Basic Study

Upregulated adenosine 2A receptor accelerates post-infectious irritable bowel syndrome by promoting CD4+ T cells' T helper 17 polarization

Dong LW et al. Th17 polarization by CD4+ T cells regulated by A2AR in PI-IBS

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Abstract

BACKGROUND
Post-infectious irritable bowel syndrome (PI-IBS) is generally regarded as a functional disease. Several recent studies have reported the involvement of low-grade inflammation and immunological dysfunction in PI-IBS. T helper 17 (Th17) polarization occurs in IBS. Adenosine and its receptors participate in intestinal inflammation and immunity regulation.

AIM
To investigate the role of Th17 polarization by CD4+ T cells regulated by adenosine 2A receptor (A2AR) in PI-IBS.

METHODS
PI-IBS model was established by infecting mice with Trichinella spiralis. The intestinal A2AR and CD4+ T lymphocytes were detected by immunohistochemistry, and the inflammatory cytokines were detected by enzyme-linked immunoassay. CD4+ T lymphocytes present in the animal’s spleen were separated and cultured with or
without A2AR agonist and antagonist. Western blotting and real-time quantitative polymerase chain reaction were performed to determine the effect of A2AR on the cells and intestinal tissue. Cytokine production was determined. The protein and mRNA levels of A2AR associated signaling pathway molecules were also evaluated. Furthermore, A2AR agonist and antagonist were injected into the mouse model and the clinical features were observed.

RESULTS
The PI-IBS mouse model showed the increased expression of ATP and A2AR ($P < 0.05$), and inhibition of A2AR improved the clinical features in PI-IBS, including abdominal withdrawal reflex and colon transportation test ($P < 0.05$). The number of intestinal CD4$^+$ T cells and interleukin-17 (IL-17) protein levels increased during PI-IBS, which was reversed by the administration of the A2AR antagonist ($P < 0.05$). CD4$^+$ T cells expressed A2AR and produced IL-17 in vitro, which was regulated by the A2AR agonist and antagonist. A2AR antagonist increased the production of IL-17 by CD4$^+$ T cells via Janus kinase-signal transducer and activator of transcription- receptor-related orphan receptor $\gamma$ signaling pathway.

CONCLUSION
The results of the present study suggested that the upregulation of A2AR increases PI-IBS by promoting the Th17 polarization of CD4$^+$ T cells.

**Key Words:** Adenosine 2A receptor; CD4$^+$ T cells; T helper 17 polarization; Post-infectious irritable bowel syndrome; Regulation

Core Tip: T helper 17 (Th17) polarization occurs during the development of irritable bowel syndrome (IBS). Adenosine and its receptors participate in the development of intestinal inflammation and immunity regulation. Here, we found that the intestinal levels of ATP, adenosine 2A receptor (A2AR), and interleukin-17 (IL-17) and the number of CD4+ T cells increased in post-infectious IBS (PI-IBS) mice. In addition, A2AR promoted CD4+ T cells’ IL-17 production. Upregulated A2AR accelerated PI-IBS by promoting CD4+ T cells’ Th17 polarization, which was reversed by the A2AR’s antagonist. Our results thus prove that the regulation of CD4+ T cells’ Th17 polarization by A2AR plays a pathogenetic role in the development of PI-IBS.

