell surface, and enhance our understanding of the CD39/CD73/adenosine pathway.

Materials and methods

CLP and cell culture. All the experiments were performed based on the guidelines of our institute and the Regulation for the Administration of Affairs Concerning Experimental Animals (approved by the State Council of China). Clean grade BALB/c mice weighing 20 to 35 g (aged 30 to 40 days) were purchased from HFK Bioscience (Beijing, China) and raised for 2 days for acclimatization. Before the CLP operation, the mice were starved for 12 h. A total of 10 mice was randomly selected for the operation and anesthetized with xylazine (10 mg per kg body weight). CLP was performed as previously described (16). At day 7 post-operation, the death rate was approximately 50%. Treg cells ($CD4^+$ and $CD25^+$) were isolated from the peritoneal lavage and splenocytes of the 5 living mice using a flow cytometer and the Mouse Regulatory T cell Staining kit (eBioscience, San Diego, CA, USA), and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 200 μg/ml streptomycin and 0.25 μg/ml amphotericin (Invitrogen). The cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C, and the medium was changed every 2 days.

High performance liquid chromatography (HPLC) analysis. At 48 h post-transfection, the cells were treated with ATP (substrate of CD39) or AMP (substrate of CD73; Sigma-Aldrich, Shanghai, China) at a concentration of 1 mM for 1 h. The supernatant was then collected for the detection of the ATP, AMP and adenosine concentrations using the HPLC SpectraSYSTEM (Thermo Fisher Scientific, Waltham, MA, USA). Tests were conducted in the octadecyl silane-C18 column at room temperature. The injection volume of each test was 20 μl. Potassium phosphate buffer (50 mM, pH 6.5) was used as the mobile phase, with a flow velocity of 1 ml/min. The ultraviolet radiation wavelength for detection was 254 nm.

Flow cytometry. Peritoneal lavage and splenocytes were collected from the mice based on the methods of a previous study (17). Briefly, the peritoneal lavage and the spleen tissue of the 5 living mice were collected after the mice were anesthetized and sacrificed. For the collection of the peritoneal lavage, 5 ml phosphate-buffered saline was injected intraperitoneally and samples were obtained after 30 min of gently pressing the abdomen. The cells were then isolated and cultured according to the abovementioned method. Treg cells were isolated using the $CD4^+CD25^+$ Regulatory T Cell Isolation kit (Miltenyi Biotec, Teterow, Germany). αCD4 (RM4-5) fluorochrome-labeled antibody (BD Biosciences, Heidelberg, Germany), CD25 (PC61.5) antibody and Armenian and Syrian hamster IgG (BioLegend, San Diego, CA, USA) were used to label the Treg cells. The purity of the Treg cells was detected using a flow cytometer (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Drug treatment and siRNA transfection. The Treg cells were treated with CGS21680 (100 nM, Biochempartner, Shanghai, China), or adenosine (10 μM, MCE, Shanghai, China) at 37˚C. Transcription factors in the promoter sequences of CD39 and CD73 were predicted using UCSC genome browser (http://genome.ucsc.edu) and VISTA Enhancer Brower (http://genome.lbl.gov/vista/index.shtml). The E2F-1- and CREB-specific siRNA and negative control siRNA were purchased from Cell Signaling Technology (CST, Boston, MA, USA). The Treg cells were plated on 60-mm dishes and transfected with the corresponding siRNA treated with Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) after 24 h of incubation at the concentration of 1 pmol per 0.3 μl reagent.

Luciferase assay. The cells were transfected with Cignal E2F Reporter (luc) kit or CREB-luc (Qiagen, Shanghai, China) using Lipofectamine 2000 transfection reagent (Invitrogen). The transfected cells were lysed and the luciferase activities were analyzed using the Dual-Luciferase® reporter assay system (Promega, Madison, WI, USA) and measured as relative light units using a luminometer (Turner Designs, Sunnyvale, CA, USA).

