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 Takagi[1], Toshimasa Yamamoto[2], and Masaaki Kawano[1,*]

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

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

[3]Allergy 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 adenyl cyclase, protein kinase A, and cAMP response element binding protein (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.

38 **Introduction**

39

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

61 A1R and A3R (13). In addition, adenosine induces Th17 differentiation by activating A2bR on
62 $CD4^+$ T cells (14). By contrast, several reports suggest that adenosine suppresses Th17
63 differentiation via activation of A2aR on $CD4^+$ T cells (15-17). Considering that the G protein
64 downstream of A2aR is Gs, and those of A2bR are Gs and Gq (5), it is assumed that induction of
65 A2bR-mediated Th17 differentiation is induced through simultaneous activation of Gq and
66 suppression of Gs, or via other unknown mechanisms. Therefore, the precise molecular
67 mechanism(s) underlying the effect of adenosine on Th cells is unclear. Here, we show that
68 adenosine promotes IL-17A production in a two-way mixed lymphocyte reaction (MLR). In
69 addition, an A2aR agonist (PSB0777) induced IL-17A production, and an A2aR antagonist
70 (Istradefylline) inhibited production, induced by adenosine, suggesting that activation of A2aR
71 plays a role in adenosine-mediated IL-17A production. This notion was further supported by the
72 observation that inhibitors of AC, PKA, and CREB, which are signaling molecules downstream
73 of the Gs protein (18), also suppressed adenosine-mediated IL-17A production. Immune subset
74 studies suggested that Th17 cells play a role in adenosine-mediated hypersecretion of IL-17A.
75 Administration of an A2R antagonist to mice with experimental autoimmune encephalomyelitis
76 (EAE) (19,20) markedly ameliorated symptoms. Taken together, the data indicate that
77 adenosine-dependent hypersecretion of IL-17A by Th17 cells contributes not only to
78 antibacterial defense but also to neutrophilic autoimmune diseases, and that suppressing this
79 process may be an effective therapy for the latter.

80 **Methods**

81

82 *Mice*

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

88

89 *Two-way MLR*

90 Splenic lymphocytes were collected by lyzing tissue in a Dounce homogenizer, followed by
91 layering over Ficoll Paque (GE health care, Chicago, IL, USA), as described previously (21).
92 BALB/c splenic lymphocytes (3×10^6) were mixed with SJL/J splenic lymphocytes (3×10^6) in
93 2 mL of DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 μg/mL streptomycin, 2
94 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (D10 medium) in 12
95 well plates in the presence of adenosine (0–1 mM) (Sigma, St. Louis, MO, USA); in the presence
96 of each adenosine receptor agonist (0–10 μM) (A1R: 2-Chloro-N6-cyclopentyladenosine
97 (CCPA), Tocris, Bristol, UK; A2aR: PSB0777, Tocris; A2bR: BAY 60-6583, Tocris; A3R:
98 HEMADO, Tocris); in the presence of A2aR antagonist (0–1 nM) (Istradefylline, Sigma) plus
99 adenosine (100 μM); in the presence of an AC inhibitor (0–1 μM) (MDL-12330A, Enzo Life
100 Sciences, Farmingdale, NY, USA), a PKA inhibitor (0–1 μM) (H-89, Tocris) plus adenosine
101 (100 μM), or a CREB inhibitor (0.1–0.6 μM) (666-15, Cayman Chemical Company, Ann Arbor,
102 MI, USA) plus adenosine (100 μM) (to inhibit A2aR signaling); in the presence of an activator

103 of AC (0.06–0.6 μM) (Forskolin, Sigma); or in the presence of a CD39 inhibitor (0–1 μM)
104 (ARL67156, Tocris) or a CD73 inhibitor (0–1 μM) (adenosine 5'-(α, β-methylene) diphosphate
105 (AMP-CP; Tocris) plus ATP (100 μM) (GE healthcare). After mixing, the plates were incubated
106 for 7 days at 37°C. The supernatants were collected for use in cytokine ELISAs.
107
108 *Isolation of $CD4^+$, $CD4^+CD62L^+$ T cells, and B cells*
109 $CD4^+$ T cells within the BALB/c and SJL/J splenocyte populations were isolated from the
110 mixture prepared previously after rupturing red blood cells (22). Cells were isolated by positive
111 selection of $CD4^+$ T cells using magnetic-activated cell sorting (MACS) (Miltenyi Biotec,
112 Bergisch Gladbach, Germany), according to the manufacturer's instructions. $CD4^+CD62L^+$ T
113 cells were isolated from BALB/c splenocytes by a combination of negative and positive selection
114 by MACS. During positive selection of $CD62L^+$ T cells, negatively isolated $CD4^+$ T cells were
115 collected as the flow through fraction. B cells were isolated from BALB/c splenocytes by
116 positive selection using MACS. Cells were resuspended in 1 mL of D10 medium and counted.
117 The purity and viability of $CD4^+$ T cells, $CD4^+CD62L^+$ T cells, and B cells were >90% (Sup. Fig.
118 1). The purity and viability of cells in the flow through fraction collected during isolation of
119 $CD4^+CD62L^+$ T cells are shown in Supplementary Figure 1.
120
121 *Cell sorting*
122 BALB/c $CD4^+$ T cells (prepared as described above) were labeled for 30 min at 4°C with
123 PE-conjugated anti-mouse CCR3, CCR5, CCD6, CD25, or CD62L antibodies (BioLegend). The
124 cells were then washed and sorted using a FACS Aria II flow cytometer (BD Biosciences). The
125 purity and viability of the sorted cells are Supplementary Figure 1.

126

127 *CD3/CD28 stimulation*

128 $CD4^+$ T cells (1×10^6) were stimulated for 7 days at 37°C with anti-mouse CD3 (BioLegend; 1
129 μg/mL) and CD28 (BioLegend; 0.5 μg/mL) antibodies (CD3/CD28) in the presence of adenosine
130 (0–1 mM), each adenosine receptor agonist (0–10 μM; A1R: 2-Chloro-N6-cyclopentyladenosine
131 (CCPA), Tocris, Bristol, UK; A2aR: PSB0777, Tocris; A2bR: BAY 60-6583, Tocris; and A3R:
132 HEMADO, Tocris), an A2aR antagonist (Istradefylline; 0–1 nM) plus adenosine (600 μM), an
133 AC inhibitor (0–1 μM; MDL-12330A), a PKA inhibitor (0–1 μM) (H-89), a CREB inhibitor
134 (0.1–0.6 μM; 666-15) plus adenosine (600 μM), or an activator of AC (0.06–0.6 μM; Forskolin)
135 in 500 μL of D10 medium. After cell sorting, cells (3×10^5) were plated in 24 well plates and
136 stimulated for 7 days at 37°C with anti-mouse CD3 (1 μg/mL) and CD28 (0.5 μg/mL) antibodies
137 in the presence of adenosine (600 μM) in 100 μL of D10 medium. After stimulation, the
138 supernatants were collected for use in cytokine ELISAs.

139

140 *Adenosine or ATP ELISAs*

141 MLR was performed by mixing BALB/c lymphocytes (6×10^6) with SJL/J lymphocytes ($6 \times$
142 10^6) in a 15 mL tube for 0–24 h at 37°C in the presence of a CD39 inhibitor (ARL67156) (0–1
143 μM) and a CD73 inhibitor (AMP-CP) (0–1 μM) in 200 μL of D10 medium. CD3/CD28
144 stimulation was performed for 24 h at 37°C in a 15 mL tube by incubating BALB/c $CD4^+$ T cells
145 (1×10^7) with anti-mouse CD3 (1 μg/mL) and CD28 (0.5 μg/mL) antibodies plus ARL67156 (1
146 μM) or AMP-CP (1 μM) in 200 μL of D10 medium. Simulation with LPS or an anti-mouse
147 CD40 antibody (Ab) was performed for 24 h at 37°C in a 15 mL tube; briefly, BALB/c B cells (1

148 × 10^7) or BALB/c bone marrow (BM)-derived dendritic cells (BM-DCs) (1 × 10^7), generated
149 from mouse bone marrow cells as described previously (23), were incubated with LPS (Sigma;
150 0.5 μg/mL) or anti-mouse CD40 Ab (5 μg/mL; BioLegend) plus ARL67156 (1 μM) or AMP-CP
151 (1 μM) in 200 μL of D10 medium. The purity and viability of BM-DCs were >90% (Sup. Fig. 1).
152 After incubation, the supernatants were collected and tested in adenosine or ATP ELISAs
153 (Biovision, Milpitas, CA, USA).
154
155 *Differentiation of naïve $CD4^+$ T cells*
156 Naïve $CD4^+$ T cells (3 × 10^5) in 500 μL of D10 medium in 24 well plates were stimulated for 7
157 days at 37°C with anti-mouse CD3 (1 μg/mL) and CD28 (0.5 mg/mL) antibodies plus mouse
158 IL-6 (20 ng/mL) (Peprotech, Rocky Hill, NJ), and human TGF-β1 (2 ng/mL) (Peprotech) in the
159 presence of an A2aR antagonist (Istradefylline) (0–1 nM). After incubation, the supernatants
160 were collected for use in cytokine ELISAs, and total RNA was extracted from the cells using a
161 Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) for quantitative PCR. In another
162 experiment, cells in 500 μL of D10 medium were stimulated for another 7 days at 37°C with
163 anti-mouse CD3 (1 μg/mL) and CD28 (0.5 μg/mL) antibodies in the presence of Istradefylline
164 (0.1–1 nM) plus adenosine (600 μM). Supernatants were collected for use in cytokine ELISAs.
165
166 *Quantitative PCR*
167 cDNA was synthesized from total RNA prepared using Superscript IV VILO master mix
168 (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions. Quantitative
169 PCR (qPCR) was performed as described previously (24) using the primer pairs described below,
170 Power SYBR green PCR Master mix (Applied Biosystems, Waltham, MA, USA), and a