INTRODUCTION
The predominant clinical features of irritable bowel syndrome (IBS) include abdominal pain, abnormal bowel habit, and stool changes. IBS occurs predominantly in developed countries. The incidence rate is about 10%-60%. During the past three decades, the incidence of IBS has rapidly increased in underdeveloped countries[1]. IBS can occur after an acute gastrointestinal infection, which is defined as post-infectious IBS (PI-IBS)[2]. The patients suffer from refractory discomfort, live with problems, and have to undergo high medical costs for their treatment. The laboratory reports of the patients do not show morphological findings, which leads to a shortage of time in targeting treatment[3]. Low-grade inflammation and immune activation in the colonic mucosa are observed during IBS[4]. PI-IBS might be alleviated due to the regulation of inflammatory response[5]. Upregulated ephrin type-A receptor 2 and activated nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway can alleviate PI-IBS[6]. Moreover, non-coding RNAs such as micro RNA can modulate inflammation and immune response by targeting peroxiredoxin-1 in PI-IBS[7]. Recently, interleukin-17 (IL-17) raised concerns in PI-IBS. For instance, rifaximin protects mice against PI-IBS by suppressing the production of IL-17[8]. Our previous studies reported that heat shock protein 70 alleviated PI-IBS by upregulating T helper 17 (Th17) response by γδ T cells[9,10].
Adenosine originates from ATP in the local tissue during inflammation and regulates tissue blood flow[13]. As an endogenous molecule, adenosine exhibits anti-inflammatory and immunosuppressive effects by binding to the adenosine receptors (ARs), which are located on some immune and tissue cells and function as a G-protein coupled receptor[12-14]. ARs consist of the following four subtypes: A1R, adenosine A2a receptor, adenosine A2b receptor, and A3R, which are expressed at various locations of the intestine[15]. Different receptors induce different pathophysiological roles. A2BR inhibits the release of inflammatory cytokines, tumor necrotic factor-α, IL-6, and IL-12[16-18]. We previously reported that ARs contribute to the modulation of immune response by γδ T cells[19,20]. In the intestine, adenosine and its receptors regulate the enteric nervous system and visceral hypersensitivity. Endogenous adenosine affects the secretion of hybricarbinate by dendeulm, whereas extracellular ATP induces the apoptosis of intestinal epithelial cells[21,22]. Adenosine inhibits the inflammatory damages in the intestine, protects the intestine against ischemia, and reduces lesions in chronic septic mice by A1AR, A2AR, and A2BR[23,24]. A3R regulates distal colonic neuro-muscle function in rats with colitis[25]. Adenosine receptors also contribute to the inhibition of visceral pain by electroacupuncture in IBS[26]. The role of ARs in intestinal functions shows that they can be a potential target for intestinal inflammatory diseases. However, ARs are not widely studied in IBS, especially in PI-IBS. A2AR is reported to protect the enteric Glias barrier function against intestinal injury induced by hypoxia[27].

The relationship between A2AR, CD4+ T cells, and IL-17 is unknown. Whether A2AR is expressed in CD4+ T cells is not known. Whether A2AR affects IL-17 production directly or through other indirect pathways is unknown. Furthermore, the relationship between A2AR, IL-17, and CD4+ T cells in PI-IBS is unknown. Therefore, we investigated the role of Th17 polarization of CD4+ T cells regulated by A2AR in PI-IBS.

MATERIALS AND METHODS

Animals grouping
57BL/6 mice (Female, mean age: 56 d, average weight: 15 g) were provided by the Hainan Provincial Experimental Animal Center and fed in a pathogen-free, room temperature condition under a normal 12-h light/dark diurnal rhythm. The animals were provided with a standard diet and water ad libitum. Four groups of mice were formed: control, PI-IBS, PI-IBS+Sch58261, and PI-IBS+CGS21680 (n = 24 in each group). Twelve mice were processed for the measurement of the levels of A2AR, cytokines, and signaling molecules protein and mRNA. Six mice were sacrificed for isolating, purifying, and culturing their CD4+ T cells. The visceral hypersensitivity and intestinal motility were evaluated in the remaining 6 animals.

**PI-IBS modeling**

Infection with *Trichinella spiralis* (Lanzhou Veterinary Research Institute, Lanzhou, China) was performed to establish the PI-IBS model[28]. After 60 d of infection, the parasite larvae were digested from Sprague-Dawley rats with 1.5% gastric pepsin (Invitrogen, Carlsbad, CA, United States) and suspended in 0.2 mL of saline. The mice were fed with larvae (300 Larvae/mouse).

**Histopathological examination**

On the 56th day after infection, the animals’ ileum was fixed in 10% formalin and embedded in paraffin wax. The tissues were mounted on slides after creating 5-μm-thick sections, followed by dewaxing and dehydration. The slide was stained with hematoxylin-eosin and used for further evaluation of the inflammatory score following the previous scoring standards[29].