Western blot analysis. The protein samples were extracted from the cells using lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P 40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), and 0.5% sodium deoxycholate. The proteins of the same amounts were then separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. The blots were blocked with 5% skim milk at 4˚C overnight and incubated with the primary antibodies [GAPDH (sc-365062), CD39 (sc-33558), CD73 (sc-14684), E2F-1 (sc-251), signal transducer and activator of transcription (STAT)5 (sc-836), p-STAT5 (sc-12893), CREB (sc-377154) or p-CREB-specific (sc-7978), Santa Cruz Biotechnology, Dallas, TX, USA] at room temperature for 2 h, followed by incubation with peroxidase-conjugated secondary antibody (anti-β-actin antibody, Sigma-Aldrich) at

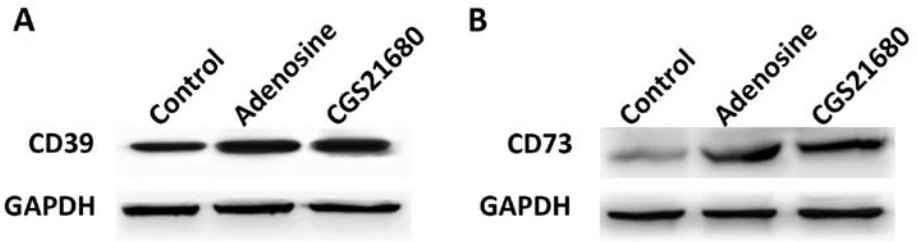

Figure 1. Effects of adenosine and CGS21680 on CD39 and CD73 expression. (A) Adenosine and CGS21680 promoted the expression of CD39. (B) Adenosine and CGS21680 promoted the expression of CD73. GAPDH was used as an internal reference. CD39, ectonucleoside triphosphate diphosphohydrolase 1; CD73, 5'-ectonucleotidase; CGS21680, the specific adenosine A_{2A} receptor agonist, 2-p-(2-carboxyethyl) phenylethylamino-5'-N-ethylcarboxamidoadenosine.

room temperature for 1 h. GAPDH was used as the internal reference. Positive bands were detected using enhanced chemiluminescence reagents and analyzed using the Kodak Digital Imaging System (Rochester, NY, USA) in triplicate.

Statistical analysis. All experiments were repeated 3 times and the results are presented as the means ± standard deviation (SD). Statistical analyses were performed using Statistical Product and Service Solutions (SPSS) 19.0 software and one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P<0.05$.

Results

Adenosine and CGS21680 upregulate CD39 and CD73. Western blot analysis was used to examine the effects of adenosine and CGS21680 on the expression of CD39 and CD73. At 24 h post-treatment with adenosine or CGS21680, both the CD39 and CD73 expression levels were upregulated compared to the control group (Fig. 1A and B), indicating that CD39 and CD73 are upregulated by adenosine and CGS21680. We then performed further experiments to determine the direct regulatory factors of CD39 and CD63.

Adenosine and CGS21680 upregulate E2F-1 and CREB. The predicted transcription factors, E2F-1 and STAT5 in the CD39 promoter, and CREB in the CD73 promoter (data not shown), were selected for this study. We conjectured that these factors may be regulated by adenosine or CGS21680, and may thus regulate CD39 and CD73. We thus performed a serious of experiments for verification. First, the expression levels of E2F-1 and STAT5 were measured by western blot analysis to verify whether they are regulated by adenosine or CGS21680 (Fig. 2A). The results revealed that adenosine upregulated the expression of p-E2F-1 and E2F-1. CGS21680 upregulated p-E2F-1; however, it did not affect E2F-1 expression. On the contrary, the expression patterns of p-STAT5 and STAT5 were not affected by either adenosine or CGS21680, indicating that STAT5 is not regulated by adenosine or CGS21680. Similarly, the expression levels of CREB and p-CREB were both upregulated by adenosine and CGS21680 (Fig. 2B). Thus, we only referred to E2F-1 and CREB in the following experiments. Dual-luciferase reporter assay indicated that E2F-1 was expressed at significantly higher levels in the Treg cells treated with adenosine or CGS21680 ($P<0.01$, Fig. 2C), which further confirmed tht E2F-1 is regulated by adenosine and CGS21680. Furthermore, the expression of CREB was significantly increased by adenosine and CGS21680 ($P<0.01$, Fig. 2D), inferring that adenosine and CGS21680 upregulate CREB. Taken together, these data indicate that the two transcription regulatory factors, E2F-1 and CREB, may be regulated by adenosine and CGS21680.