171 QuantStudio 12K Flex (Applied Biosystems) equipped with QuantStudio 12K Flex software
172 (Applied Biosystems). Then, the ΔCt was calculated. The following forward and reverse primers,
173 respectively, were used (all from Eurofins Scientific, Luxembourg): GAPDH,
174 5'-AGCTTGTCATCAACGGGAAG-3' and 5'-TTTGATGTTAGTGGGGTCTCG-3'; IL-17A,
175 5'-TGTGAAGGTCAACCTCAAAGTCT-3' and
176 5'-GAGGGATATCTATCAGGGTCTTCAT-3'.
177
178 *EAE model*
179 EAE was induced as described previously (19). Briefly, SJL/J mice received a subcutaneous
180 inguinal injection (100 μg/mouse) of the proteolipid protein (PLP) peptide (PLP139-151, Tocris)
181 emulsified in complete Freund's adjuvant (CFA) containing mycobacterium tuberculosis H37Ra
182 (100 μg/mouse; Difco, Detroit, MI, USA). Mice also received oral water or an A2aR antagonist
183 (Istradefylline; 6 μg/mouse) once every 2 days from Day –7 to Day +18 after immunization with
184 PLP peptide (Day 0). Mice were examined daily for signs of EAE, which were graded as
185 described (25).
186
187 *Histological analysis of spinal cord samples*
188 On Day 18 post-induction, histological analysis was performed by excising the lumbar, thoracal,
189 and cervical parts of the spinal cord. Paraffin-embedded spinal cord sections were stained with
190 hematoxylin and eosin. T cells were labeled with an anti-mouse CD3ε Ab (Cell Signaling
191 technology, Danvers, MA, USA). Tissue sections were observed using the light microscopy
192 mode of an inverted fluorescence phase-contrast microscope (BZ-9000, Keyence, Osaka, Japan).
193

194 *Peptide pulse assay*

195 At 7 days post-subcutaneous immunization with PLP peptide emulsified in CFA, splenocytes (2
196 × 10^6 in 200 μL of D10 medium) were seeded in 96-well round plates and pulsed for 3 days at
197 37°C with PLP peptide (10 μM) in the presence of adenosine (600 μM) and an A2aR antagonist
198 (Istradefylline; 0–1 nM). For the oral administration experiments using the A2a antagonist,
199 splenocytes (2 × 10^6 in 200 μL of D10 medium) or inguinal lymph node lymphocytes, prepared
200 as described previously (26) (1 × 10^6 in 200 μL of D10 medium) were seeded in 96-well round
201 plates and pulsed for 3 days at 37°C; this was done at 7 days post-subcutaneous immunization
202 with PLP peptide emulsified in CFA. Mice also received oral water, an A2aR agonist (PSB0777)
203 (6 μg/mouse), or an A2aR antagonist (Istradefylline; 6 μg/mouse) once every 2 days from Day –
204 7 to Day +7 after immunization with PLP peptide (Day 0). At 18 days post-subcutaneous
205 immunization with PLP peptide emulsified in CFA, spinal cord cells prepared as described
206 previously (26) (100 μL of resuspended in 500 μL of D10 medium/mouse) were seeded in
207 96-well round plates and pulsed for 3 days at 37°C with PLP peptide (10 μM). The supernatants
208 were collected for use in cytokine ELISAs.

209

210 *Flow cytometry analysis*

211 The MLR was performed for 7 days in the presence or absence of adenosine (100 μM) (as
212 described above). For the $CD4^+IL\text{-}17A^+$ study, cells were blocked with anti-mouse CD16/CD32
213 antibodies (BioLegend, San Diego, CA, USA) and stained for 30 min at 4°C with a fluorescein
214 isothiocyanate (FITC)-conjugated anti-mouse CD4 Ab (BioLegend). The cells were then fixed,
215 permeabilized, and stained with a phycoerythrin (PE)-conjugated anti-mouse IL-17A Ab
216 (BioLegend). For the Th subset study, after 0, 3, and 7 days of the MLR assay, cells were

217 blocked with anti-mouse CD16/CD32 antibodies (BioLegend) and then stained for 30 min at 4°C
218 with a FITC-conjugated anti-mouse CD4 Ab (BioLegend) and PE-conjugated anti-mouse CCR5
219 (a Th1 marker (27)), CCR3 (a Th2 marker (28)), or CCR6 (a Th17 marker (29,30)) antibodies
220 (BioLegend). For the MHC, costimulatory molecule, and A2aR expression studies in APCs,
221 BALB/c B cells (3×10^5) or BALB/c BM-DCs (3×10^5) seeded in the wells of a 96-well in 200
222 μL of D10 medium were incubated with LPS (0.5 μg/mL; Sigma) or an anti-mouse CD40 Ab (5
223 μg/mL) (BioLegend) for 1 day. Next, supernatants were collected for cytokine ELISAs, or
224 blocked with anti-mouse CD16/CD32 antibodies (BioLegend) and then stained for 30 min at 4°C
225 with FITC-conjugated anti-mouse CD80, CD86, or CD40 Abs and corresponding isotype
226 controls (BioLegend) (for the costimulatory study), with PE-conjugated anti-mouse H-2 or
227 I-A/I-E Ab and corresponding isotype controls (BioLegend) (for the MHC study), or fixed,
228 permeabilized, and stained with a PE-conjugated anti-A2aR Ab (Novus Biologicals, Minneapolis,
229 MN, USA) and corresponding isotype control (for the A2aR expression study). For the A2aR
230 expression study in $CD4^+$ T cells, $CD4^+$ T cells (1×10^6) were stimulated for 7 days at 37°C with
231 anti-mouse CD3 (1 μg/mL) and CD28 (0.5 μg/mL) antibodies in the presence of adenosine (0–1
232 mM). At 0–7 days post-stimulation, cells were blocked with anti-mouse CD16/CD32 antibodies
233 (BioLegend) and then stained for 30 min at 4°C with a FITC-conjugated anti-mouse CD4 Ab
234 (BioLegend) before fixation, permeabilization, and staining with a PE-conjugated anti-A2aR Ab
235 (Novus Biologicals). For EAE analysis at 18 days post-subcutaneous immunization with PLP
236 peptide emulsified in CFA, splenocytes (1×10^6), inguinal lymph node lymphocytes (1×10^6),
237 and spinal cord cells (100 μL of resuspended in 500 μL of D10 medium/mouse) were blocked
238 with anti-mouse CD16/CD32 antibodies (BioLegend) and then stained for 30 min at 4°C with a
239 FITC-conjugated anti-mouse CD4 Ab (BioLegend) and PE-conjugated anti-mouse CCR5 (a Th1

240 marker (27)), CCR3 (a Th2 marker (28)), or CCR6 (a Th17 marker (29,30)) antibodies
241 (BioLegend). Finally, the cells were washed and analyzed on a FACSCanto II flow cytometer
242 (BD Biosciences, Franklin Lakes, NJ) using FACSDiva acquisition software (BD Biosciences).
243
244 *Cytokine ELISAs*
245 The concentrations of IFN-γ, IL-5, IL-6, IL-17A, IL-17F, IL-22, IL-23, and TGF-β1, in cell
246 supernatants were measured using specific ELISA kits (DuoSet Kit, R&D, Minneapolis, MN,
247 USA). Any value below the lower limit of detection (15.6 pg/mL) was set to 0. No cytokine
248 cross-reactivity was observed within the detection ranges of the kits. If necessary, samples were
249 diluted appropriately so that the measurements fell within the appropriate detection range for
250 each cytokine.
251
252 *Statistical analysis*
253 Differences between two groups were analyzed using an unpaired Student's t-tests. Differences
254 between three or more groups were analyzed using one-way ANOVA with Tukey's post-hoc test.
255 Clinical scores were analyzed using a non-parametric Mann-Whitney *U*-test. All calculations
256 were performed using KaleidaGraph software (Synergy software, Reading, PA, USA). A P value
257 < 0.05 was considered statistically significant.