**Abdominal withdrawal reflex**

The visceral hypersensitivity was evaluated with the abdominal withdrawal reflex (AWR)[30]. The anesthetized animals’ anus was inserted with a gasbag, distended in volume from 0.25 mL to 0.65 mL for 15 min, thrice, followed by a recording of the animals’ behavior. The AWR score was found to be in accordance with the standard
from 0 to 4 points, as described previously\cite{30}. The AWR scoring standard: when stimulated, the animals are in stable mood, 0 point; if the animals are in unstable mood, twisting their heads once in a while, 1 point; slightly contracting their abdomen and back muscles, 2 points; intensively contracting their abdomen muscles and uplifting the abdomen from the ground, 3 points; intensively contracting abdomen muscles, bowing abdomen and uplifting the abdomen and perineum, 4 points.

**Colon transportation test**

The intestinal motility was evaluated with the colon transportation test (CTT): 0.4 mL of active carbon was filled into the animal’s stomach, and the animal’s initial time duration of black stool elimination was recorded. After 8 h, the Bristol stool scoring system was used to assess the total stool’s characteristics\cite{31}. Normal shaped stool, 1 point; soft or deformed stool, 2 points; water-like stool, 3 points.

**Cellular Immunohistochemistry**

Cellular immunohistochemistry was performed as reported elsewhere\cite{32}. The suspending cells were fixed in 4% paraformaldehyde and uniformly placed onto slides. The sections were then incubated with primary antibody rabbit anti-mouse A2AR monoclonal antibody and a secondary antibody goat anti-rabbit HRP antibody (ab60032 and ab6721; Abcam, Cambridge, United Kingdom). DAB reagent sets (Beijing Sequoia Jinqiao, Beijing, China) were used to determine the reactivity. The quantitative expression of A2AR in the cellular membrane was calculated by using a semiquantitative integral method. The staining intensity was scored on a scale of 0-3. The percentage area of positively stained cells was classified as 0-4 corresponding to the values of < 5% to > 75%. The evaluation with a score of ≥ 6 was expressed as a positive expression. The stain was analyzed by the Nikon DR-Si2 cell count software and digital image analysis; two senior pathologists verified the results.

**Western blotting**
Western blotting (Wuhan Boster Corporation, Wuhan, China) was performed to evaluate the protein level. RIPA (R0278; Sigma-Aldrich, Saint Louis, MO, United States) buffer was used to extract the protein from the ground ileum tissue samples. Bradford Assay was performed to determine the protein concentration. After separation on SDS page gel electrophoresis, 40 μg of the sample was transferred onto a polyvinylidene fluoride membrane. The following transmembrane conditions were set during the assay: (1) β-actin and A2AR: running at 200 mA for 90 min; (2) Janus kinase (JAK) and p-JAK: running at 200 mA for 120 min, followed by running at 300 mA for 30 min; (3) Signal transducer and activator of transcription (STAT3) and p-STAT3: running at 200 mA for 20 min, followed by running at 300 mA for 15 min; and (4) Receptor-related orphan receptor-γt (RORγt): running at 200 mA for 120 min.

The dilution rate of the primary antibodies rabbit anti-mouse multiple-clone antibodies is shown in Table 1.

Blotted within TBST for 60 min, the membrane was incubated with rabbit anti-mouse protein antibodies (ab2787; Abcam, United States) as the primary antibodies and with goat anti-rabbit antibodies as the secondary antibodies (ab8226; Abcam) at 4 °C for 12 h. ECL chemiluminescent assay was performed to autographed the membrane. The detected gray-scale ratio of protein/β-actin represented the relative protein level.

**Real-time quantitative PCR**

Total RNA in the ileum tissues was extracted with the Trizol reagent set (Invitrogen). The primers were designed by Invitrogen (Table 2). β-actin served as an internal control.

RT-PCR was performed under the following protocol conditions: The samples were run by a pre-denaturation program (5 min at 94 °C), followed by a denaturation program (1 min at 94 °C). The amplification and qualification program was repeated for 30 cycles (50 s at 57 °C, 20 s at 60 °C), followed by a prolonging program (7 min at 72 °C).

The relative expression was expressed as the ratio of the target gene to the control.
**Determination of ATP**

The ileum tissue sample was ultrasonically cracked and centrifuged at 4 °C for 15 min, followed by the addition of double-distilled water (1:9). The 10% homogenate was heated in a boiling water bath for 10 min and then centrifuged at 3000 rpm for 10 min. The supernatant was collected for enumeration.