Adenosine and CGS21680 upregulate CD39 and CD73 via E2F-1 and CREB. We then verified the effects of E2F-1 and CREB on CD39 and CD73 using their specific siRNA. The knockdown of E2F-1 led to the downregulation of both p-E2F-1 and E2F-1 (Fig. 3A), and the knockdown of CREB inhibited the expression of p-CREB and CREB protein as expected (Fig. 3B). We then examined the effect of E2F-1 or CREB knockdown on CD39 and CD73, and found that the inhibition of E2F-1 resulted in the downregulation of CD39 (Fig. 3C). There was no change in CD39 expression when the transfected cells were treated with adenosine (si-E2F-1 + adenosine vs. si-E2F-1), while CGS21680 compensated to some extent for the inhibition of E2F-1. These results indicate that CGS21680 and E2F-1 are both positive regulators of CD39. As for CREB, its knockdown also downregulated the expression of CD73 (Fig. 3D), indicating that CREB regulates CD73. Adenosine and CGS21680 slightly increased the expression of CD73 in the transfected cells (si-CREB + adenosine or si-CREB + CGS21680 vs. si-CREB), implying that adenosine and CGS21680 upregulated CD73 by promoting CREB. Taken together, our data indicate that adenosine and CGS21680 promote CD39 and CD73 by upregulating E2F-1 or CREB, constituting the CD39/CD73/adenosine pathway. Therefore, the regulatory functions of adenosine, CGS21680, E2F-1 and CREB are the possible reasons for the high expression of CD39 and CD73 on the Treg cell surface.

Adenosine and CGS21680 promote adenosine generation. After the cells were treated with ATP, the production of ATP and AMP in the supernatant was detected and compared, as shown in Fig. 4A. The amount of ATP decreased sharply in both the CGS21680-treated and untreated cells when detected at 5 min after ATP treatment. In particular, the changes in the amount of ATP in the CGS21680-treated cells were more significant than those in the untreated cells ($P<0.05$), lasting until the final detection at 80 min post-ATP treatment. Furthermore, the amount of AMP, the hydrolysate of ATP, kept increasing until 40 min post-ATP treatment and the CGS21680-treated cells exhibited