258 ***Results***

259

260 ***Adenosine promotes IL-17A production by CD4$^+$ T cells in an MLR***

261 First, we analyzed the effect of adenosine on CD4$^+$ T cells during T cell-APC interactions in an
262 MLR (31). We found that CD4$^+$ T cells exposed to adenosine secreted IL-17A in a
263 dose-dependent manner (Fig. 1A–C). Since both agonist-mediated IL-17A production and
264 antagonist-mediated suppression of adenosine-mediated IL-17A production were observed in the
265 presence of an adenosine A2aR agonist (PSB0777) and an antagonist (Istradefylline),
266 respectively (Fig. 1D and Sup. Fig. 2A), we hypothesized that IL-17A in the MLR was produced
267 by CD4$^+$ T cells stimulated via the A2aR. This notion was supported by the finding that AC,
268 PKA, and CREB inhibitors of signaling molecules downstream of the A2aR also suppressed
269 IL-17A production (Fig. 1E). Consistent with this notion, activation of A2aR downstream of AC
270 upregulated IL-17A production (Fig. 1E). In the MLR, the Th1 and Th17 cell populations
271 increased slightly but significantly (by approximately 1.7-fold) in the presence of 100 μM
272 adenosine at Day 7 (Sup. Fig. 2C). Considering that 100 μM adenosine increased IL-17A
273 secretion by approximately 10-fold compared with medium (Fig. 1B), it appears that
274 adenosine-mediated upregulation of IL-17A is the main contributor to upregulation of the
275 IL-17A production by CD4$^+$ T cells. Furthermore, ATP induced IL-17A production in the MLR,
276 which was suppressed by the A2aR antagonist and by inhibitors of CD39 and CD73 (Fig. 1F),
277 suggesting that adenosine plays a role in IL-17A production. Since the A2aR antagonist and
278 inhibitors of CD39 and CD73 also inhibited production of IL-17A in the MLR (Fig. 1G), we
279 postulate that *de novo* adenosine production is induced in the MLR.

280

281 ***Adenosine production in the MLR***

282 CD39 and CD73 expressed on the surface of endothelial cells (32,33) and immune cells (10,11),
283 including T cells and DCs, are critical for production of adenosine from ATP. Since we found
284 that inhibitors of CD39 and CD73 inhibited basal IL-17A production in the MLR (Fig. 1G), we
285 next addressed the source of adenosine production in the MLR. As shown in Figure 2A,
286 production of adenosine and ATP was time-dependent. In accordance with suppression of
287 IL-17A production, inhibitors of CD39 and CD73 suppressed adenosine production at 24 h after
288 the start of the MLR (Fig. 2B and C). Since the MLR induces activation of $CD4^+$ T cells by
289 APCs (31), we also addressed adenosine production by activated $CD4^+$ T cells, B cells, and
290 BM-DCs (Fig. 2D–F). Production of both adenosine and ATP by activated $CD4^+$ T cells, B cells,
291 and BM-DCs was observed; inhibitors of CD39 and CD73 suppressed production by activated
292 $CD4^+$ T cells although production of both adenosine and ATP by $CD4^+$ T cells, B cells, and
293 BM-DCs was observed without LPS or anti CD40 Ab stimulation (Fig. 2D). This indicates the
294 possibility that adenosine stimulation is mediated by the $CD4^+$ T cell to other immune cell
295 contact and adenosine produced by $CD4^+$ T cells or other immune cells such as B cells may
296 induce adenosine-mediated IL-17A hypersecretion by $CD4^+$ T cells in the MLR.

297

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

299 Next, we tried to identify the Th subset that generated IL-17A in the presence of adenosine. First,
300 we confirmed that $CD4^+$ T cells expressed the A2aR and secreted IL-17A by stimulating them
301 with agonistic anti-CD3/CD28 antibodies in the presence of adenosine (Fig. 3A). Time course
302 studies showed that adenosine-mediated IL-17A production was detected from 3 days
303 post-CD3/CD28 stimulation (Fig. 3B and Sup. Fig. 3). Administration of adenosine within 6 h of

304 Ab stimulation triggered IL-17A production; however, administration at 24 h post-stimulation
305 did not (Fig. 3C). We noticed that A2aR expression by $CD4^+$ T cells was upregulated
306 significantly (2–4.7 fold) by anti-mouse CD3 and CD28 Abs at 1–7 days after stimulation, and
307 that this upregulation was further (slightly but significantly) enhanced (by approximately
308 1.5-fold) by increasing amounts of adenosine (Sup. Fig. 4A). As in the MLR, $CD4^+$ T cells also
309 produced IL-17A after stimulation with anti-CD3/CD28 antibodies in the presence of an A2aR
310 agonist (Fig. 3D and Sup. Fig. 2B). Adenosine-mediated IL-17A production by
311 CD3/CD28-stimulated $CD4^+$ T cells was suppressed by an A2aR antagonist (Fig. 3D and Sup.
312 Fig. 2B). This was supported by data showing that inhibitors of signaling molecules downstream
313 of the A2R also suppressed IL-17A production, whereas activation of A2aR downstream of AC
314 upregulated the production (Fig. 3E). Production of other Th17-related cytokines was also
315 induced by adenosine through the A2aR (Fig. 4). This again suggests that activated $CD4^+$ T cells
316 produce IL-17A upon A2aR activation. Furthermore, we noticed that $CD4^+CD62L^-$, but not
317 $CD4^+CD62L^+$, cells produced IL-17A after CD3/CD28 stimulation in the presence of adenosine,
318 suggesting that adenosine induces IL-17A production by effector Th cells (Fig. 3F). Therefore,
319 we performed immune subset studies after separating $CD4^+$ T cells using anti-chemokine
320 receptor (CCR) antibodies. As shown in Figure 3G, $CD4^+CCR6^{hi}$ T cells produced IL-17A upon
321 stimulation of CD3/CD28, and production was strongly upregulated in the presence of adenosine.
322 Since CCR6 is a typical marker of Th17 cells (29,30), this suggests that activated Th17 cells
323 hypersecrete IL-17A in the presence of adenosine.

324 To address the effect of adenosine on APCs, we analyzed production of IL-6, IL-23,
325 and, TGF-β1 by B cells and BM-DCs (Sup. Fig. 4B). Also, we analyzed the effect of adenosine
326 on expression of CD80/CD86/CD40, MHC class I/class II, and A2aR by B cells and BM-DCs

327 (Sup. Fig. 4C). Increasing amounts of adenosine suppressed IL-6 production by LPS-stimulated
328 B cells. and IL-6 and IL-23 production by LPS-stimulated BM-DCs. Expression of
329 CD80/CD86/CD40, MHC class I/class II, and A2aR by LPS or anti-CD40 Ab-stimulated-B cells
330 and -BM-DCs in the presence of 100 μM and 600 μM adenosine was 0.70- and 1.31-fold higher
331 than that by cells stimulated by LPS or anti-CD40 Ab alone. These data indicate that adenosine
332 mainly affects $CD4^+$ T cells to activate Th17 function.
333
334 ***An adenosine A2aR antagonist ameliorates IL-17A-related autoimmune EAE responses***
335 The above results raise the possibility that adenosine-mediated hypersecretion of IL-17A by
336 Th17 cells contributes to Th17-related autoimmune diseases. This hypothesis is supported by a
337 report showing that CD73 knockout mice are resistant to EAE (34), a Th17-mediated
338 autoimmune disease (20). We expected, therefore, that A2aR antagonist-mediated suppression of
339 Th17 responses should improve EAE. To address this, we examined the efficacy of an A2aR
340 antagonist in EAE model SJL/J mice (19). EAE was induced by immunization of mice with an
341 $I\text{-}A^s$ restricted helper peptide derived from a myelin PLP peptide comprising amino acids 139–
342 151 (HSLGKWLGHPDKF). The peptide was emulsified in CFA. First, we confirmed that the
343 A2aR antagonist suppressed adenosine-mediated IL-17A production by CD3/CD28-stimulated
344 $CD4^+$ T cells from SJL/J strain mice (Fig. 5A). The A2aR antagonist also significantly
345 suppressed adenosine-mediated IL-17A production after differentiation of Th17 cells from naïve
346 $CD4^+$ T cells (Fig. 5B, *right*). The A2aR antagonist did not suppress IL-17A expression or
347 production during differentiation of Th17 cells from naïve $CD4^+$ T cells (Fig. 5B, *left and center*).
348 By contrast, and in agreement with Figure 3G, adenosine administration did not induce IL-17A
349 production during and after differentiation of Th1, Th2, and Treg cells from naïve $CD4^+$ T cells

