Extracellular adenosine induces hypersecretion of IL-17A by T-helper 17 cells through the adenosine A2a receptor to promote neutrophilic inflammation

Mieko Tokano^{1,2}, Sho Matsushita^{1,3}, Rie Takagi¹, Toshimasa Yamamoto², and Masaaki Kawano^{1,*}

¹Department of Allergy and Immunology, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama, Saitama 350-0495, Japan

²Department of Neurology, Saitama Medical University, 38 Morohongo, Moroyama, Saitama, 350-0495, Japan

³Allergy Center, Saitama Medical University, 38 Morohongo, Moroyama, Saitama 350-0495, Japan

*Correspondence: Department of Allergy and Immunology, Faculty of Medicine, Saitama Medical University, 38 Morohongo, Moroyama, Saitama 350-0495, Japan

Tel.: +81 49 276 1173; Fax: +81 49 294 2274

E-mail address: mkawano@saitama-med.ac.jp

Running title: Adenosine modulates neutrophilic inflammation

Key words: Adenosine, Adenosine A2a receptor, CD4⁺ T cells, IL-17A, Th17 cells, EAE

Abstract

Extracellular adenosine, produced from ATP secreted by neuronal or immune cells, may play a role in endogenous regulation of inflammatory responses. However, the underlying molecular mechanisms are largely unknown. Here, we show that adenosine primes hypersecretion of interleukin (IL)-17A by CD4⁺ T cells via T cell receptor activation. This hypersecretion was also induced by an adenosine A2a receptor (A2aR) agonist, PSB0777. In addition, an A2aR antagonist, Istradefylline, and inhibitors of adenylylase and protein kinase A (both of which are signaling molecules downstream of the Gs protein coupled with the A2aR), suppressed IL-17A production, suggesting that activation of A2aR induces IL-17A production by CD4⁺ T cells. Furthermore, immune subset studies revealed that adenosine induced hypersecretion of IL-17A by T-helper (Th)17 cells. These results indicate that adenosine is an endogenous modulator of neutrophilic inflammation. Administration of an A2aR antagonist to mice with experimental autoimmune encephalomyelitis led to marked amelioration of symptoms, suggesting that suppression of adenosine-mediated IL-17A production is an effective treatment for Th17-related autoimmune diseases.

Introduction

T-helper (Th)17 cells are a subset of T-helper cells induced by stimulation of naïve CD4⁺ T cells with both tumor growth factor (TGF)- β and interleukin (IL)-6 in the presence of T cell receptor signaling. IL-17A production by Th17 cells increases neutrophilic inflammation (1-3); however, not all neutrophilic inflammatory diseases are explained by known Th17 responses (4). Indeed, it is likely that as-yet-unknown Th17 or neutrophilic inflammatory responses occur. Here, we show that adenosine induces IL-17A production by CD4⁺ T cells directly. Extracellular adenosine is one of the first “signals” identified during regulation of a large number of physiological and pathological processes, including bulging of an artery (5), sleep promotion (6), and regulation of nerve action (7,8). Extracellular adenosine is produced from secreted ATP that undergoes rapid stepwise dephosphorylation by ectonucleotidases such as the E-NTPDase CD39, which converts ATP or ADP to ADP or AMP, respectively, and the 5'-nucleotidase CD73, which dephosphorylates AMP to adenosine (9); both CD39 and CD73 are expressed by activated CD4⁺ T cells and antigen presenting cells (APCs) (10,11). Extracellular adenosine stimulates adenosine receptors (A1R, A2aR, A2bR, and A3R) belonging to a superfamily of membrane proteins called the G protein-coupled receptor family of class A seven-transmembrane domain receptors. A2aR and A2bR signal the Gs protein to trigger cAMP synthesis, which in turn activates adenylyl cyclase and protein kinase A. By contrast, A1R and A3R signal the Gi protein to trigger cAMP degradation. In addition, A2bR also signals the Gq protein, which in turn activates phospholipase C. In an immunological context, adenosine receptors are expressed by various immune cells, including T cells and APCs (12). It is also suggested that adenosine stimulates neutrophil chemotaxis and phagocytosis via A1R and A3R (13). In addition, adenosine induces Th17

61 differentiation by activating A2bR on CD4⁺ T cells (14). By contrast, several reports suggest that
62 adenosine suppresses Th17 differentiation via activation of A2aR on CD4⁺ T cells (15-17).
63 Considering that the G protein downstream of A2aR is Gs, and those of A2bR are Gs and Gq (5),
64 it is assumed that induction of A2bR-mediated Th17 differentiation is induced through
65 simultaneous activation of Gq and suppression of Gs, or via other unknown mechanisms.
66 Therefore, the precise molecular mechanism(s) underlying the effect of adenosine on Th cells is
67 unclear. Here, we show that adenosine promotes IL-17A production in a two-way mixed
68 lymphocyte reaction (MLR). In addition, an A2aR agonist (PSB0777) induced IL-17A
69 production, and an A2aR antagonist (Istradefylline) inhibited production, induced by adenosine,
70 suggesting that activation of A2aR plays a role in adenosine-mediated IL-17A production. This
71 notion was further supported by the observation that inhibitors of adenylylase and protein
72 kinase A, both of which are signaling molecules downstream of the Gs protein (18), also
73 suppressed adenosine-mediated IL-17A production. Immune subset studies suggested that Th17
74 cells play a role in adenosine-mediated hypersecretion of IL-17A. Administration of an A2R
75 antagonist to mice with experimental autoimmune encephalomyelitis (EAE) (19,20) markedly
76 ameliorated symptoms. Taken together, the data indicate that adenosine-dependent
77 hypersecretion of IL-17A by Th17 cells contributes not only to antibacterial defense but also to
78 neutrophilic autoimmune diseases, and that suppressing this process may be an effective therapy
79 for the latter.

Methods

Mice

Balb/c mice were obtained from Japan SLC, Inc. SJL/J mice were obtained from Charles River Laboratories Japan, Inc. Mice were housed in appropriate animal care facilities at Saitama Medical University and handled according to international guidelines for experiments with animals. All experiments were approved by the Animal Research Committee of Saitama Medical University.

Two-way MLR

Splenic lymphocytes were collected by lyzing tissue in a Dounce homogenizer, followed by layering over Ficoll Paque (GE health care, Chicago, IL, USA), as described previously (21). Balb/c splenic lymphocytes (3×10^6) were mixed with SJL/J splenic lymphocytes (3×10^6) in 500 μ L of DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μ M 2-mercaptoethanol (D10 medium) in 24 well plates in the presence of adenosine (0–1 mM) (Sigma, St. Louis, MO, USA); in the presence of each adenosine receptor agonist (0–10 μ M) (A1R: 2-Chloro-N6-cyclopentyladenosine (CCPA), Tocris, Bristol, UK; A2aR: PSB0777, Tocris; A2bR: BAY 60-6583, Tocris; A3R: HEMADO, Tocris); in the presence of A2aR antagonist (0–1 nM) (Istradefylline, Sigma) plus adenosine (100 μ M); in the presence of an adenylyl cyclase inhibitor (0–1 μ M) (MDL-12330A, Enzo Life Sciences, Farmingdale, NY, USA) or a protein kinase A inhibitor (0–1 μ M) (H-89, Tocris) plus adenosine (100 μ M) to inhibit A2aR signaling; or in the presence of a CD39 inhibitor (0–1 μ M) (ARL67156, Tocris) or a CD73 inhibitor (0–1 μ M) (adenosine 5'-(α ,

β -methylene) diphosphate (AMP-CP; Tocris) plus ATP (100 μ M) (GE healthcare). After mixing, the plates were incubated for 7 days at 37°C. The supernatants were collected for use in cytokine ELISAs.