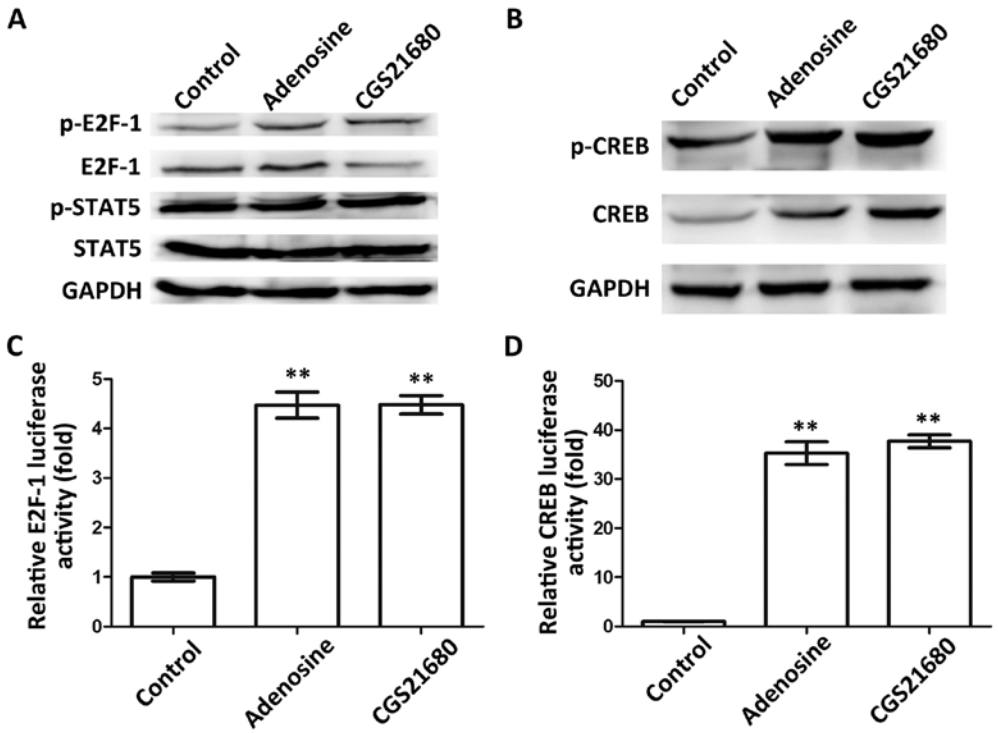

Figure 2. Regulation of E2F-1 and CREB by adenosine and CGS21680. (A) Adenosine and CGS21680 promoted the expression of E2F-1 but not STAT5. (B) Adenosine and CGS21680 promoted the expression of CREB. (C) Dual-luciferase report assay indicated that E2F-1 was significantly upregulated by adenosine or CGS21680. **P<0.01 compared to the control group. (D) Dual-luciferase report assay indicated that CREB was significantly upregulated by adenosine or CGS21680. **P<0.01 compared to the control group. GAPDH was the internal reference in western blot analysis. E2F-1, E2F transcription factor 1; p-E2F-1, phosphorylated E2F-1; STAT5, signal transducer and activator of transcription 5; p-STAT5, phosphorylated STAT5; CREB, cAMP responsive element-binding protein; p-CREB, phosphorylated CREB; CGS21680, specific adenosine A_{2A} receptor agonist.

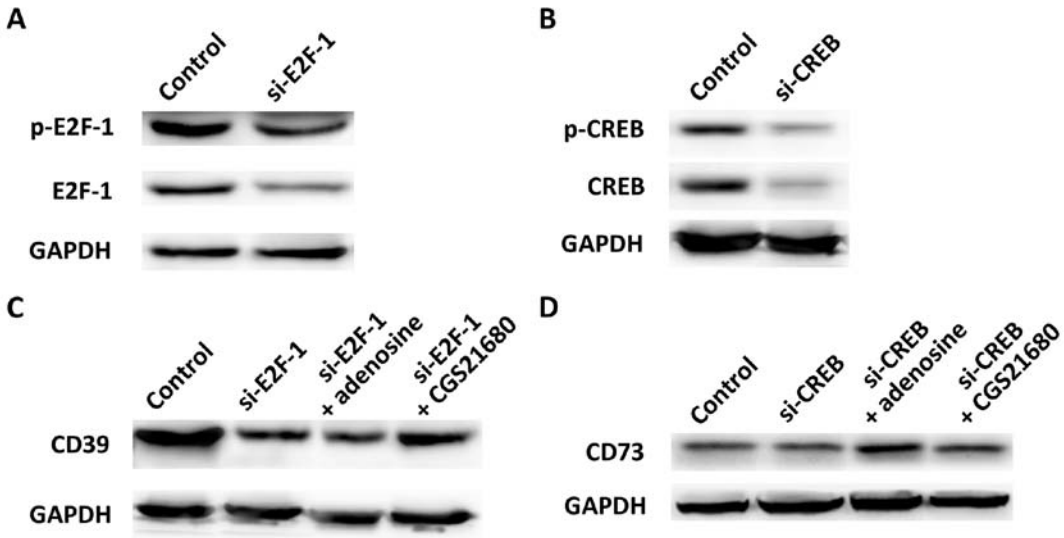

Figure 3. Regulation of CD39 and CD73 by E2F-1 and CREB. (A) E2F-1-specific siRNA inhibited the expression of p-E2F-1 and E2F-1. (B) CREB-specific siRNA inhibited the expression of p-CREB and CREB. (C) E2F-1, adenosine and CGS21680 regulated CD39. (D) CREB, adenosine and CGS21680 regulated CD73. GAPDH was the internal reference in western blot analysis. E2F-1, E2F transcription factor 1; p-E2F-1, phosphorylated E2F-1; CREB, cAMP responsive element-binding protein; p-CREB, phosphorylated CREB; si-E2F-1, E2F-1-specific siRNA; si-CREB, CREB-specific siRNA; CGS21680, specific adenosine A_{2A} receptor agonist; CD39, ectonucleoside triphosphate diphosphohydrolase 1; CD73, 5'-ectonucleotidase.