350 (data not shown). Next, we pulsed splenocytes with the PLP peptide after immunization to
351 confirm that IL-17A production was induced in a peptide-dependent manner, and that production
352 was upregulated by adenosine. As expected, IL-17A production occurred in a peptide-dependent
353 manner and was upregulated by adenosine (Fig. 5C). Furthermore, the A2aR antagonist
354 suppressed production of IL-17A, suggesting that the A2aR antagonist inhibits IL-17A
355 production by $CD4^+$ T cells induced by immunization with the PLP peptide. We next addressed
356 the effect of an oral A2aR antagonist on production of IL-17A by $CD4^+$ T cells induced by
357 immunization with the PLP peptide (Fig. 5D). Oral administration of PSB0777 or Istradefylline
358 did not significantly induce or suppress IL-17A production by PLP peptide-pulsed splenocytes
359 and inguinal lymph node lymphocytes. These results indicate that the A2aR antagonist may not
360 inhibit PLP peptide-specific Th17-generation but rather suppress adenosine-mediated IL-17A
361 hypersecretion by PLP peptide-specific Th17 cells. Since oral administration of PSB0777 did not
362 up-regulate IL-17A production, adenosine-mediated IL-17A production may be saturated by *de*
363 *novo* extracellular adenosine in the body. Hence, we expect that the A2aR antagonist may
364 suppress adenosine-mediated IL-17A hypersecretion by PLP peptide-specific Th17 cells during
365 migration to the inflammatory site, which may suppress EAE symptoms. Finally, the A2aR
366 antagonist was administered orally to mice before and during EAE induction (Fig. 5E and F). As
367 shown, the clinical scores of mice receiving the A2aR antagonist were markedly lower than
368 those of control mice (receiving water) at 18 days post-immunization with the PLP peptide (Fig.
369 5E). Accordingly, histological studies showed that the numbers of central nervous
370 system-infiltrating $CD3^+$ cells in mice receiving the A2aR antagonist were much lower than
371 those in mice receiving water (Fig. 5F). Consistent with these data, flow cytometry analysis
372 showed that the proportion of $CD4^+$ cells in total counts in the spinal cord were reduced

373 significantly by the oral A2aR antagonist; in particular, the percentage of Th17 cells in the spinal
374 cord was reduced significantly (Fig. 5G), suggesting the A2aR antagonist suppressed migration
375 of $CD4^+$ T cells to, and retained Th17 cells within, the spinal cord during EAE induction.
376 Accordingly, PLP peptide-dependent production of IL-17A was observed in spinal cord cells
377 after oral administration of water; production was suppressed significantly by the oral A2aR
378 antagonist (Fig. 5H). These results suggest that the A2aR antagonist suppresses hypersecretion of
379 IL-17A by PLP peptide-specific Th17-cells, resulting in impaired migration from the draining
380 lymph nodes to the inflammatory site, and subsequent retention at the inflammatory site. These
381 phenomena may suppress IL-17A-mediated EAE symptoms.

382 **Discussion**

383 Here, we showed that adenosine induces hypersecretion of IL-17A by Th17 cells. Addition of
384 adenosine (1 mM) to a two-way MLR increased IL-17A production to > 25 times the basal level;
385 however, both basal production and increased production of IL-17A were suppressed by an
386 A2aR antagonist, and by CD39/CD73 inhibitors. This indicates that hypersecretion of IL-17A in
387 the presence of adenosine occurs by other mechanisms in addition to T cell-APC interactions.
388 Since endothelial cells and nervous system cells also express CD39/CD73 (32,33,35,36) and
389 produce adenosine (8,37), and activated $CD4^+$ T cells in the present study hypersecreted IL-17A
390 at 6 h post-CD3/CD28 stimulation, it is possible that activated Th17 cells also receive adenosine
391 from endothelial and neuronal cells to induce hypersecretion of IL-17A.

392 We hypothesize that adenosine affects multiple immunological events as follows: After
393 APCs take up antigen they move to the secondary lymphoid organs. Followed by an innate
394 immune response in the secondary lymphoid organs, APCs induce Th differentiation from naïve
395 $CD4^+$ T cells. In particular, APCs induce Th17 differentiation by secreting IL-6 and TGF-β1.
396 Then, the transcription factor RORγt switches on the IL-17A gene by binding to the IL-17A
397 promoter (38). During this response, adenosine rarely stimulates naïve $CD4^+$ T cells due to low
398 levels of A2aR expression (Sup. Fig. 4A). Following an adaptive immune response, APCs in the
399 secondary lymphoid organs stimulates effector Th17 cells and drives expression of the IL-17A
400 gene by recruiting transcription factors such as NF-κB and NFAT downstream of the TCR
401 signaling pathway (39). In this response, A2aR expression is also upregulated (Sup. Fig. 4A),
402 thereby increasing and sensitivity of A2aR signaling in response to adenosine. After stimulation,
403 Th17 cells migrate toward the inflammatory site. During migration, activated Th17 cells receive
404 adenosine from immune cells, neural cells, endothelial cells, or in an autocrine manner. During

405 this process, Th17 cells further up-regulate expression of the IL-17A gene by recruiting
406 transcription factors such as CREB downstream of the A2aR-cAMP-PKA pathway (40). Then,
407 Th17 cells induce IL-17A hypersecretion at the inflammatory site. In humans, the transcription
408 factor cAMP-responsive element modulator α increases transcription of the human *Il-17* gene by
409 binding to the promoter region (41). The A2aR antagonist may suppress adenosine-mediated
410 IL-17A hypersecretion by adenosine-stimulated Th17 cells at the inflammatory site and
411 subsequently suppress neutrophil-mediated inflammatory responses and Th cell migration. This
412 hypothesis is consistent with a previous EAE study showing that CD73 expression and adenosine
413 receptor signaling are required for efficient entry of lymphocytes into the central nervous system
414 during EAE development (34); our own data suggest that CD73 expression and adenosine
415 receptor signaling may be required to induce IL-17A hypersecretion by Th17 cells around the
416 inflammatory site. γδ T cells, which are categorized mainly into two subpopulations ($CD8\alpha\alpha^+$
417 and $CD8^-$ cells), express RORγt and are also a source of IL-17A (42,43). We hypothesize that
418 the A2aR antagonist will also suppress adenosine-mediated IL-17A production by
419 adenosine-stimulated γδ T cells through A2aR signaling (44).

420 During the sequence of immunological events described above, TCR signaling
421 up-regulates phosphodiesterase activity and suppresses cAMP signaling during the adaptive
422 responses in the secondary lymphoid organs (45). The cAMP–PKA signaling pathway plays a
423 major role in regulating immune responses, and cAMP is the most potent and acute inhibitor of T
424 cell activation (45). Our data indicate that up-regulation of A2aR expression is induced after
425 TCR stimulation (Sup. Fig. 4A); therefore, adenosine-mediated IL-17A hypersecretion is
426 induced after stimulation of TCR signaling as seen in Fig. 3C. Therefore, extracellular adenosine
427 may not block TCR signaling in the body. Also, a specific amount of Forskolin may not block

428 TCR signaling, leading to subsequent upregulation of IL-17A secretion by TCR-activated $CD4^+$
429 T cells (Fig. 1E and 3E). We hypothesize that extracellular adenosine-mediated IL-17A
430 hypersecretion is induced after activation of TCR signaling during migration from the secondary
431 lymphoid organs to the inflammatory site. In the MLR and the PLP peptide pulse experiment,
432 both activated Th17 cells and adenosine-stimulated Th17 cells might produce IL-17A during the
433 T-APC interaction. Thus, inhibitors of CD39/CD73 and an A2aR antagonist may suppress
434 adenosine-mediated hypersecretion by adenosine-stimulated activated Th17 cells.

435 It is suggested that physiological concentrations of adenosine are lower than 1 μM;
436 however, they can be increased by stimuli such as high K^+ levels, electrical stimulation,
437 glutamate receptor agonists, hypoxia, hypoglycemia, and ischemia (46). To obtain sufficient
438 adenosine (> 100 μM) to trigger hypersecretion of IL-17A, activated Th17 cells may need to
439 make contact with non-immune cells such as adenosine-producing endothelial cells (47) and
440 neuronal cells (48) to form a microenvironment with a high adenosine concentration (as
441 observed during T cell-APC interactions at immunological synapses) (49). Thus, A2aR
442 antagonists, rather than CD39/CD73 inhibitors, might be more effective at inhibiting *de novo*
443 adenosine-mediated hypersecretion of IL-17 by Th17 cells. A previous study suggests that
444 intracellular adenosine is transported out of cells by efficient equilibrative transporters (50);
445 CD39/CD73 inhibitors would not suppress this type of *de novo* adenosine production.

446 With regard to the effect of adenosine on other Th subsets, our observations were
447 different from those of previous reports (51,52); here, we observed that adenosine upregulated
448 IFN-γ (a Th1-related cytokine) secretion at 5 and 7 days and had no significant effect on IL-5 (a
449 Th2-related cytokine) secretion by $CD4^+$ T cells after CD3/CD28 stimulation with 600 μM of
450 adenosine, although IL-17A production was significant (Sup. Fig. 3). This suggests that

451 adenosine induces hypersecretion of IL-17A by Th17 cell but does not suppress Th1 and Th2
452 activity. However, previous studies report that adenosine-mediated suppression of IFN-γ and
453 IL-5 was observed 1 day after T cell receptor-mediated stimulation of $CD4^+$ T cells (51,52). This
454 may indicate that in the short term adenosine prioritizes stimulation of Th17 cell activity rather
455 than that of Th1 and Th2 cells, and that it does not suppress effector Th activity in the long term.