Flow cytometry analysis

The MLR was performed for 7 days in the presence or absence of adenosine (100 μ M) (as described above). After 7 days, cells were blocked with anti-mouse CD16/CD32 antibodies (BioLegend, San Diego, CA, USA) and then stained for 30 min at 4°C with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 antibody (BioLegend). The cells were then fixed, permeabilized, and stained with a phycoerythrin (PE)-conjugated anti-mouse IL-17A antibody (BioLegend). Finally, the cells were washed and analyzed on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ) using FACSDiva acquisition software (BD Biosciences).

Isolation of CD4⁺, CD4⁺CD62L⁺ T cells, and B cells

CD4⁺ T cells within the Balb/c and SJL/J splenocyte populations were isolated from the mixture prepared previously after rupturing red blood cells (22). Cells were isolated by positive selection of CD4⁺ T cells using magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. CD4⁺CD62L⁺ T cells were isolated from Balb/c splenocytes by a combination of negative and positive selection by MACS. During positive selection of CD62L⁺ T cells, negatively isolated CD4⁺ T cells were collected as the flow through fraction. B cells were isolated from Balb/c splenocytes by positive selection using MACS. Cells were resuspended in 1 mL of D10 medium and counted. The purity and

viability of CD4⁺ T cells, CD4⁺CD62L⁺ T cells, and B cells were >90% (Sup. Fig. 1). The purity and viability of cells in the flow through fraction collected during isolation of CD4⁺CD62L⁺ T cells are shown in Supplementary Figure 1.

Cell sorting

Balb/c CD4⁺ T cells (prepared as described above) were labeled for 30 min at 4°C with PE-conjugated anti-mouse CCR3, CCR5, CCD6, CD25, or CD62L antibodies (BioLegend). The cells were then washed and sorted using a FACS Aria II flow cytometer (BD Biosciences). The purity and viability of the sorted cells are Supplementary Figure 1.

CD3/CD28 stimulation

CD4⁺ T cells (1×10^6) were stimulated for 7 days at 37°C with anti-mouse CD3 (BioLegend) (1 µg/mL) and CD28 (BioLegend) (0.5 µg/mL) antibodies (CD3/CD28) in the presence of adenosine (0–1 mM), each adenosine receptor agonist (0–10 µM) (A1R: 2-Chloro-N6-cyclopentyladenosine (CCPA), Tocris, Bristol, UK; A2aR: PSB0777, Tocris; A2bR: BAY 60-6583, Tocris; A3R: HEMADO, Tocris), an A2aR antagonist (Istradefylline; 0–1 nM) plus adenosine (600 µM), or an adenylyl cyclase inhibitor (0–1 µM) (MDL-12330A) or a protein kinase A inhibitor (0–1 µM) (H-89) plus adenosine (600 µM) in 500 µL of D10 medium. After cell sorting, cells (3×10^5) were plated in 24 well plates and stimulated for 7 days at 37°C with anti-mouse CD3 (1 µg/mL) and CD28 (0.5 µg/mL) antibodies in the presence of adenosine (600 µM) in 100 µL of D10 medium. After stimulation, the supernatants were collected for use in cytokine ELISAs.

Adenosine or ATP ELISAs

MLR was performed by mixing Balb/c lymphocytes (6×10^6) with SJL/J lymphocytes (6×10^6) in a 15 mL tube for 0–24 h at 37°C in the presence of a CD39 inhibitor (ARL67156) (0–1 μ M) and a CD73 inhibitor (AMP-CP) (0–1 μ M) in 200 μ L of D10 medium. CD3/CD28 stimulation was performed for 24 h at 37°C in a 15 mL tube by incubating Balb/c CD4⁺ T cells (1×10^7) with anti-mouse CD3 (1 μ g/mL) and CD28 (0.5 μ g/mL) antibodies plus ARL67156 (1 μ M) or AMP-CP (1 μ M) in 200 μ L of D10 medium. LPS stimulation was performed for 24 h at 37°C in a 15 mL tube by incubating Balb/c B cells (1×10^7) or Balb/c bone marrow (BM) derived-dendritic cells (BM-DCs) (1×10^7) generated from mouse bone marrow cells as described previously (23) with LPS (Sigma) (0.5 μ g/ml) plus ARL67156 (1 μ M) or AMP-CP (1 μ M) in 200 μ L of D10 medium. The purity and viability of BM-DCs were >90% (Sup. Fig. 1). After incubation, the supernatants were collected and tested in adenosine or ATP ELISAs (Biovision, Milpitas, CA, USA).

Differentiation of naïve CD4⁺ T cells

Naïve CD4⁺ T cells (3×10^5) in 500 μ L of D10 medium in 24 well plates were stimulated for 7 days at 37°C with anti-mouse CD3 (1 μ g/mL) and CD28 (0.5 mg/mL) antibodies plus mouse IL-6 (20 ng/mL) (Peprotech, Rocky Hill, NJ), and human TGF- β 1 (2 ng/mL) (Peprotech) in the presence of an A2aR antagonist (Istradefylline) (0–1 nM). After incubation, cells in 500 μ L of D10 medium were stimulated for another 7 days at 37°C with anti-mouse CD3 (1 μ g/mL) and CD28 (0.5 μ g/mL) antibodies in the presence of Istradefylline (0–1 nM). Supernatants were collected for use in cytokine ELISAs.

EAE model

EAE was induced as described previously (19). Briefly, SJL/J mice received a subcutaneous inguinal injection (100 µg/mouse) of the proteolipid protein (PLP) peptide (PLP139-151, Tocris) emulsified in complete Freund's adjuvant (CFA) containing mycobacterium tuberculosis H37Ra (100 µg/mouse; Difco, Detroit, MI, USA). Mice also received oral PBS(-) or an A2aR antagonist (Istradefylline) (6 µg/mouse) once every 2 days from Day -7 to Day +18 after immunization with PLP peptide (Day 0). Mice were examined daily for signs of EAE, which were graded as described (24).

Peptide pulse assay

At 7 days post-subcutaneous immunization with PLP peptide emulsified in CFA, splenocytes (2×10^6 in 200 µL of D10 medium) were seeded in 96-well round plates and pulsed for 3 days at 37°C with PLP peptide (10 µM) in the presence of adenosine (600 µM) and an A2aR antagonist (Istradefylline; 0–1 nM). Supernatants were collected for use in cytokine ELISAs.

Cytokine ELISAs

The concentrations of IFN-γ, IL-5, IL-17A, IL-17F, and IL-22 in cell supernatants were measured using specific ELISA kits (DuoSet Kit, R&D, Minneapolis, MN, USA). Any value below the lower limit of detection (15.6 pg/mL) was set to 0. No cytokine cross-reactivity was observed within the detection ranges of the kits. If necessary, samples were diluted appropriately so that the measurements fell within the appropriate detection range for each cytokine.