The concentration of ATP was measured by a colorimetric method. Briefly, the tube’s OD was measured at the wavelength of 636 nm. The ATP concentration in the sample (µmol/gprot) = (measured OD - Control OD)/(Standard OD - OD)* standard concentration (1000 µmol/mL)/sample concentration (gprot/L).

**Isolation and purification of CD4+ T cells**

CD4+ T cells were isolated from the animals' spleen\(^ {33} \). Briefly, the animals were sacrificed and their spleens were collected and washed with PBS. The red blood cells were rinsed with red blood cell lysis buffer. The lymphocytes liquid overflowing from the cleaned spleen were repeatedly washed with PBS, centrifugated at 1960 rpm, and incubated with CD4 (FITC) 0.25 µg/test; the CD4+ T cells were selected by fluorescence-activated cell sorting (FACS).

**Evaluation of CD4+ T cells' function**

The effect of A2AR on CD4 T cells' function, including proliferation, apoptosis, ATP production, and Th17 polarization response was evaluated by the CCK8 assay, Annexin V staining assay, and ELISA. Furthermore, the intro-cellular signaling pathway was investigated by Western blotting and RT-PCR.

**Proliferation**

The CCK8 assay was performed to determine the proliferation of the isolated CD4+ T cells. The cell density was set to 5 × 10^5/mL. The cells were added to a 96 flat culture plate at the concentration of 100 µL (5 × 10^4 cells), followed by treatment with 100
nmol/L of CGS-21680 and 1 μmol/L Sch58261, cultured for 0.5 h at 37 °C under 5% CO₂ atmosphere. Then, 20 μL/well of CCK8 was added into the system and continuously culture for 2 h at 37 °C under 5% CO₂ atmosphere. The OD₄₅₀ of the cultured cells was measured with an enzyme-labeled instrument.

**Apoptosis**
The rate of apoptosis of CD4+ T cells was measured by Annexin V staining assay. Briefly, the collected CD4+ T cells were incubated in a 6-well plate (5 × 10⁶ cells/well), to which 100 nmol/L of CGS-21680 and 1 μmol/L of Sch58261 were added and cultured at 37 °C under 5% CO₂ for 0.5 h. Then, 5 μL of Annexin V-APC and 5 μL of 7-AAD staining liquid were added to the wells. FACS was performed to detect the rate of apoptosis.

**Th17 polarization**
ELISA was performed to determine the concentration of cytokines in the supernatant of CD4+ T cells. High-sensitivity (sensitivity range: 0.25-16 pg/mL) ELISA kit (R&D Systems, Minneapolis, Minnesota, United States) was used to analyze the IL-17 and IFNγ protein concentrations according to the manufacturer’s protocol.

**Signaling pathway**
The protein and mRNA of the signaling-associated molecules were determined with Western blotting and RT-qPCR, as described previously.

**Statistical Analysis**
Kolmogorov-Smirnov test was performed using the SPSS 22.0 software to indicate a normality distribution of all data. Data were expressed as the mean ± standard error of the mean. The differences between the two groups were analyzed by Student’s t-test. Values in the same row with different superscripts indicate significance (P < 0.05), otherwise no significance (P > 0.05).
**Ethical considerations**

The experimental protocol was approved by the Animal Care and Use Committee of Hainan Province and in conducted accordance with the Chinese guidelines for animal welfare.

**RESULTS**

**PI-IBS mouse model and the effect of A2AR on the clinical features of PI-IBS**

On the 56th day of infection with *T. spiralis*, the experimental mice showed no significant colonic inflammation (Figure 1), accompanied by a higher AWR score, longer CTT, and higher Bristol stool grade (Tables 3 and 4), suggesting that the PI-IBS mouse model was successfully established. Injection with the A2AR agonist CGS21680 could significantly increase the clinical features and slightly accelerate intestinal inflammation. On the other hand, the A2AR antagonist Sch58261 remarkably improved the model animal's clinical manifestation. These results together confirm the important role of A2AR in the development of PI-IBS. However, the underlying mechanism behind this role warrants further investigation.