456 It is also suggested that the A2aR agonist, CGS21680, suppresses Th17 differentiation
457 (15-17). This result is opposite to ours; one reason for this may be differences in the source of
458 the A2aR agonist. The A2aR agonist CGS 21680 is much less selective than the A2aR agonist
459 that we used in this study (PSB0777); this is because CGS21680 binds not only to the A2aR but
460 also to A1R and A3R, which are associated with the Gi protein (which has opposite effects to the
461 Gs protein) (53,54). Therefore, it is probable that CGS21680 may cancel out any agonist effects
462 by activating A1R and A3R. Also, it is suggested that the A2aR antagonist, SCH58261,
463 up-regulates Th17 differentiation in mice (17). We hypothesized that up-regulation of Th17
464 differentiation by SCH58261 may be induced through relative downregulation of A2aR activity
465 compared with that of A2bR; this relative increase in A2bR activity induces Th17 differentiation
466 in mice (55,56). Also, it is probable that SCH58261 induces relative increase in the activity of G
467 protein-coupled receptors other than adenosine receptors (e.g., dopamine receptors) to induce
468 Th17 differentiation in mice (57). We also hypothesize that although SCH58261 may induce
469 Th17 differentiation *in vivo*, it may not stimulate Th17 activity; this is because our data show
470 that an A2a antagonist (Istradefylline) suppressed IL-17A secretion by differentiated Th17 cells
471 but did not suppress Th17 differentiation (Fig. 5B). This hypothesis is supported by previous
472 data showing that SCH58261 markedly suppresses symptoms of EAE, a typical Th17-mediated
473 disease (34).

474 Our data suggest that production of IL-17A is relatively higher after exposure to an
475 A2aR agonist, PSB0777, than after exposure to an A2bR agonist, BAY 60-6583. PSB0777 is a
476 potent adenosine A2aR agonist (Ki = 44.4 nM for rat brain striatal A2aR) (54), and BAY
477 60-6583 is a potent adenosine A2bR agonist (Ki = 100 nM for rat A2bR) (58). By assuming that
478 the Ki values of PSB0777 and BAY 60-6583 are comparable, we thought that production of
479 IL-17A mediated by activation of the A2aR might be higher than that mediated by activation of
480 the A2bR. This hypothesis is supported by the notion that the A2aR is a high affinity receptor
481 with activity in the low to mid-nanomolar range, whereas the A2bR has a much lower affinity for
482 adenosine (micromolar) (5); this suggests that adenosine activates the A2aR rather than the
483 A2bR.

484 A2aR antagonists have been developed for treatment of Parkinsonism (59) and
485 malignancies (60). In addition, inhibitors of CD39/CD73 have been developed as anti-tumor
486 drugs (61,62). Regarding the effects of adenosine on tumor immunity, a previous study suggests
487 that adenosine suppresses effector T cell function since tumor cells express both CD39 and
488 CD73 and secrete adenosine (63). By contrast, several reports suggest that IL-17A promotes
489 emergence of pro-tumorigenic neutrophil phenotypes (64,65). Neutrophils in mouse tumor
490 models promote tumor metastasis (66-68), and observations in cancer patients have linked
491 elevated neutrophil counts in blood with increased risk of metastasis (69). Therefore, it is
492 probable that tumor-produced adenosine induces IL-17A secretion by $CD4^{+}CCR6^{hi}$ T cells
493 followed by neutrophilic inflammation, which promotes tumor metastasis. Cancer vaccines may
494 need to be administered along with an A2aR antagonist to suppress hypersecretion of IL-17A by
495 tumor-specific Th17 cells induced by tumor-produced adenosine, and to inhibit neutrophilic
496 inflammation at the tumor site.

497 The results presented herein indicate that these drugs may also be effective treatments
498 for Th17-mediated diseases (4) such as psoriasis, neutrophilic bronchial asthma, severe atopic
499 dermatitis, and autoimmune diseases by suppressing hypersecretion of IL-17A by Th17 cells.
500 Moreover, these drugs might be effective treatments for diseases caused by neutrophilic
501 inflammation of unknown cause in the dermis; such diseases include Behcet uveitis (70) and
502 vasculitis of adenosine deaminase 2 deficiency (71). This is because endothelial cells express
503 CD39/CD73 and produce adenosine, which could induce hypersecretion of IL-17A by Th17 cells,
504 thereby contributing to inflammation.

505 **References**

506

507 1 Matsuzaki, G. and Umemura, M. 2007. Interleukin-17 as an effector molecule of innate
508 and acquired immunity against infections. *Microbiol Immunol* 51:1139.

509 2 Manel, N., Unutmaz, D., and Littman, D. R. 2008. The differentiation of human T(H)-17
510 cells requires transforming growth factor-beta and induction of the nuclear receptor
511 RORgammat. *Nat Immunol* 9:641.

512 3 Ivanov, II, Zhou, L., and Littman, D. R. 2007. Transcriptional regulation of Th17 cell
513 differentiation. *Semin Immunol* 19:409.

514 4 Tesmer, L. A., Lundy, S. K., Sarkar, S., and Fox, D. A. 2008. Th17 cells in human
515 disease. *Immunol Rev* 223:87.

516 5 Cronstein, B. N. and Sitkovsky, M. 2017. Adenosine and adenosine receptors in the
517 pathogenesis and treatment of rheumatic diseases. *Nat Rev Rheumatol* 13:41.

518 6 Jones, B. E. 2009. Glia, adenosine, and sleep. *Neuron* 61:156.

519 7 Boison, D., Chen, J. F., and Fredholm, B. B. 2010. Adenosine signaling and function in
520 glial cells. *Cell Death Differ* 17:1071.

521 8 Sebastiao, A. M. and Ribeiro, J. A. 2009. Tuning and fine-tuning of synapses with
522 adenosine. *Curr Neuropharmacol* 7:180.

523 9 Yegutkin, G. G. 2008. Nucleotide- and nucleoside-converting ectoenzymes: Important
524 modulators of purinergic signalling cascade. *Biochim Biophys Acta* 1783:673.

525 10 Whiteside, T. L., Mandapathil, M., and Schuler, P. 2011. The role of the adenosinergic
526 pathway in immunosuppression mediated by human regulatory T cells (Treg). *Curr Med*
527 *Chem* 18:5217.

528 11 Allard, B., Longhi, M. S., Robson, S. C., and Stagg, J. 2017. The ectonucleotidases CD39
529 and CD73: Novel checkpoint inhibitor targets. *Immunol Rev* 276:121.
530 12 Junger, W. G. 2011. Immune cell regulation by autocrine purinergic signalling. *Nat Rev*
531 *Immunol* 11:201.
532 13 Barletta, K. E., Ley, K., and Mehrad, B. 2012. Regulation of neutrophil function by
533 adenosine. *Arteriosclerosis, thrombosis, and vascular biology* 32:856.
534 14 Wilson, J. M., Kurtz, C. C., Black, S. G., Ross, W. G., Alam, M. S., Linden, J., and Ernst,
535 P. B. 2011. The A2B adenosine receptor promotes Th17 differentiation via stimulation of
536 dendritic cell IL-6. *Journal of immunology* 186:6746.
537 15 Alchera, E., Rolla, S., Imarisio, C., Bardina, V., Valente, G., Novelli, F., and Carini, R.
538 2017. Adenosine A2a receptor stimulation blocks development of nonalcoholic
539 steatohepatitis in mice by multilevel inhibition of signals that cause immunolipotoxicity.
540 *Transl Res* 182:75.
541 16 Wang, L., Wan, H., Tang, W., Ni, Y., Hou, X., Pan, L., Song, Y., and Shi, G. 2018.
542 Critical roles of adenosine A2A receptor in regulating the balance of Treg/Th17 cells in
543 allergic asthma. *Clin Respir J* 12:149.
544 17 Ansari, M. A., Nadeem, A., Attia, S. M., Bakheet, S. A., Raish, M., and Ahmad, S. F.
545 2017. Adenosine A2A receptor modulates neuroimmune function through
546 Th17/retinoid-related orphan receptor gamma t (RORgammat) signaling in a BTBR T(+)
547 Itpr3(tf)/J mouse model of autism. *Cell Signal* 36:14.
548 18 Hasko, G., Linden, J., Cronstein, B., and Pacher, P. 2008. Adenosine receptors:
549 therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* 7:759.
550 19 Nakano, K., Higashi, T., Hashimoto, K., Takagi, R., Tanaka, Y., and Matsushita, S. 2008.