Statistical analysis

195 Differences between two groups were analyzed using an unpaired Student's t-tests. Differences
 196 between three or more groups were analyzed using one-way ANOVA with Tukey's post-hoc test.
 197 Clinical scores were analyzed using a non-parametric Mann-Whitney *U*-test. All calculations
 198 were performed using KaleidaGraph software (Synergy software, Reading, PA, USA). A P value
 199 < 0.05 was considered statistically significant.

Results

Adenosine promotes IL-17A production by CD4⁺ T cells in an MLR

First, we analyzed the effect of adenosine on CD4⁺ T cells during T cell-APC interactions in an MLR (25). We found that CD4⁺ T cells exposed to adenosine secreted IL-17A in a dose-dependent manner (Fig. 1A–C). Since both agonist-mediated IL-17A production and antagonist-mediated suppression of adenosine-mediated IL-17A production were observed in the presence of an adenosine A2aR agonist (PSB0777) and an antagonist (Istradefylline), respectively (Fig. 1D and Sup. Fig. 2), we hypothesized that IL-17A in the MLR was produced by CD4⁺ T cells stimulated via the A2aR. This notion was supported by the finding that inhibitors of signaling molecules downstream of the A2aR also suppressed IL-17A production (Fig. 1E). Furthermore, ATP induced IL-17A production in the MLR, which was suppressed by the A2aR antagonist and by inhibitors of CD39 and CD73 (Fig. 1F), suggesting that adenosine plays a role in IL-17A production. Since the A2aR antagonist and inhibitors of CD39 and CD73 also inhibited basal production of IL-17A in the MLR (Fig. 1G), we postulate that *de novo* adenosine production is induced in the MLR.

Adenosine production in the MLR

CD39 and CD73 expressed on the surface of endothelial cells (26,27) and immune cells (10,11), including T cells and DCs, are critical for production of adenosine from ATP. Since we found that inhibitors of CD39 and CD73 inhibited basal IL-17A production in the MLR (Fig. 1G), we next addressed the source of adenosine production in the MLR. As shown in Figure 2A, production of adenosine and ATP was time-dependent. In accordance with suppression of

IL-17A production, inhibitors of CD39 and CD73 suppressed adenosine production at 24 h after the start of the MLR (Fig. 2B and C). Since the MLR induces activation of CD4⁺ T cells by APCs (25), we also addressed adenosine production by activated CD4⁺ T cells, B cells, and BM-DCs (Fig. 2D–F). Production of both adenosine and ATP by activated CD4⁺ T cells, B cells, and BM-DCs was observed; inhibitors of CD39 and CD73 suppressed production by activated CD4⁺ T cells (Fig. 2D). This suggests that adenosine produced by CD4⁺ T cells may induce spontaneous IL-17A secretion by CD4⁺ T cells in the MLR.

Th17 cells hypersecrete IL-17A in the presence of anti-CD3/CD28 antibodies and adenosine

Next, we tried to identify the Th subset that generated IL-17A in the presence of adenosine. First, we confirmed that CD4⁺ T cells expressed the A2aR and secreted IL-17A by stimulating them with agonistic anti-CD3/CD28 antibodies in the presence of adenosine (Fig. 3A). Time course studies showed that adenosine-mediated IL-17A production was detected from 3 days post-CD3/CD28 stimulation (Fig. 3B and Sup. Fig. 3). Administration of adenosine within 6 h of antibody stimulation triggered IL-17A production; however, administration at 24 h post-stimulation did not (Fig. 3C). As in the MLR, CD4⁺ T cells also produced IL-17A after stimulation with anti-CD3/CD28 antibodies in the presence of an A2aR agonist (Fig. 3D and Sup. Fig. 4). Adenosine-mediated IL-17A production by CD3/CD28-stimulated CD4⁺ T cells was suppressed by an A2aR antagonist (Fig. 3D and Sup. Fig. 4). This was supported by data showing that inhibitors of signaling molecules downstream of the A2R also suppressed IL-17A production (Fig. 3E). Production of other Th17-related cytokines was also induced by adenosine through the A2aR (Fig. 4). This again suggests that activated CD4⁺ T cells produce IL-17A upon A2aR activation. Furthermore, we noticed that CD4⁺CD62L⁻, but not CD4⁺CD62L⁺, cells

produced IL-17A after CD3/CD28 stimulation in the presence of adenosine, suggesting that adenosine induces IL-17A production by effector Th cells (Fig. 3F). Therefore, we performed immune subset studies after separating CD4⁺ T cells using anti-chemokine receptor (CCR) antibodies. As shown in Figure 3G, CD4⁺CCR6^{hi} T cells produced IL-17A upon stimulation of CD3/CD28, and production was strongly up-regulated in the presence of adenosine. Since CCR6 is a typical marker of Th17 cells (28,29), this suggests that activated Th17 cells hypersecrete IL-17A in the presence of adenosine.

An adenosine A2aR antagonist ameliorates IL-17A-related autoimmune EAE responses

The above results raise the possibility that adenosine-mediated hypersecretion of IL-17A by Th17 cells contributes to Th17-related autoimmune diseases. This hypothesis is supported by a report showing that CD73 knockout mice are resistant to EAE (30), a Th17-mediated autoimmune disease (20). We expected, therefore, that A2aR antagonist-mediated suppression of Th17 responses should improve EAE. To address this, we examined the efficacy of an A2aR antagonist in EAE model SJL/J mice (19). EAE was induced by immunization of mice with an I-A^s restricted helper peptide derived from a myelin PLP peptide comprising amino acids 139–151 (HSLGKWLGHDPKF). The peptide was emulsified in CFA. First, we confirmed that the A2aR antagonist suppressed adenosine-mediated IL-17A production by CD3/CD28-stimulated CD4⁺ T cells from SJL/J strain mice (Fig. 5A). The A2aR antagonist also significantly suppressed adenosine-mediated IL-17A production after, but not during, differentiation of Th17 cells from naïve CD4⁺ T cells (Fig. 5B). By contrast, and in agreement with Figure 3G, adenosine administration did not induce IL-17A production during and after differentiation of Th1, Th2, and Treg cells from naïve CD4⁺ T cells (data not shown). Next, we

269 pulsed splenocytes with the PLP peptide after immunization to confirm that IL-17A production
 270 was induced in a peptide-dependent manner, and that production was up-regulated by adenosine.
 271 As expected, IL-17A production occurred in a peptide-dependent manner and was up-regulated
 272 by adenosine (Fig. 5C). Furthermore, the A2aR antagonist suppressed production of IL-17A,
 273 suggesting that the A2aR antagonist inhibits IL-17A production by CD4⁺ T cells induced by
 274 immunization with the PLP peptide. Finally, the A2aR antagonist was administered orally to
 275 mice before and during EAE induction (Fig. 5D and E). As shown, the clinical scores of mice
 276 receiving the A2aR antagonist were markedly lower than those of control mice (receiving water)
 277 at 18 days post-immunization with the PLP peptide (Fig. 5D). Accordingly, histological studies
 278 showed that the numbers of central nervous system-infiltrating CD3⁺ cells in mice receiving the
 279 A2aR antagonist were much lower than those in mice receiving water (Fig. 5E). This suggests
 280 that the A2aR antagonist suppresses IL-17A-mediated autoimmune responses by suppressing
 281 hypersecretion of IL-17A by Th17 cells.