**The changes in the intestinal ATP and A2AR in the PI-IBS mouse**

When compared with the control group, the intestinal ATP significantly increased in the PI-IBS mouse (*P* < 0.05; Figure 2) along with an increase in the A2AR protein expression (*P* < 0.05; Figure 3A and B). The expression of the A2AR antagonist Sch58261 significantly decreased the intestinal ATP and A2AR protein levels, while the A2AR antagonist CGS21680 significantly increased them (*P* < 0.05; Figure 2 and Figure 3A and B). These results together hint that adenosine and A2AR are involved in the development of PI-IBS.

**CD4+ T cells’ isolation and A2AR expression**
CD4+ T cells were isolated and purified from the PI-IBS mouse’s spleen by FACS (Figure 3A) for further functional evaluation in vitro. Cellular immunohistochemistry study results revealed that the CD4+ T cells from PI-IBS mice obviously increased their A2AR protein and mRNA expression, which could have been regulated by the A2AR’s antagonist and agonist respectively (P < 0.05; Figure 3C-E). These results suggest that CD4+ T cells could express A2AR, and their capability of expressing was regulated by A2AR.

CD4+ T cells from the PI-IBS mouse showed obvious proliferation, decreased apoptosis, and increased ATP and IL-17 production, which could have been regulated by the A2AR’s antagonist and agonist, respectively (P < 0.05; Figure 4A-D). These results suggest that CD4+ T cells’ function, especially Th17 polarization was regulated by A2AR.

**CD4+ T cells’ signaling pathway**

The JAK/P-JAK protein level and the mRNA expression of CD4+ T cells from the PI-IBS mouse were detected by Western blotting and RT-PCR. The A2AR antagonist Sch58261 decreased the JAK/P-JAK protein and mRNA expression, while the A2AR agonist CGS21680 increased the JAK/P-JAK protein and mRNA expression. These results suggest that, during PI-IBS, the JAK pathway participates in CD4+ T cells’ A2AR-associated biological behavior (Figure 5A and B).

The STAT3/P-STAT3 protein level and the mRNA expression of CD4+ T cells from PI-IBS mice were detected by Western blotting and RT-PCR. The A2AR antagonist Sch58261 decreased the STAT3/P-STAT3 protein and mRNA expression, while the A2AR agonist CGS21680 increased the STAT3/P-STAT3 protein and mRNA expression. These results suggest that, during PI-IBS, the STAT3 pathway participated in CD4+ T-cells’ A2AR-associated biological behavior (Figure 5A and B).

The RORγt protein level and the mRNA expression of CD4+ T cells from PI-IBS mice were detected by Western blotting and RT-PCR. The A2AR antagonist Sch58261 decreased the RORγt protein and mRNA expression, while the A2AR agonist CGS21680
increased the RORγt protein and mRNA expression. These results together suggest that, during PI-IBS, the RORγt pathway participated in CD4+ T cells’ A2AR-associated biological behavior (Figure 5A and B).

DISCUSSION

Lasting low degree inflammation occurs in the intestine in IBS and especially in PI-IBS because of complex immune abnormalities. Therefore, the patients show continuous clinical symptoms and not distinct biochemical and pathological changes. However, the precise mechanism underlying the immune regulation in PI-IBS is unclear. Therefore, the relationship between ARs and IL-17 response in the inflammation and immune response in PI-IBS should be investigated.

Our results showed that the increasing intestinal ATP level in PI-IBS was accompanied by increasing intestinal A2AR level, which suggested that ATP and A2AR could participate in the pathogenesis of PI-IBS. The quantitative changes that lead to the development of PI-IBS, resulted from PI-IBS, or increased PI-IBS is not known. The animal’s intestinal tissue remain unchanged after injecting A2AR antagonists but the clinical symptoms improved. This result was contradictory to the previous hypotheses which state that adenosine and its receptors play a protective role in inflammation. During the inflammatory process, adenosine and its receptors could trigger another unknown pathway to maintain the balance of inflammation and immune response, otherwise, a prolonged presence of increased concentrations of adenosine can be harmful and can induce an immunosuppressed environment that is ideal for the onset and development of neoplasia[34]. Our study reports the complex condition in PI-IBS.