551 Antagonizing dopamine D1-like receptor inhibits Th17 cell differentiation: preventive
552 and therapeutic effects on experimental autoimmune encephalomyelitis. *Biochem*
553 *Biophys Res Commun* 373:286.
554 20 Kleinewietfeld, M., Manzel, A., Titze, J., Kvakan, H., Yosef, N., Linker, R. A., Muller, D.
555 N., and Hafler, D. A. 2013. Sodium chloride drives autoimmune disease by the induction
556 of pathogenic TH17 cells. *Nature* 496:518.
557 21 Higashi, T., Shimojo, N., Suzuki, S., Nakaya, M., Takagi, R., Hashimoto, K., Nakagome,
558 K., Nakamura, K., Kohno, Y., and Matsushita, S. 2011. Coenzyme A contained in
559 mothers' milk is associated with the potential to induce atopic dermatitis. *Int Immunol*
560 23:741.
561 22 Kawano, M., Takagi, R., Saika, K., Matsui, M., and Matsushita, S. 2018. Dopamine
562 regulates cytokine secretion during innate and adaptive immune responses. *Int Immunol*
563 30:591.
564 23 Lutz, M. B., Kukutsch, N., Ogilvie, A. L., Rossner, S., Koch, F., Romani, N., and Schuler,
565 G. 1999. An advanced culture method for generating large quantities of highly pure
566 dendritic cells from mouse bone marrow. *Journal of immunological methods* 223:77.
567 24 Joseph, J., Bittner, S., Kaiser, F. M., Wiendl, H., and Kissler, S. 2012. IL-17 silencing
568 does not protect nonobese diabetic mice from autoimmune diabetes. *Journal of*
569 *immunology* 188:216.
570 25 Podojil, J. R., Padval, M. V., and Miller, S. D. 2011. Combination treatment of mice with
571 CRx-153 (nortriptyline and desloratadine) decreases the severity of experimental
572 autoimmune encephalomyelitis. *Cell Immunol* 270:237.
573 26 Barthelmes, J., Tafferner, N., Kurz, J., de Bruin, N., Parnham, M. J., Geisslinger, G., and

574 Schiffmann, S. 2016. Induction of Experimental Autoimmune Encephalomyelitis in Mice
575 and Evaluation of the Disease-dependent Distribution of Immune Cells in Various
576 Tissues. *J Vis Exp*.
577 27 Takeuchi, A., Usui, Y., Takeuchi, M., Hattori, T., Kezuka, T., Suzuki, J., Okunuki, Y.,
578 Iwasaki, T., Haino, M., Matsushima, K., and Usui, M. 2005. CCR5-deficient mice
579 develop experimental autoimmune uveoretinitis in the context of a deviant effector
580 response. *Invest Ophthalmol Vis Sci* 46:3753.
581 28 Ma, W., Bryce, P. J., Humbles, A. A., Laouini, D., Yalcindag, A., Alenius, H., Friend, D.
582 S., Oettgen, H. C., Gerard, C., and Geha, R. S. 2002. CCR3 is essential for skin
583 eosinophilia and airway hyperresponsiveness in a murine model of allergic skin
584 inflammation. *J Clin Invest* 109:621.
585 29 Acosta-Rodriguez, E. V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M.,
586 Lanzavecchia, A., Sallusto, F., and Napolitani, G. 2007. Surface phenotype and antigenic
587 specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol*
588 8:639.
589 30 Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente,
590 E., Fili, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F.,
591 Maggi, E., and Romagnani, S. 2007. Phenotypic and functional features of human Th17
592 cells. *J Exp Med* 204:1849.
593 31 McDevitt, H. O. 2000. Discovering the role of the major histocompatibility complex in
594 the immune response. *Annu Rev Immunol* 18:1.
595 32 Kaczmarek, E., Koziak, K., Sevigny, J., Siegel, J. B., Anrather, J., Beaudoin, A. R., Bach,
596 F. H., and Robson, S. C. 1996. Identification and characterization of CD39/vascular ATP

597 diphosphohydrolase. *J Biol Chem* 271:33116.

598 33 Jalkanen, S. and Salmi, M. 2008. VAP-1 and CD73, endothelial cell surface enzymes in
599 leukocyte extravasation. *Arteriosclerosis, thrombosis, and vascular biology* 28:18.

600 34 Mills, J. H., Thompson, L. F., Mueller, C., Waickman, A. T., Jalkanen, S., Niemela, J.,
601 Airas, L., and Bynoe, M. S. 2008. CD73 is required for efficient entry of lymphocytes
602 into the central nervous system during experimental autoimmune encephalomyelitis.
603 *Proceedings of the National Academy of Sciences of the United States of America*
604 105:9325.

605 35 Braun, N., Sevigny, J., Robson, S. C., Enjyoji, K., Guckelberger, O., Hammer, K., Di
606 Virgilio, F., and Zimmermann, H. 2000. Assignment of ecto-nucleoside triphosphate
607 diphosphohydrolase-1/cd39 expression to microglia and vasculature of the brain. *Eur J*
608 *Neurosci* 12:4357.

609 36 Kulesskaya, N., Voikar, V., Peltola, M., Yegutkin, G. G., Salmi, M., Jalkanen, S., and
610 Rauvala, H. 2013. CD73 is a major regulator of adenosinergic signalling in mouse brain.
611 *PLoS One* 8:e66896.

612 37 Gunther, G. R. and Herring, M. B. 1991. Inhibition of neutrophil superoxide production
613 by adenosine released from vascular endothelial cells. *Ann Vasc Surg* 5:325.

614 38 Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua,
615 D. J., and Littman, D. R. 2006. The orphan nuclear receptor RORgammat directs the
616 differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121.

617 39 Gaud, G., Lesourne, R., and Love, P. E. 2018. Regulatory mechanisms in T cell receptor
618 signalling. *Nat Rev Immunol* 18:485.

619 40 Hernandez, J. B., Chang, C., LeBlanc, M., Grimm, D., Le Lay, J., Kaestner, K. H., Zheng,

620 Y., and Montminy, M. 2015. The CREB/CRTC2 pathway modulates autoimmune disease
621 by promoting Th17 differentiation. *Nat Commun* 6:7216.
622 41 Rauen, T., Hedrich, C. M., Juang, Y. T., Tenbrock, K., and Tsokos, G. C. 2011.
623 cAMP-responsive element modulator (CREM)α protein induces interleukin 17A
624 expression and mediates epigenetic alterations at the interleukin-17A gene locus in
625 patients with systemic lupus erythematosus. *J Biol Chem* 286:43437.
626 42 Khairallah, C., Chu, T. H., and Sheridan, B. S. 2018. Tissue Adaptations of Memory and
627 Tissue-Resident Gamma Delta T Cells. *Frontiers in immunology* 9:2636.
628 43 Kadivar, M., Petersson, J., Svensson, L., and Marsal, J. 2016. CD8αβ+ γδ T Cells: A
629 Novel T Cell Subset with a Potential Role in Inflammatory Bowel Disease. *Journal of*
630 *immunology* 197:4584.
631 44 Liang, D., Shao, H., Born, W. K., O'Brien, R. L., Kaplan, H. J., and Sun, D. 2018. High
632 level expression of A2ARs is required for the enhancing function, but not for the
633 inhibiting function, of γδ T cells in the autoimmune responses of EAU. *PLoS One*
634 13:e0199601.
635 45 Bjørgo, E., Moltu, K., and Taskén, K. 2011. Phosphodiesterases as targets for modulating
636 T-cell responses. *Handb Exp Pharmacol*:345.
637 46 Latini, S. and Pedata, F. 2001. Adenosine in the central nervous system: release
638 mechanisms and extracellular concentrations. *J Neurochem* 79:463.
639 47 Carman, C. V. and Martinelli, R. 2015. T Lymphocyte-Endothelial Interactions:
640 Emerging Understanding of Trafficking and Antigen-Specific Immunity. *Frontiers in*
641 *immunology* 6:603.
642 48 Siffrin, V., Radbruch, H., Glumm, R., Niesner, R., Paterka, M., Herz, J., Leuenberger, T.,

643 Lehmann, S. M., Luenstedt, S., Rinnenthal, J. L., Laube, G., Luche, H., Lehnardt, S.,
644 Fehling, H. J., Griesbeck, O., and Zipp, F. 2010. In vivo imaging of partially reversible
645 th17 cell-induced neuronal dysfunction in the course of encephalomyelitis. *Immunity*
646 33:424.
647 49 Monks, C. R., Kupfer, H., Tamir, I., Barlow, A., and Kupfer, A. 1997. Selective
648 modulation of protein kinase C-theta during T-cell activation. *Nature* 385:83.
649 50 Fredholm, B. B. 2007. Adenosine, an endogenous distress signal, modulates tissue
650 damage and repair. *Cell Death Differ* 14:1315.
651 51 Csoka, B., Himer, L., Selmeczy, Z., Vizi, E. S., Pacher, P., Ledent, C., Deitch, E. A.,
652 Spolarics, Z., Nemeth, Z. H., and Hasko, G. 2008. Adenosine A2A receptor activation
653 inhibits T helper 1 and T helper 2 cell development and effector function. *FASEB J*
654 22:3491.
655 52 Lappas, C. M., Rieger, J. M., and Linden, J. 2005. A2A adenosine receptor induction
656 inhibits IFN-gamma production in murine CD4+ T cells. *Journal of immunology*
657 174:1073.
658 53 Klotz, K. N. 2000. Adenosine receptors and their ligands. *Naunyn Schmiedebergs Arch*
659 *Pharmacol* 362:382.
660 54 El-Tayeb, A., Michael, S., Abdelrahman, A., Behrenswerth, A., Gollos, S., Nieber, K.,
661 and Muller, C. E. 2011. Development of Polar Adenosine A2A Receptor Agonists for
662 Inflammatory Bowel Disease: Synergism with A2B Antagonists. *ACS Med Chem Lett*
663 2:890.
664 55 Kenakin, T. 2001. Inverse, protean, and ligand-selective agonism: matters of receptor
665 conformation. *FASEB J* 15:598.