Discussion

Here, we showed that adenosine induces hypersecretion of IL-17A by Th17 cells. Addition of adenosine (1 mM) to a two-way MLR increased IL-17A production to > 25 times the basal level; however, both basal production and increased production of IL-17A were suppressed by an A2aR antagonist, and by CD39/CD73 inhibitors. This indicates that hypersecretion of IL-17A in the presence of adenosine occurs by other mechanisms in addition to T cell-APC interactions. Since endothelial cells and nervous system cells also express CD39/CD73 (26,27,31,32) and produce adenosine (8,33), and activated CD4⁺ T cells in the present study hypersecreted IL-17A at 6 h post-CD3/CD28 stimulation, it is possible that activated Th17 cells also receive adenosine from endothelial and neuronal cells to induce hypersecretion of IL-17A.

It is suggested that physiological concentrations of adenosine are lower than 1 μ M, but can be increased by stimuli such as high K⁺ levels, electrical stimulation, glutamate receptor agonists, hypoxia, hypoglycemia, and ischemia (34). To obtain sufficient adenosine (> 100 μ M) to trigger hypersecretion of IL-17A, activated Th17 cells may need to make contact with non-immune cells such as adenosine-producing endothelial cells (35) and neuronal cells (36) to form a microenvironment with a high adenosine concentration (as observed during T cell-APC interactions at immunological synapses) (37). Thus, A2aR antagonists rather than CD39/CD73 inhibitors might be more effective at inhibiting *de novo* adenosine-mediated hypersecretion of IL-17 by Th17 cells. A previous study suggests that intracellular adenosine is transported out of cells by efficient equilibrative transporters (38); CD39/CD73 inhibitors would not suppress this type of *de novo* adenosine production.

With regard to the effect of adenosine on other Th subsets, our observations were

different from those of previous reports (39,40); here, we observed that adenosine up-regulated IFN- γ (a Th1-related cytokine) secretion at 5 and 7 days and had no significant effect on IL-5 (a Th2-related cytokine) secretion by CD4⁺ T cells after CD3/CD28 stimulation with 600 μ M of adenosine, although IL-17A production was significant (Sup. Fig. 3). This suggests that adenosine induces hypersecretion of IL-17A by Th17 cell but does not suppress Th1 and Th2 activity. However, previous studies report that adenosine-mediated suppression of IFN- γ and IL-5 was observed 1 day after T cell receptor-mediated stimulation of CD4⁺ T cells (39,40). This may indicate that in the short term adenosine prioritizes stimulation of Th17 cell activity rather than that of Th1 and Th2 cells, and that it does not suppress effector Th activity in the long term.

It is also suggested that the A2aR agonist, CGS21680, suppresses Th17 differentiation (15-17). This result is opposite to ours; one reason for this may be differences in the source of the A2aR agonist. The A2aR agonist CGS 21680 is much less selective than the A2aR agonist we used in this study (PSB0777); this is because CGS21680 not only binds to the A2aR but also to A1R and A3R, which are associated with the Gi protein (which has opposite effects to the Gs protein) (41,42). Therefore, it is probable that CGS21680 may cancel out any agonist effects by activating A1R and A3R. Also, it is suggested that the A2aR antagonist, SCH58261, up-regulates Th17 differentiation in mice (17). We hypothesized that up-regulation of Th17 differentiation by SCH58261 may be induced through relative downregulation of A2aR activity compared with that of A2bR; this relative increase in A2bR activity induces Th17 differentiation in mice (43,44). Also, it is probable that SCH58261 induces relative increase in activity of G protein-coupled receptors other than adenosine receptors (e.g., dopamine receptors) to induce Th17 differentiation in mice (45). We also hypothesize that although SCH58261 may induce Th17 differentiation *in vivo*, it may not stimulate Th17 activity; this is because our data show

that an A2a antagonist (Istradefylline) suppressed IL-17A secretion by differentiated Th17 cells but did not suppress Th17 differentiation (Fig. 5B). This hypothesis is supported by previous data showing that SCH58261 markedly suppresses symptoms of EAE, a typical Th17-mediated disease (30).

Our data suggest that production of IL-17A is relatively higher after exposure to an A2aR agonist, PSB0777, than after exposure to an A2bR agonist, BAY 60-6583. PSB0777 is a potent adenosine A2aR agonist ($K_i = 44.4$ nM for rat brain striatal A2aR) (42), and BAY 60-6583 is a potent adenosine A2bR agonist ($K_i = 100$ nM for rat A2bR) (46). By assuming that the K_i values of PSB0777 and BAY 60-6583 are comparable, we thought that production of IL-17A mediated by activation of the A2aR might be higher than that mediated by activation of the A2bR. This hypothesis is supported by the notion that the A2aR is a high affinity receptor with activity in the low to mid-nanomolar range, whereas the A2bR has a much lower affinity for adenosine (micromolar) (5); this suggests that adenosine activates the A2aR rather than the A2bR.

A2aR antagonists have been developed for treatment of Parkinsonism (47) and malignancies (48). In addition, inhibitors of CD39/CD73 have been developed as anti-tumor drugs (49,50). Regarding the effects of adenosine on tumor immunity, a previous study suggests that adenosine suppresses effector T cell function since tumor cells express both CD39 and CD73 and secrete adenosine (51). By contrast, several reports suggest that IL-17A promotes emergence of pro-tumorigenic neutrophil phenotypes (52,53). Neutrophils in mouse tumor models promote tumor metastasis (54-56), and observations in cancer patients have linked elevated neutrophil counts in blood with increased risk of metastasis (57). Therefore, it is probable that tumor-produced adenosine induces IL-17A secretion by $CD4^+CCR6^{hi}$ T cells

followed by neutrophilic inflammation, which promotes tumor metastasis.

The results presented herein indicate that these drugs may also be effective treatments for Th17-mediated diseases (4) such as psoriasis, neutrophilic bronchial asthma, severe atopic dermatitis, and autoimmune diseases by suppressing hypersecretion of IL-17A by Th17 cells. Moreover, these drugs might be effective treatments for diseases caused by neutrophilic inflammation of unknown cause in the dermis; such diseases include Behcet uveitis (58) and vasculitis of adenosine deaminase 2 deficiency (59). This is because endothelial cells express CD39/CD73 and produce adenosine, which could induce hypersecretion of IL-17A by Th17 cells, thereby contributing to inflammation.

References

- 1 Matsuzaki, G. and Umemura, M. 2007. Interleukin-17 as an effector molecule of innate and acquired immunity against infections. *Microbiol Immunol* 51:1139.
- 2 Manel, N., Unutmaz, D., and Littman, D. R. 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* 9:641.
- 3 Ivanov, II, Zhou, L., and Littman, D. R. 2007. Transcriptional regulation of Th17 cell differentiation. *Semin Immunol* 19:409.
- 4 Tesmer, L. A., Lundy, S. K., Sarkar, S., and Fox, D. A. 2008. Th17 cells in human disease. *Immunol Rev* 223:87.
- 5 Cronstein, B. N. and Sitkovsky, M. 2017. Adenosine and adenosine receptors in the pathogenesis and treatment of rheumatic diseases. *Nat Rev Rheumatol* 13:41.
- 6 Jones, B. E. 2009. Glia, adenosine, and sleep. *Neuron* 61:156.
- 7 Boison, D., Chen, J. F., and Fredholm, B. B. 2010. Adenosine signaling and function in glial cells. *Cell Death Differ* 17:1071.
- 8 Sebastiao, A. M. and Ribeiro, J. A. 2009. Tuning and fine-tuning of synapses with adenosine. *Curr Neuropharmacol* 7:180.
- 9 Yegutkin, G. G. 2008. Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* 1783:673.
- 10 Whiteside, T. L., Mandapathil, M., and Schuler, P. 2011. The role of the adenosinergic pathway in immunosuppression mediated by human regulatory T cells (Treg). *Curr Med Chem* 18:5217.