We elucidated the mechanism underlying the role of A2AR in PI-IBS. CD4+ T cells act as an important regulator in inflammation and immune response. Th17 polarization is one of the most important pathways of the immune event associated with CD4+ T cells. We first found that the number of CD4+ T cells distinctly increased, accompanied by an increase in the expression of A2AR on them. We then found that the agonist of A2AR increased the number of CD4+ T cells accompanied by an increase in the expression of
A2AR on them. A2AR agonist increased the proliferation of CD4+ T cells and inhibited their apoptosis. On the contrary, the A2AR antagonist decreased the number of CD4+ T cells and inhibited their function. These results suggested that CD4+ T cells participate in the A2AR induced intestinal inflammation.

Th17 polarization was interestingly associated with A2AR. IL-17 plays a dual role in inflammation and immune response. IL-17 can improve inflammation in some conditions. For instance, HSP70 induces γδ T cells to produce IL-17 that protects mice against PI-IBS[10]. IL-17 protects mice against colitis induced by adherent-invasive Escherichia coli[35]. In other conditions, IL-17 can increase inflammation. For instance, Th17 cells and IL-17 promotes skin and lung inflammation and fibrosis in the bleomycin-induced murine model of systemic sclerosis[36].

Unfortunately, the studies involving animal models have not determined the efficacy of anti-IL-17 antibodies in improving intestinal inflammation[37]. Our results showed that the increase in A2AR expression and chronic inflammation is associated with the Th17 polarization of CD4+ T cells, which suggested that the A2AR-CD4+ T cells-IL-17 polarization pathway is present in PI-IBS.

We investigated the intracellular signaling pathway of the Th17 polarization of CD4+ T cells induced by A2AR. We found that JAK and STAT3 protein and their phosphorylated product were significantly increased in CD4+ T cells of the PI-IBS mouse model. The A2AR antagonist reduced the level of protein and mRNA of JAK and STAT3, which was increased by the A2AR agonist. Our results suggested that the JAK-STAT3 pathway is an important intracellular signaling pathway of the A2AR-induced Th17 polarization by CD4+ T cells in PI-IBS.

The present study has some limitations. The type of visceral hypersensitivity in the PI-IBS mouse model was not investigated. The water content of the Bristol stool was not analyzed. The direct isolation of CD4+ T cells from the intestine is difficult. Therefore, we used the CD4+ T cells isolated from the spleen, which might have resulted in some variation compared with the CD4+ T cells of the intestine.
CONCLUSION
In the present study, we established mouse model of PI-IBS and administrated it with A2AR’s agonist and antagonist. Interestingly, we found that A2AR increased in PI-IBS accompanied with CD4+ T cells’ activation, proliferation and production of IL-17. A2AR antagonist improved the clinical symptoms and the cellular and molecular events during PI-IBS. To conclude, the present study showed that the upregulation of A2AR increases PI-IBS by promoting the Th17 polarization by CD4+ T cells.

ARTICLE HIGHLIGHTS

Research background
It is proved that low-grade inflammation and immunological dysfunction are involved in post-infectious irritable bowel syndrome (PI-IBS). T helper 17 (Th17) polarization occurs in IBS. Adenosine and its receptors participate in intestinal inflammation and immunity regulation.

Research motivation
To elucidate the pathogenesis of PI-IBS and find some potential target for the treatment of this disease.

Research objectives
This study aims to explore the role of adenosine 2A receptor (A2AR) in PI-IBS and its underlying mechanism, especially the relationship between A2AR and Th17 response.

Research methods
PI-IBS model was established by infecting mice with Trichinella spiralis. The expression and function of A2AR and CD4+ T lymphocytes were examined. Furthermore, the effect of A2AR on CD4+ T lymphocytes’ Th17 polarization was observed and the clinical features of PI-IBS were evaluated.
Research results

The main results can be summarized as follow: (1) expression of ATP and A2AR and inhibition of A2AR improved the clinical features in PI-IBS; (2) CD4+ T cells expressed A2AR and produced IL-17 in vitro, which was regulated by the A2AR agonist and antagonist; and (3) A2AR antagonist increased the production of IL-17 by CD4+ T cells via Janus kinase-signal transducer and activator of transcription-receptor-related orphan receptor γ signaling pathway.

Research conclusions

The upregulation of A2AR increases PI-IBS by promoting the Th17 polarization of CD4+ T cells.

Research perspectives

The present study could open a new pathway to elucidate pathogenesis of PI-IBS and explore a novel therapy target for this disease.

REFERENCES


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