666 56 Prather, P. L. 2004. Inverse agonists: tools to reveal ligand-specific conformations of G
667 protein-coupled receptors. *Sci STKE* 2004:pe1.
668 57 Nakano, K., Yamaoka, K., Hanami, K., Saito, K., Sasaguri, Y., Yanagihara, N., Tanaka,
669 S., Katsuki, I., Matsushita, S., and Tanaka, Y. 2011. Dopamine induces IL-6-dependent
670 IL-17 production via D1-like receptor on CD4 naive T cells and D1-like receptor
671 antagonist SCH-23390 inhibits cartilage destruction in a human rheumatoid
672 arthritis/SCID mouse chimera model. *Journal of immunology* 186:3745.
673 58 Alnouri, M. W., Jepards, S., Casari, A., Schiedel, A. C., Hinz, S., and Muller, C. E. 2015.
674 Selectivity is species-dependent: Characterization of standard agonists and antagonists at
675 human, rat, and mouse adenosine receptors. *Purinergic Signal* 11:389.
676 59 Kanda, T., Jackson, M. J., Smith, L. A., Pearce, R. K., Nakamura, J., Kase, H., Kuwana,
677 Y., and Jenner, P. 1998. Adenosine A2A antagonist: a novel antiparkinsonian agent that
678 does not provoke dyskinesia in parkinsonian monkeys. *Ann Neurol* 43:507.
679 60 Ohta, A., Gorelik, E., Prasad, S. J., Ronchese, F., Lukashev, D., Wong, M. K., Huang, X.,
680 Caldwell, S., Liu, K., Smith, P., Chen, J. F., Jackson, E. K., Apasov, S., Abrams, S., and
681 Sitkovsky, M. 2006. A2A adenosine receptor protects tumors from antitumor T cells.
682 *Proceedings of the National Academy of Sciences of the United States of America*
683 103:13132.
684 61 Kobie, J. J., Shah, P. R., Yang, L., Rebhahn, J. A., Fowell, D. J., and Mosmann, T. R.
685 2006. T regulatory and primed uncommitted CD4 T cells express CD73, which
686 suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine.
687 *Journal of immunology* 177:6780.
688 62 Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F.,

689 Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B., and Robson, S. C. 2007.
690 Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells
691 mediates immune suppression. *J Exp Med* 204:1257.
692 63 Moesta, A. K., Li, X. Y., and Smyth, M. J. 2020. Targeting CD39 in cancer. *Nat Rev*
693 *Immunol*.
694 64 Tuting, T. and de Visser, K. E. 2016. CANCER. How neutrophils promote metastasis.
695 *Science* 352:145.
696 65 Coffelt, S. B., Kersten, K., Doornebal, C. W., Weiden, J., Vrijland, K., Hau, C. S.,
697 Verstegen, N. J. M., Ciampricotti, M., Hawinkels, L., Jonkers, J., and de Visser, K. E.
698 2015. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast
699 cancer metastasis. *Nature* 522:345.
700 66 Bald, T., Quast, T., Landsberg, J., Rogava, M., Glodde, N., Lopez-Ramos, D., Kohlmeyer,
701 J., Riesenberg, S., van den Boorn-Konijnenberg, D., Homig-Holzel, C., Reuten, R.,
702 Schadow, B., Weighardt, H., Wenzel, D., Helfrich, I., Schadendorf, D., Bloch, W.,
703 Bianchi, M. E., Lugassy, C., Barnhill, R. L., Koch, M., Fleischmann, B. K., Forster, I.,
704 Kastenmuller, W., Kolanus, W., Holzel, M., Gaffal, E., and Tuting, T. 2014.
705 Ultraviolet-radiation-induced inflammation promotes angiotropism and metastasis in
706 melanoma. *Nature* 507:109.
707 67 El Rayes, T., Catena, R., Lee, S., Stawowczyk, M., Joshi, N., Fischbach, C., Powell, C.
708 A., Dannenberg, A. J., Altorki, N. K., Gao, D., and Mittal, V. 2015. Lung inflammation
709 promotes metastasis through neutrophil protease-mediated degradation of Tsp-1.
710 *Proceedings of the National Academy of Sciences of the United States of America*
711 112:16000.

712 68 Wculek, S. K. and Malanchi, I. 2015. Neutrophils support lung colonization of
713 metastasis-initiating breast cancer cells. *Nature* 528:413.
714 69 Templeton, A. J., McNamara, M. G., Seruga, B., Vera-Badillo, F. E., Aneja, P., Ocana,
715 A., Leibowitz-Amit, R., Sonpavde, G., Knox, J. J., Tran, B., Tannock, I. F., and Amir, E.
716 2014. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic
717 review and meta-analysis. *J Natl Cancer Inst* 106:dju124.
718 70 Yazici, H., Seyahi, E., Hatemi, G., and Yazici, Y. 2018. Behcet syndrome: a
719 contemporary view. *Nat Rev Rheumatol* 14:107.
720 71 Meyts, I. and Aksentijevich, I. 2018. Deficiency of Adenosine Deaminase 2 (DADA2):
721 Updates on the Phenotype, Genetics, Pathogenesis, and Treatment. *J Clin Immunol*
722 38:569.
723
724

725 **Abbreviations:** Th, T-helper; TGF, tumor growth factor; IL, interleukin; APCs, antigen
726 presenting cells; AC, adenyl cyclase; PKA, protein kinase A; CREB, cAMP response element
727 binding protein; MLR, two-way mixed lymphocyte reaction; EAE, experimental autoimmune
728 encephalomyelitis; CCPA, 2-Chloro-N6-cyclopentyladenosine; AMP-CP, adenosine 5'-(α,
729 β-methylene) diphosphate; BM, bone marrow; BM-DC, bone-marrow-derived dendritic cell;
730 CD3/CD28, agonistic anti-CD3/CD28 antibodies; PLP, myelin proteolipid protein; PLP peptide,
731 I-A^s restricted helper peptide derived from the PLP; CFA, complete Freund's adjuvant; CCR,
732 chemokine receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; MACS,
733 magnetic-activated cell sorting; Ab, antibody; n, number of repeat experiments; SD, standard
734 deviation.
735

736 **Acknowledgments**

737 This work was supported by a Grant-in-Aid for Scientific Research (C) (no. 19K07201),
738 awarded to M.K., a Grant-in-Aid for Young Scientists (B) (no. 18K15327) to R.T., and a
739 Grant-in-Aid for Scientific Research (C) (no. 19K08887) awarded to S.M. by the Japanese
740 Society for the Promotion of Science. This work was also supported by the 44th and 45th
741 Science Research Promotion Fund, awarded to M.K. by the Promotion and Mutual Aid
742 Corporation for Private Schools of Japan.
743

744 **Author contributions**

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

748

749 **Conflicts of interest**

750 Sho Matsushita is an employee of iMmno, Inc.

751 The other authors have no conflicts of interest to declare.

752 **Figure legends**

753

754 **Fig. 1. Adenosine induces hypersecretion of IL-17A by CD4$^+$ T cells in an MLR. A–C**, An
755 MLR was performed for 7 days in the presence of adenosine (0–1 mM). After 7 days, cells were
756 stained with anti-CD4 (x-axis) and IL-17A (y-axis) antibodies (Abs), followed by flow
757 cytometry analysis (**A**, n (number of repeat experiments) = 4). The percentage of
758 IL-17A-producing CD4$^+$ T cells within the total CD4$^+$ T cell population is shown (**B**, n = 6–9).
759 Cells supernatants were analyzed in an IL-17A ELISA (**C**, n = 6–9). **D**, The effects of the A2aR
760 on IL-17A production in the presence of PSB0777 (an A2aR agonist) (*left*, n = 6–9),
761 Istradefylline (an A2aR antagonist) plus adenosine (100 μM) (*right*, n = 6–9). **E**, The effects of
762 A2aR signaling on IL-17A production in the presence of MDL-12330A (an adenyl cyclase
763 inhibitor) plus adenosine (100 μM) (*first panel*, n = 4–6), H-89 (a protein kinase A inhibitor)
764 plus adenosine (100 μM) (*second panel*, n = 4–6), 666-15 (a cAMP response element binding
765 protein inhibitor) plus adenosine (100 μM) (*third panel*, n = 4–6), and Forskolin (an activator of
766 adenyl cyclase) (*fourth panel*, n = 4–6) were analyzed in an IL-17A ELISA. **F**, The effects of
767 CD39/CD73 inhibitors on IL-17A production in the presence of ARL67156 (a CD39 inhibitor)
768 plus ATP (100 μM) (*left*, n = 4 – 6) or AMP-CP (a CD73 inhibitor) plus ATP (100 μM) (*right*, n
769 = 4–6) were analyzed in an IL-17A ELISA. **G**, The effects of an A2aR antagonist and
770 CD39/CD73 inhibitors on basal IL-17A production in the presence of Istradefylline (*left*, n = 4–
771 7), ARL67156 (*center*, n = 4–7), or AMP-CP (*right*, n = 4–7) were analyzed in an IL-17A
772 ELISA. Data are expressed as the mean ± standard deviation (SD) and were compared using an
773 unpaired Student's t-test (**B**) or one-way ANOVA with Tukey's post-hoc test (**C**–**G**). *P < 0.05
774 and **P < 0.01, compared with medium (**C**, **D**, *left*; **E**, *fourth panel*; and **G**), adenosine (100 μM)

775 (**D**, *right*, **E**, *first–third panels*), or ATP (100 μM) (**F**).