383 11 Allard, B., Longhi, M. S., Robson, S. C., and Stagg, J. 2017. The ectonucleotidases CD39
384 and CD73: Novel checkpoint inhibitor targets. *Immunol Rev* 276:121.

385 12 Junger, W. G. 2011. Immune cell regulation by autocrine purinergic signalling. *Nat Rev*
386 *Immunol* 11:201.

387 13 Barletta, K. E., Ley, K., and Mehrad, B. 2012. Regulation of neutrophil function by
388 adenosine. *Arteriosclerosis, thrombosis, and vascular biology* 32:856.

389 14 Wilson, J. M., Kurtz, C. C., Black, S. G., Ross, W. G., Alam, M. S., Linden, J., and Ernst,
390 P. B. 2011. The A2B adenosine receptor promotes Th17 differentiation via stimulation of
391 dendritic cell IL-6. *Journal of immunology* 186:6746.

392 15 Alchera, E., Rolla, S., Imarisio, C., Bardina, V., Valente, G., Novelli, F., and Carini, R.
393 2017. Adenosine A2a receptor stimulation blocks development of nonalcoholic
394 steatohepatitis in mice by multilevel inhibition of signals that cause immunolipotoxicity.
395 *Transl Res* 182:75.

396 16 Wang, L., Wan, H., Tang, W., Ni, Y., Hou, X., Pan, L., Song, Y., and Shi, G. 2018.
397 Critical roles of adenosine A2A receptor in regulating the balance of Treg/Th17 cells in
398 allergic asthma. *Clin Respir J* 12:149.

399 17 Ansari, M. A., Nadeem, A., Attia, S. M., Bakheet, S. A., Raish, M., and Ahmad, S. F.
400 2017. Adenosine A2A receptor modulates neuroimmune function through
401 Th17/retinoid-related orphan receptor gamma t (RORgammat) signaling in a BTBR T(+)
402 Itpr3(tf)/J mouse model of autism. *Cell Signal* 36:14.

403 18 Hasko, G., Linden, J., Cronstein, B., and Pacher, P. 2008. Adenosine receptors:
404 therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* 7:759.

405 19 Nakano, K., Higashi, T., Hashimoto, K., Takagi, R., Tanaka, Y., and Matsushita, S. 2008.

Antagonizing dopamine D1-like receptor inhibits Th17 cell differentiation: preventive and therapeutic effects on experimental autoimmune encephalomyelitis. *Biochem Biophys Res Commun* 373:286.

20 Kleinewietfeld, M., Manzel, A., Titze, J., Kvakan, H., Yosef, N., Linker, R. A., Muller, D. N., and Hafler, D. A. 2013. Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells. *Nature* 496:518.

21 Higashi, T., Shimojo, N., Suzuki, S., Nakaya, M., Takagi, R., Hashimoto, K., Nakagome, K., Nakamura, K., Kohno, Y., and Matsushita, S. 2011. Coenzyme A contained in mothers' milk is associated with the potential to induce atopic dermatitis. *Int Immunol* 23:741.

22 Kawano, M., Takagi, R., Saika, K., Matsui, M., and Matsushita, S. 2018. Dopamine regulates cytokine secretion during innate and adaptive immune responses. *Int Immunol* 30:591.

23 Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S., Koch, F., Romani, N., and Schuler, G. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *Journal of immunological methods* 223:77.

24 Podojil, J. R., Padval, M. V., and Miller, S. D. 2011. Combination treatment of mice with CRx-153 (nortriptyline and desloratadine) decreases the severity of experimental autoimmune encephalomyelitis. *Cell Immunol* 270:237.

25 McDevitt, H. O. 2000. Discovering the role of the major histocompatibility complex in the immune response. *Annu Rev Immunol* 18:1.

26 Kaczmarek, E., Koziak, K., Sevigny, J., Siegel, J. B., Anrather, J., Beaudoin, A. R., Bach, F. H., and Robson, S. C. 1996. Identification and characterization of CD39/vascular ATP

diphosphohydrolase. *J Biol Chem* 271:33116.

27 Jalkanen, S. and Salmi, M. 2008. VAP-1 and CD73, endothelial cell surface enzymes in leukocyte extravasation. *Arteriosclerosis, thrombosis, and vascular biology* 28:18.

28 Acosta-Rodriguez, E. V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., Sallusto, F., and Napolitani, G. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8:639.

29 Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E., and Romagnani, S. 2007. Phenotypic and functional features of human Th17 cells. *J Exp Med* 204:1849.

30 Mills, J. H., Thompson, L. F., Mueller, C., Waickman, A. T., Jalkanen, S., Niemela, J., Airas, L., and Bynoe, M. S. 2008. CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences of the United States of America* 105:9325.

31 Braun, N., Sevigny, J., Robson, S. C., Enjoji, K., Guckelberger, O., Hammer, K., Di Virgilio, F., and Zimmermann, H. 2000. Assignment of ecto-nucleoside triphosphate diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. *Eur J Neurosci* 12:4357.

32 Kuleshkaya, N., Voikar, V., Peltola, M., Yegutkin, G. G., Salmi, M., Jalkanen, S., and Rauvala, H. 2013. CD73 is a major regulator of adenosinergic signalling in mouse brain. *PLoS One* 8:e66896.

- 33 Gunther, G. R. and Herring, M. B. 1991. Inhibition of neutrophil superoxide production by adenosine released from vascular endothelial cells. *Ann Vasc Surg* 5:325.
- 34 Latini, S. and Pedata, F. 2001. Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem* 79:463.
- 35 Carman, C. V. and Martinelli, R. 2015. T Lymphocyte-Endothelial Interactions: Emerging Understanding of Trafficking and Antigen-Specific Immunity. *Frontiers in immunology* 6:603.
- 36 Siffrin, V., Radbruch, H., Glumm, R., Niesner, R., Paterka, M., Herz, J., Leuenberger, T., Lehmann, S. M., Luenstedt, S., Rinnenthal, J. L., Laube, G., Luche, H., Lehnardt, S., Fehling, H. J., Griesbeck, O., and Zipp, F. 2010. In vivo imaging of partially reversible th17 cell-induced neuronal dysfunction in the course of encephalomyelitis. *Immunity* 33:424.
- 37 Monks, C. R., Kupfer, H., Tamir, I., Barlow, A., and Kupfer, A. 1997. Selective modulation of protein kinase C-theta during T-cell activation. *Nature* 385:83.
- 38 Fredholm, B. B. 2007. Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ* 14:1315.
- 39 Csoka, B., Himer, L., Selmeczy, Z., Vizi, E. S., Pacher, P., Ledent, C., Deitch, E. A., Spolarics, Z., Nemeth, Z. H., and Hasko, G. 2008. Adenosine A2A receptor activation inhibits T helper 1 and T helper 2 cell development and effector function. *FASEB J* 22:3491.
- 40 Lappas, C. M., Rieger, J. M., and Linden, J. 2005. A2A adenosine receptor induction inhibits IFN-gamma production in murine CD4+ T cells. *Journal of immunology* 174:1073.