significant differences before this time point when compared with the untreated cells (P<0.05). Similarly, after adding AMP to the cells, AMP decreased gradually and adenosine, the hydrolysate of ATP, increased accordingly (Fig. 4B). Significant differences were observed between the CGS21680-treated and untreated cells in the changes in the amount of both AMP and adenosine (P<0.05). These results indicated that the adenosine cycle was accelerated in the presence of CGS21680.

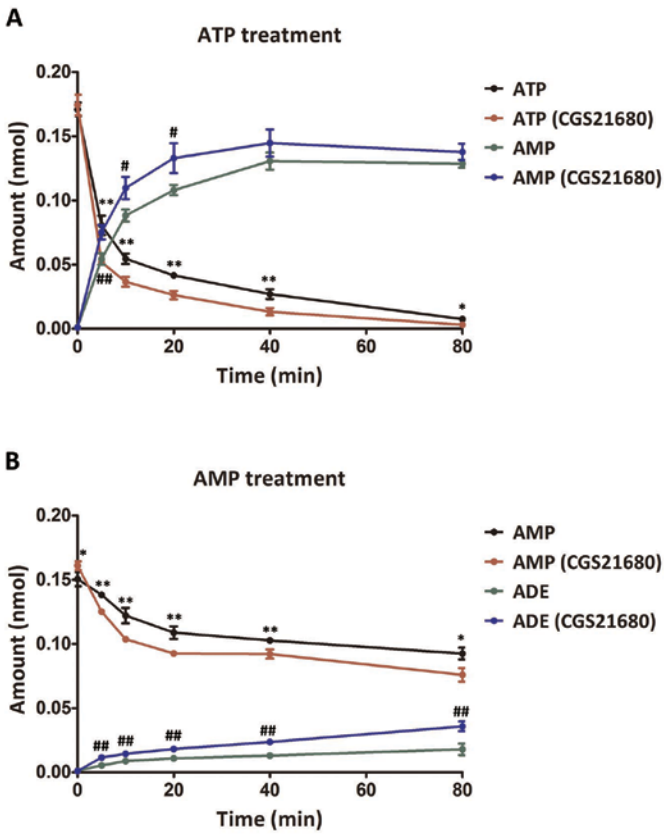

Figure 4. Amount of ATP, AMP and ADE in the supernatant after the addition of ATP or AMP to the CGS21680-treated cells. (A) Changes in the amount of ATP and AMP after the addition of ATP. * and ** indicate extremely significant differences (P<0.01) and significant differences (P<0.05) in the amount of ATP between the control group and the CGS21680-treated group. # and ## indicate extremely significant differences (P<0.01) and significant differences (P<0.05) in the amount of AMP between the control group and the CGS21680-treated group, respectively. (B) Changes in the amount of AMP and adenosine after AMP treatment. * and ** indicate extremely significant differences (P<0.01) and significant differences (P<0.05) in the amount of AMP between the control group and the CGS21680-treated group, respectively. ## indicates extremely significant differences (P<0.01) in the amount of adenosine between the control group and the CGS21680-treated group. Amounts were detected at 0, 5, 10, 20, 40, 60 and 80 min post-ATP or -AMP treatment. ATP, adenosine triphosphate; AMP, adenosine monophosphate; ADE, adenosine; CGS21680, specific adenosine A_{2A} receptor agonist.