475 41 Klotz, K. N. 2000. Adenosine receptors and their ligands. *Naunyn Schmiedebergs Arch*
476 *Pharmacol* 362:382.

477 42 El-Tayeb, A., Michael, S., Abdelrahman, A., Behrenswerth, A., Gollos, S., Nieber, K.,
478 and Muller, C. E. 2011. Development of Polar Adenosine A2A Receptor Agonists for
479 Inflammatory Bowel Disease: Synergism with A2B Antagonists. *ACS Med Chem Lett*
480 2:890.

481 43 Kenakin, T. 2001. Inverse, protean, and ligand-selective agonism: matters of receptor
482 conformation. *FASEB J* 15:598.

483 44 Prather, P. L. 2004. Inverse agonists: tools to reveal ligand-specific conformations of G
484 protein-coupled receptors. *Sci STKE* 2004:pe1.

485 45 Nakano, K., Yamaoka, K., Hanami, K., Saito, K., Sasaguri, Y., Yanagihara, N., Tanaka,
486 S., Katsuki, I., Matsushita, S., and Tanaka, Y. 2011. Dopamine induces IL-6-dependent
487 IL-17 production via D1-like receptor on CD4 naive T cells and D1-like receptor
488 antagonist SCH-23390 inhibits cartilage destruction in a human rheumatoid
489 arthritis/SCID mouse chimera model. *Journal of immunology* 186:3745.

490 46 Alnouri, M. W., Jepards, S., Casari, A., Schiedel, A. C., Hinz, S., and Muller, C. E. 2015.
491 Selectivity is species-dependent: Characterization of standard agonists and antagonists at
492 human, rat, and mouse adenosine receptors. *Purinergic Signal* 11:389.

493 47 Kanda, T., Jackson, M. J., Smith, L. A., Pearce, R. K., Nakamura, J., Kase, H., Kuwana,
494 Y., and Jenner, P. 1998. Adenosine A2A antagonist: a novel antiparkinsonian agent that
495 does not provoke dyskinesia in parkinsonian monkeys. *Ann Neurol* 43:507.

496 48 Ohta, A., Gorelik, E., Prasad, S. J., Ronchese, F., Lukashev, D., Wong, M. K., Huang, X.,
497 Caldwell, S., Liu, K., Smith, P., Chen, J. F., Jackson, E. K., Apasov, S., Abrams, S., and

498 Sitkovsky, M. 2006. A2A adenosine receptor protects tumors from antitumor T cells.
499 *Proceedings of the National Academy of Sciences of the United States of America*
500 103:13132.

501 49 Kobie, J. J., Shah, P. R., Yang, L., Rebhahn, J. A., Fowell, D. J., and Mosmann, T. R.
502 2006. T regulatory and primed uncommitted CD4 T cells express CD73, which
503 suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine.
504 *Journal of immunology* 177:6780.

505 50 Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F.,
506 Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B., and Robson, S. C. 2007.
507 Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells
508 mediates immune suppression. *J Exp Med* 204:1257.

509 51 Moesta, A. K., Li, X. Y., and Smyth, M. J. 2020. Targeting CD39 in cancer. *Nat Rev*
510 *Immunol.*

511 52 Tuting, T. and de Visser, K. E. 2016. CANCER. How neutrophils promote metastasis.
512 *Science* 352:145.

513 53 Coffelt, S. B., Kersten, K., Doornebal, C. W., Weiden, J., Vrijland, K., Hau, C. S.,
514 Verstegen, N. J. M., Ciampricotti, M., Hawinkels, L., Jonkers, J., and de Visser, K. E.
515 2015. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast
516 cancer metastasis. *Nature* 522:345.

517 54 Bald, T., Quast, T., Landsberg, J., Rogava, M., Glodde, N., Lopez-Ramos, D., Kohlmeyer,
518 J., Riesenberger, S., van den Boorn-Konijnenberg, D., Homig-Holzel, C., Reuten, R.,
519 Schadow, B., Weighardt, H., Wenzel, D., Helfrich, I., Schadendorf, D., Bloch, W.,
520 Bianchi, M. E., Lugassy, C., Barnhill, R. L., Koch, M., Fleischmann, B. K., Forster, I.,

- Kastenmuller, W., Kolanus, W., Holzel, M., Gaffal, E., and Tuting, T. 2014. Ultraviolet-radiation-induced inflammation promotes angiotropism and metastasis in melanoma. *Nature* 507:109.
- 55 El Rayes, T., Catena, R., Lee, S., Stawowczyk, M., Joshi, N., Fischbach, C., Powell, C. A., Dannenberg, A. J., Altorki, N. K., Gao, D., and Mittal, V. 2015. Lung inflammation promotes metastasis through neutrophil protease-mediated degradation of Tsp-1. *Proceedings of the National Academy of Sciences of the United States of America* 112:16000.
- 56 Wculek, S. K. and Malanchi, I. 2015. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature* 528:413.
- 57 Templeton, A. J., McNamara, M. G., Seruga, B., Vera-Badillo, F. E., Aneja, P., Ocana, A., Leibowitz-Amit, R., Sonpavde, G., Knox, J. J., Tran, B., Tannock, I. F., and Amir, E. 2014. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst* 106:dju124.
- 58 Yazici, H., Seyahi, E., Hatemi, G., and Yazici, Y. 2018. Behcet syndrome: a contemporary view. *Nat Rev Rheumatol* 14:107.
- 59 Meyts, I. and Aksentijevich, I. 2018. Deficiency of Adenosine Deaminase 2 (DADA2): Updates on the Phenotype, Genetics, Pathogenesis, and Treatment. *J Clin Immunol* 38:569.

Abbreviations: Th, T-helper; TGF, tumor growth factor; IL, interleukin; APCs, antigen presenting cells; MLR, two-way mixed lymphocyte reaction; EAE, experimental autoimmune encephalomyelitis; BM, bone marrow; BM-DC, bone-marrow-derived dendritic cell; CD3/CD28, agonistic anti-CD3/CD28 antibodies; PLP, myelin proteolipid protein; PLP peptide, I-A^s restricted helper peptide derived from the PLP; CFA, complete Freund's adjuvant; CCR, chemokine receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; MACS, magnetic-activated cell sorting; Ab, antibody; n, number of repeat experiments; SD, standard deviation.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (C) (no. 19K07201), awarded to M.K., a Grant-in-Aid for Young Scientists (B) (no. 18K15327) to R.T., and a Grant-in-Aid for Scientific Research (C) (no. 19K08887) awarded to S.M. by the Japanese Society for the Promotion of Science. This work was also supported by the 44th and 45th Science Research Promotion Fund, awarded to M.K. by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

Author contributions

M.T., R.T., S.M., and M.K., performed the experiments. M.T., S.M., and M.K., conceived and designed the experiments. M.T., S.M., T.Y., and M.K., wrote the manuscript. All authors discussed the results and commented on the manuscript.

Conflicts of interest

- 565 Sho Matsushita is an employee of iMmno, Inc.
- 566 The other authors have no conflicts of interest to declare.

Figure legends

Fig. 1. Adenosine induces hypersecretion of IL-17A by CD4⁺ T cells in an MLR. A–C, An MLR was performed for 7 days in the presence of adenosine (0–1 mM). After 7 days, cells were stained with anti-CD4 (x-axis) and IL-17A (y-axis) antibodies (Abs), followed by flow cytometry analysis (A, n (number of repeat experiments) = 4). The percentage of IL-17A-producing CD4⁺ T cells within the total CD4⁺ T cell population is shown (B, n = 6–9). Cells supernatants were analyzed in an IL-17A ELISA (C, n = 6–9). D, The effects of the A2aR on IL-17A production in the presence of PSB0777 (an A2aR agonist) (*left*, n = 6–9), Istradefylline (an A2aR antagonist) plus adenosine (100 μM) (*right*, n = 6–9). E, The effects of A2aR signaling on IL-17A production in the presence of MDL-12330A (an adenylyl cyclase inhibitor) plus adenosine (100 μM) (*left*, n = 4–6), or H-89 (a protein kinase A inhibitor) plus adenosine (100 μM) (*right*, n = 4–6) were analyzed in an IL-17A ELISA. F, The effects of CD39/CD73 inhibitors on IL-17A production in the presence of ARL67156 (a CD39 inhibitor) plus ATP (100 μM) (*left*, n = 4–6) or AMP-CP (a CD73 inhibitor) plus ATP (100 μM) (*right*, n = 4–6) were analyzed in an IL-17A ELISA. G, The effects of an A2aR antagonist and CD39/CD73 inhibitors on basal IL-17A production in the presence of Istradefylline (*left*, n = 4–7), ARL67156 (*center*, n = 4–7), or AMP-CP (*right*, n = 4–7) were analyzed in an IL-17A ELISA. Data are expressed as the mean ± standard deviation (SD) and were compared using an unpaired Student's t-test (B) or one-way ANOVA with Tukey's post-hoc test (C–G). *P < 0.05 and **P < 0.01, compared with medium.

Fig. 2. Adenosine production in the MLR. A, Concentrations of adenosine or ATP in MLR

supernatants were measured in an adenosine (*left*, $n = 4-6$) or ATP (*right*, $n = 4-6$) ELISA (0–24 h). **B** and **C**, The effects of CD39 (**B**, ARL67156, $n = 4-6$) and CD73 (**C**, AMP-CP, $n = 4-6$) inhibitors on production of adenosine or ATP in the MLR were analyzed in an adenosine (*left*) or ATP (*right*) ELISA. **D**, **E** and **F**, Levels of adenosine and ATP in the supernatants of CD3/CD28-stimulated CD4⁺ T cells (**D**, $n = 4$), LPS-stimulated B cells (**E**, $n = 4$), and LPS-stimulated BM-DCs (**F**, $n = 4$) were analyzed in adenosine and ATP ELISAs at 24 h post-stimulation in the presence of CD39 or CD73 inhibitors. Data are expressed as the mean $\pm$ SD and were compared using one-way ANOVA with Tukey's post-hoc test. * $P < 0.05$ and ** $P < 0.01$, compared with medium (**B** and **C**), CD3/CD28 stimulation (**D**), or LPS stimulation (**E** and **F**).

Fig. 3. Adenosine induces hypersecretion of IL-17A by Th17 cells. **A** and **B**, CD4⁺ T cells were stimulated for 1–7 days with anti-CD3/CD28 antibodies in the presence of adenosine (0–1 mM). After stimulation, the supernatants were analyzed in an IL-17A ELISA ($n = 4-6$). **C**, Adenosine (600 μ M) was added at 0–3 days after CD3/CD28 stimulation. At 7 days post-CD3/CD28 stimulation, supernatants were analyzed in an IL-17A ELISA ($n = 4$). **D**, Effects of the A2aR on IL-17A production in the presence of PSB0777 (an A2aR agonist) (*left*, $n = 4-6$), Istradefylline (an A2aR antagonist) plus adenosine (600 μ M) (*right*, $n = 4-6$). **E**, Effects of A2aR signaling on IL-17A production in the presence of MDL-12330A (an adenylyl cyclase inhibitor) plus adenosine (600 μ M) (*left*, $n = 4-6$), or H-89 (a protein kinase A inhibitor) plus adenosine (600 μ M) (*right*, $n = 4-6$) were analyzed in an IL-17A ELISA. **F**, CD4⁺CD62L⁺ and CD4⁺CD62L⁺FT cells were stimulated for 7 days with anti-CD3/CD28 antibodies in the presence of adenosine (0–1 mM). After 7 days, supernatants were analyzed in an IL-17A ELISA

(n = 4). **G**, Subsets of CD4⁺ T cells were stimulated for 7 days by anti-CD3/CD28 antibodies in the presence of adenosine (600 μM) after isolation of each CCR cell type (high (hi) and low (lo) expression) (n = 4). Data are expressed as the mean ± SD and were compared using an unpaired Student's t-test (**g**) or one-way ANOVA with Tukey's post-hoc test (**A-F**). *P < 0.05 and **P < 0.01, compared with CD3/CD28 stimulation (**A-C**, **D**, *left*, and **F**) or CD3/28 stimulation plus adenosine (600 μM) (**D**, *right* and **E**).

Fig. 4. Effect of adenosine and an A2aR antagonist on production of Th17-related cytokines.

A, An MLR was performed for 7 days in the presence of adenosine (0–1 mM) (*top row*), PSB0777 (an A2aR agonist) (*second row*), or Istradefylline (an A2aR antagonist) plus adenosine (100 μM) (*bottom row*). At 7 days post-incubation, the supernatants were analyzed in an IL-17A (*left*), IL-17F (*center*), or IL-22 (*right*) ELISA. All experiments were repeated six to nine times. **B**, CD4⁺ T cells were stimulated with an anti-CD3/CD28 antibody for 7 days in the presence of adenosine (600 μM) (*top row*), PSB0777 (an A2aR agonist) (*second row*), or Istradefylline (an A2aR antagonist) plus adenosine (600 μM) (*bottom row*). After stimulation, supernatants were analyzed in IL-17A (*left*), IL-17F (*center*), and IL-22 (*right*) ELISAs. All experiments were repeated four to six times. Data are expressed as the mean ± SD and were compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with CD3/CD28 stimulation.

Fig. 5. Suppression of adenosine-mediated hypersecretion of IL-17A ameliorates EAE. A

and **B**, SJL/J CD4⁺ T cells were stimulated for 7 days by anti-CD3/CD28 antibodies and an A2aR antagonist (**A**, n = 4). Naïve CD4⁺ T cells were stimulated for 7 days by anti-CD3/CD28