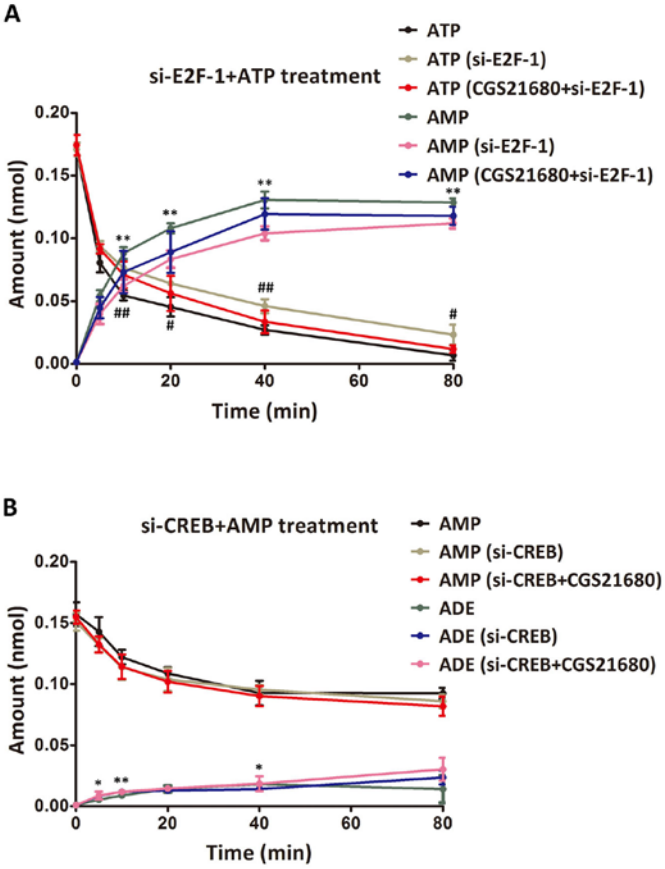

Figure 5. Amount of ATP, AMP and ADE in the supernatant after the addition of ATP or AMP to the transfected cells. (A) Changes in the amount of ATP and AMP after the addition of ATP. # and ## indicate extremely significant differences (P<0.01) and significant differences (P<0.05) in the amount of ATP between the control group and the si-E2F-1-transfected group, respectively. ** indicates extremely significant differences (P<0.01) in the amount of AMP between the control group and the si-E2F-1-transfected group. No significant difference was observed between the si-E2F-1-transfected group and the transfected group treated with CGS21680 (P>0.05). (B) Changes in the amount of AMP and adenosine after AMP treatment. ** and * indicate extremely significant differences (P<0.01) and significant differences (P<0.05) in the amount of AMP between the control group and the si-CREB-transfected group, respectively. No significant difference was observed in the other comparisons (P>0.05). Amounts are detected at 0, 5, 10, 20, 40, 60 and 80 min post-ATP or -AMP treatment. ATP, adenosine triphosphate; AMP, adenosine monophosphate; ADE, adenosine; CGS21680, specific adenosine A_{2A} receptor agonist; E2F-1, phosphorylated E2F-1; si-E2F-1, E2F-1-specific siRNA; CREB, cAMP responsive element-binding protein; si-CREB, CREB-specific siRNA.

Based on the above-mentioned results that E2F-1 may regulate CD39, and that CREB may regulate CD73, we conducted further analysis by adding ATP and AMP, the corresponding substrates of CD39 and CD73. First, the changes in the adenosine cycle were analyzed after ATP treatment in the 3 groups of cells, namely the cells with no pre-treatment, the cells transfected with si-E2F-1 and the transfected cells treated with CGS21680 (Fig. 5A). After the addition of ATP, the amount of ATP in the supernatant decreased, while the amount of AMP increased. Significant differences were observed between the untreated cells and the si-E2F-1-transfected cells (P<0.05). Slight differences were observed between the transfected cells and the transfected cells treated with CGS21680, with no significance (P>0.05). A similar detection was also performed after AMP treatment among the cells with no pre-treatment, the si-CREB-transfected cells and the transfected cells treated with CGS21680 (Fig. 5B). The trends in the changes in AMP or adenosine content were consistent among the 3 groups, with significant differences only observed in the amount of adenosine between the untreated cells and the si-CREB-transfected cells at 5, 10 and 40 min post-AMP treatment (P<0.05). Therefore, the promoting effects of CREB on the adenosine cycle were ambiguous. Overall, these results suggest that E2F-1, CREB may promote the adenosine cycle and the effects of their inhibition may be compensated by CGS21680 to a certain extent.