antibodies in the presence of IL-6, TGF- β 1, and Istradefylline (an A2aR antagonist) (0–1 nM) (**B**,
left, n = 4). Alternatively, naïve CD4⁺ T cells were stimulated for 7 days by anti-CD3/CD28
antibodies in the presence of IL-6 and TGF- β 1, followed by another 7 day incubation with
anti-CD3/CD28 antibodies and Istradefylline (0–1 nM) (**B**, *right*, n = 4). After the stimulation,
supernatants were analyzed in an IL-17A ELISA. **C**, Mice were immunized subcutaneously with
PLP peptide emulsified in CFA (PLP peptide/CFA). At 7 days post-immunization, splenocytes
were incubated for 3 days with PLP peptide in the presence of Istradefylline and adenosine (600
 μ M). After the incubation, supernatants were analyzed in an IL-17A ELISA (**c**, n = 4). **D**, To
induce EAE, SJL/J mice were immunized with PLP peptide/CFA. Before and post-immunization
with the PLP peptide (Days 0 to 18), mice received oral Istradefylline (6 μ g/mouse) or water
once every 2 days. Clinical scores were recorded every day during EAE induction (**D**, n = 15).
Data were obtained from five independent experiments (n = 3 mice/group). (**E**) At 18 days
post-immunization, spinal cord sections from mice administered oral Istradefylline (*right*) or
water (*left*) were stained with hematoxylin and eosin (*upper panels*) or with an anti-mouse CD3
antibody (*lower panels*) (Scale bar, 100 μ m). Data are representative of five independent
experiments (all with similar results) and are expressed as the mean $\pm$ SD. Data were compared
using one-way ANOVA with Tukey's post-hoc test (**A–C**), or using a non-parametric
Mann-Whitney *U*-test (**D**). *P < 0.05 and **P < 0.01, compared with CD3/CD28 stimulation
plus adenosine (600 μ M) (**A**), CD3/CD28 stimulation plus IL-6 and TGF- β 1 (**B**), or PLP peptide
pulse (**C**).

Legends for the supplementary figures

Sup. Fig. 1. Purity and viability of each immune cell subset. **A**, *First row (left)*: Splenocytes or isolated CD4⁺ T cells were stained with a FITC-conjugated anti-mouse CD4 antibody (Ab) (BioLegend) (x-axis) and a PE-conjugated anti-mouse CD3 Ab (BioLegend) (y-axis). *Second row (left)*: Splenocytes or CD4⁺CD62L⁺ T cells isolated by MACS were stained with a FITC-conjugated anti-mouse CD4 Ab (BioLegend) (x-axis) and a PE-conjugated anti-mouse CD62L Ab (BioLegend) (y-axis). *First row (right)*: Splenocytes and B cells were stained with a FITC-conjugated anti-mouse CD19 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). *Second row (right)*: Splenocytes, BM leukocytes, and BM-DCs were stained with a FITC-conjugated anti-mouse CD11c Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). The number in each panel represents the percentage of each immune subset within the total cell population. **B**, *First row*: Isolated CD4⁺ (by MACS)-, CD4⁺CD62L⁻ (by cell sorting)-, or CD4⁺CD62L⁺ (by cell sorting) T cells were analyzed with a PE-conjugated anti-mouse CD62L Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). The number in each panel represents the percentage of each immune subset (- or +) within the total cell population (- plus +). Data are representative of at least three repeat experiments. *Second row*: Isolated CD4⁺ (by MACS), CD4⁺CCR3^{low} (lo) (by cell sorting), or CD4⁺CCR3^{high} (hi) (by cell sorting) T cells were analyzed with a PE-conjugated anti-mouse CCR3 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). *Third row*: Isolated CD4⁺ (by MACS), CD4⁺CCR5^{lo} (by cell sorting), or CD4⁺CCR5^{hi} (by cell sorting) T cells were analyzed with a PE-conjugated anti-mouse CCR5 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). *Fourth row*: Isolated CD4⁺ (by MACS), CD4⁺CCR6^{lo} (by cell sorting), or CD4⁺CCR6^{hi} (by cell sorting) T

cells were analyzed with a PE-conjugated anti-mouse CCR6 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). *Fifth row*: Isolated CD4⁺ (by MACS), CD4⁺CD25^{lo} (by cell sorting), or CD4⁺CD25^{hi} (by cell sorting) T cells were analyzed with a PE-conjugated anti-mouse CD25 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). The number in each panel represents the mean percentage $\pm$ SD of each immune subset (hi or lo) within the total cell population (hi plus lo). Data are representative of at least three repeat experiments. **C**, After cell isolation, each immune cell type was mixed with Trypan blue. Viability was calculated as the number of unstained cells/(stained cells + unstained cells) $\times$ 100. Each measurement was performed at least three times. The percentage represents the mean percentage $\pm$ SD.

Sup. Fig. 2. Effects of an adenosine receptor agonist and antagonist on production of IL-17A in an MLR. MLR was performed for 7 days in the presence of each adenosine receptor agonist or each adenosine receptor antagonist plus adenosine (100 μ M). After 7 days, the supernatants were analyzed in an IL-17A ELISA. **A**: CCPA (an A1R agonist). **B**: PSB0777 (an A2aR agonist, *left*) and Istradefylline (an A2aR antagonist, *right*). **C**: BAY 60-653 (an A2bR agonist). **D**: HEMADO (an A3R agonist). All experiments were repeated six to nine times. Data are expressed as the mean $\pm$ SD and were compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with medium.

Sup. Fig. 3. Effect of adenosine and an A2aR antagonist on production of IFN- γ , IL-5, and IL-17A by CD3/CD28-stimulated CD4⁺ T cells. CD4⁺ T cells were stimulated for 1–7 days with anti-CD3/CD28 antibodies in the presence or absence of adenosine (600 μ M). After stimulation, supernatants were analyzed in IFN- γ (*top row*), IL-5 (*second row*), and IL-17A

(*bottom row*) ELISAs. Data are expressed as the mean $\pm$ SD and were compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with CD3/CD28 stimulation. All experiments were repeated four to six times.

Sup. Fig. 4. Effects of an adenosine receptor agonist and antagonist on production of IL-17A by CD3/CD28-stimulated CD4⁺ T cells. CD4⁺ T cells were stimulated with an anti-CD3/CD28 antibody for 7 days in the presence of each adenosine receptor agonist, or in the presence of each adenosine receptor antagonist plus adenosine (600 μ M). After 7 days, the supernatants were analyzed in an IL-17A ELISA. **A:** CCPA (an A1R agonist). **B:** PSB0777 (an A2aR agonist, *left*) and Istradefylline (an A2aR antagonist, *right*). **C:** BAY 60-653 (an A2bR agonist). **D:** HEMADO (an A3R agonist). All experiments were repeated six to nine times. Data are expressed as the mean $\pm$ SD and were compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with medium.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Sup. Fig. 1

A

B

C

Cell type	Viability
Splenocytes	100 ± 0
CD4 ⁺ T cells	100 ± 0
B cells	100 ± 0
BM-DCs	94.5 ± 1.67
Naive CD4 ⁺ T cells	100 ± 0
Flow through of CD4 ⁺ CD62L ⁺ T cells	100 ± 0

Cell type	Viability
CD4 ⁺ CD62L ^{lo}	95.6 ± 2.90
CD4 ⁺ CD62L ^{hi}	98.3 ± 2.50
CD4 ⁺ CCR3 ^{lo}	97.1 ± 1.91
CD4 ⁺ CCR3 ^{hi}	91.1 ± 6.37
CD4 ⁺ CCR5 ^{lo}	98.8 ± 0.89
CD4 ⁺ CCR5 ^{hi}	91.1 ± 6.40
CD4 ⁺ CCR6 ^{lo}	98.8 ± 0.80
CD4 ⁺ CCR6 ^{hi}	94.0 ± 4.24
CD4 ⁺ CD25 ^{lo}	97.1 ± 1.77
CD4 ⁺ CD25 ^{hi}	98.4 ± 1.62

Sup. Fig. 2

Sup. Fig. 3

Sup. Fig. 4