Discussion

This study discusses the molecular mechanisms resulting in the high expression of CD39 and CD73 on the Treg cell surface during sepsis to analyze the regulatory factors of the CD39/CD73/adenosine pathway. We proved that the expression of

CD39 and CD73 is upregulated by adenosine and the specific adenosine A_{2A} receptor agonist, CGS21680. Adenosine and CGS21680 upregulated E2F-1 and CREB, the predicted transcription regulatory factors of CD39 and CD73. E2F-1 and CREB were further verified to promote the expression of CD39 and CD73. Besides, the results indicated that adenosine production was accelerated in the presence of CGS21680, as well as E2F-1 and CREB. Thus, adenosine and the adenosine A_{2A} receptor play roles as signal transducer factors, upregulating E2F-1 and CREB to increase the expression of CD39 and CD73, and promote adenosine generation in Treg cells during sepsis.

CD39 and CD73 are two vital proteins catalyzing the conversion of ATP/ADP to AMP and AMP to adenosine, respectively. Previous studies have found that CD39 and CD73 can be regulated by various factors. For example, CD39 is upregulated by specificity protein 1 (Sp1) (18) and STAT3, and is suppressed by growth factor independent 1 transcription repressor (GFI-1) (19). CD73 can be induced by interferon-β-1a (20) and inhibited by GFI-1 (19). The two proteins are both expressed broadly in various cell types and tissues (21,22). Nevertheless, the reason for their high expression levels on the Treg cell surface during sepsis is the topic of this study. We selected three predicted transcription regulatory factors in the promoters of CD39 and CD73, among which E2F-1 and CREB were likely to be regulated by adenosine and CGS21680, and could promote CD39 and CD73, respectively. Therefore, E2F-1 and CREB were two direct regulatory factors leading to the high expression of CD39 and CD73 on the Treg cell surface during sepsis. In addition, adenosine and CGS21680 seemed to be two indirect activators of CD39 and CD73 by upregulating E2F-1 and CREB. As the specific adenosine A_{2A} receptor agonist, CGS21680 divides adenosine receptor A_{2A} from A_{2B} based on different affinities (23), representing the promoted activity of adenosine A_{2A} receptor in this study. Thus, it could be inferred that adenosine and adenosine A_{2A} receptor are two regulatory factors for the indirect promotion of CD39 and CD73.

CD39 and CD73 are viewed as immunological switches, shifting the immune cell activities from an ATP-driven pro-inflammatory state to an adenosine-mediated anti-inflammatory state (24). The overexpression of CD39 increases adenosine production to inhibit activated T-lymphocytes in mesenchymal stromal cells (25). The low expression of CD73 reduces adenosine generation, resulting in the enhanced severity of juvenile idiopathic arthritis (26), and suppresses pro-inflammatory responses in endothelial cells (27) and gastritis cells (28). The roles of adenosine and its receptors in inflammation have been discussed in existing studies. Patients with septic shock possess high adenosine plasma concentrations (29). The activation of adenosine A_{2A} receptor by agonists can inhibit inflammation caused by *Helicobacter* (30), and has been shown to increase survival in murine models of sepsis (31,32). For this study, adenosine, CGS21680, E2F-1 and CREB in the CD39/CD73/adenosine pathway all promoted CD39 and CD73 indirectly or directly, accelerating ATP hydrolysis and adenosine generation. Therefore, these factors may facilitate the anti-inflammatory activities of Treg cells during sepsis.

In summary, this study discusses the molecular mechanisms responsible for the high expression of CD39 and CD73 on the Treg cell surface, and the possible regulatory factors of the CD39/CD73/adenosine pathway during sepsis. Together with adenosine and adenosine A_{2A} receptor, E2F-1 and CREB upregulate CD39 and CD73, respectively, and promote adenosine generation to participate in the anti-inflammatory activities of Treg cells during sepsis. Our study offers more detailed information of the regulatory relationship of the CD39/CD73/adenosine pathway, facilitating further research on the treatment of sepsis using adenosine, adenosine receptor agonists and other methods concerning the regulation of adenosine metabolism.

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