776

777 **Fig. 2. Adenosine production in the MLR. A**, Concentrations of adenosine or ATP in MLR
778 supernatants were measured in an adenosine (*left*, n = 4–6) or ATP (*right*, n = 4–6) ELISA (0–24
779 h). **B** and **C**, The effects of CD39 (**B**, ARL67156, n = 4–6) and CD73 (**C**, AMP-CP, n = 4–6)
780 inhibitors on production of adenosine or ATP in the MLR were analyzed in an adenosine (*left*) or
781 ATP (*right*) ELISA. **D, E** and **F**, Levels of adenosine and ATP in the supernatants of
782 CD3/CD28-stimulated $CD4^+$ T cells (**D**, n = 4), LPS- or anti-mouse CD40 Ab-stimulated B cells
783 (**E**, n = 4), and LPS- or anti-mouse CD40 Ab-stimulated BM-DCs (**F**, n = 4) were analyzed in
784 adenosine and ATP ELISAs at 24 h post-stimulation in the presence of CD39 or CD73 inhibitors.
785 Data are expressed as the mean ± SD and were compared using one-way ANOVA with Tukey's
786 post-hoc test. *P < 0.05 and **P < 0.01, compared with medium (**B** and **C**), CD3/CD28
787 stimulation (**D**), or LPS or anti-mouse CD40 Ab stimulation (**E** and **F**).

788

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

798 plus adenosine (600 μM) (*second panel*, n = 4–6), 666-15 (a cAMP response element binding
799 protein inhibitor) plus adenosine (600 μM) (*third panel*, n = 4–6), or Forskolin (an activator of
800 adenyl cyclase) (*fourth panel*, n = 4–6) were analyzed in an IL-17A ELISA. **F**, $CD4^+CD62L^+$ and
801 $CD4^+CD62L^+FT$ cells were stimulated for 7 days with anti-CD3/CD28 antibodies in the
802 presence of adenosine (0–1 mM). After 7 days, supernatants were analyzed in an IL-17A ELISA
803 (n = 4). **G**, Subsets of $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28 antibodies in
804 the presence of adenosine (600 μM) after isolation of each CCR cell type (high (hi) and low (lo)
805 expression) (n = 4). Data are expressed as the mean ± SD and were compared using an unpaired
806 Student's t-test (**G**) or one-way ANOVA with Tukey's post-hoc test (**A–F**). *P < 0.05 and **P <
807 0.01, compared with CD3/CD28 stimulation (**A–C**, **D**, *left;* **E***, fourth panel;* and **F**) or CD3/28
808 stimulation plus adenosine (600 μM) (**D**, *right* and **E***, first–third panels*).
809
810 **Fig. 4. Effect of adenosine and an A2aR antagonist on production of Th17-related cytokines.**
811 **A,** An MLR was performed for 7 days in the presence of adenosine (0–1 mM) (*top row*),
812 PSB0777 (an A2aR agonist) (*second row*), or Istradefylline (an A2aR antagonist) plus adenosine
813 (100 μM) (*bottom row*). At 7 days post-incubation, the supernatants were analyzed in an
814 IL-17A (*left*), IL-17F (*center*), or IL-22 (*right*) ELISA (n = 6–9). **B,** $CD4^+$ T cells were
815 stimulated with an anti-CD3/CD28 antibody for 7 days in the presence of adenosine (600 μM)
816 (*top row*), PSB0777 (an A2aR agonist) (*second row*), or Istradefylline (an A2aR antagonist) plus
817 adenosine (600 μM) *(bottom row*). After stimulation, supernatants were analyzed in IL-17A (*left*),
818 IL-17F (*center*), and IL-22 (*right*) ELISAs (n = 4–6). Data are expressed as the mean ± SD and
819 were compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01,
820 compared with medium or CD3/CD28 stimulation (*top and second row of panels*) or adenosine

821 (100 μM) or CD3/CD28 stimulation plus adenosine (600 μM) (*third row of panels*).

822

823 **Fig. 5. Suppression of adenosine-mediated hypersecretion of IL-17A ameliorates EAE. A**
824 and **B**, SJL/J $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28 antibodies and an
825 A2aR antagonist (**A**, n = 4). Naïve $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28
826 antibodies in the presence of IL-6, TGF-β1, and Istradefylline (an A2aR antagonist) (0–1 nM) (**B**,
827 *left*, n = 4). Alternatively, naïve $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28
828 antibodies in the presence of IL-6 and TGF-β1, followed by another 7 day incubation with
829 anti-CD3/CD28 antibodies and Istradefylline (0–1 nM) (**B**, *right*, n = 4). After the stimulation,
830 supernatants were analyzed in an IL-17A ELISA. **C and D,** Mice were immunized
831 subcutaneously with PLP peptide emulsified in CFA (PLP peptide/CFA). Mice received oral
832 water, an A2aR agonist (PSB0777; 6 μg/mouse), or an A2aR antagonist (Istradefylline; 6
833 μg/mouse) once every 2 days from Day –7 to Day +7 after immunization with PLP peptide/CFA
834 (Day 0) (**D**). At 7 days post-immunization, splenocytes were incubated for 3 days with PLP
835 peptide in the presence of Istradefylline and adenosine (600 μM) (**C**, n = 4) or in the presence or
836 absence of PLP peptide (**D**, n = 4). After the incubation, supernatants were analyzed in an
837 IL-17A ELISA. **E,** To induce EAE, SJL/J mice were immunized with PLP peptide/CFA. Before
838 and post-immunization with the PLP peptide (Days 0 to 18), mice received oral Istradefylline (6
839 μg/mouse) or water once every 2 days. Clinical scores were recorded every day during EAE
840 induction (**E**, n = 15). Data were obtained from five independent experiments (n = 3 mice/group).
841 **F,** At 18 days post-immunization, spinal cord sections from mice administered oral Istradefylline
842 (*right*) or water (*left*) were stained with hematoxylin and eosin (*upper panels*) or with an
843 anti-mouse CD3 antibody (*lower panels*) (Scale bar, 100 μm) (**F**, n = 5). **G and H,** At 18 days

844 post-immunization, splenocytes (spleen, *left*), inguinal lymph node lymphocytes (inguinal lymph
845 node, *center*), and spinal cord cells (spinal cord, *right*) from mice administered oral water (*left* of
846 each panel) or Istradefylline (*right* of each panel) were stained with anti-CD4 (x-axis) and CCR5
847 (a Th1 marker; top panels, y-axis), CCR3 (a Th2 marker; second panels, y-axis), or CCR6 (a
848 Th17 marker; bottom panels, y-axis) antibodies (Abs), followed by flow cytometry analysis (**G**,
849 n = 5). Values shown in the upper and lower right of each panel denote the percentage of
850 CCR-positive counts within the total CD4-positive counts, and the percentage of CD4-positive
851 counts within the total count, respectively. *P < 0.05. Also, spinal cord cells were incubated for 3
852 days in the presence or absence of PLP peptide (**H**, n = 4). After the incubation, supernatants
853 were analyzed in an IL-17A ELISA. Data are expressed as the mean ± SD. Data were compared
854 using one-way ANOVA with Tukey's post-hoc test (**A**–**D, G, and H**), or using a non-parametric
855 Mann-Whitney *U*-test (**E**). *P < 0.05 and **P < 0.01, compared with CD3/CD28 stimulation
856 plus adenosine (600 μM) (**A**), CD3/CD28 stimulation plus IL-6 and TGF-β1 (**B**), PLP peptide
857 pulse (**C**), oral water (**G**), or PLP peptide pulse (oral water) (**H**).

858 **Legends for the supplementary figures**

859

860 **Sup. Fig. 1. Purity and viability of each immune cell subset. A**, *First row (left)*: Splenocytes
861 or isolated $CD4^+$ T cells were stained with a FITC-conjugated anti-mouse CD4 antibody (Ab)
862 (BioLegend) (x-axis) and a PE-conjugated anti-mouse CD3 Ab (BioLegend) (y-axis). *Second*
863 *row (left)*: Splenocytes or $CD4^+CD62L^+$ T cells isolated by MACS were stained with a
864 FITC-conjugated anti-mouse CD4 Ab (BioLegend) (x-axis) and a PE-conjugated anti-mouse
865 CD62L Ab (BioLegend) (y-axis). *First row (right)*: Splenocytes and B cells were stained with a
866 FITC-conjugated anti-mouse CD19 Ab (BioLegend) (x-axis) and cell numbers were counted
867 (y-axis). *Second row (right)*: Splenocytes, BM leukocytes, and BM-DCs were stained with a
868 FITC-conjugated anti-mouse CD11c Ab (BioLegend) (x-axis) and cell numbers were counted
869 (y-axis). The number in each panel represents the percentage ± SD of each immune subset within
870 the total cell population (n = 3). **B**, *First row*: Isolated $CD4^+$ (by MACS)-, $CD4^+CD62L^-$ (by cell
871 sorting)-, or $CD4^+CD62L^+$ (by cell sorting) T cells were analyzed with a PE-conjugated
872 anti-mouse CD62L Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). The
873 number in each panel represents the percentage of each immune subset (- or +) within the total
874 cell population (- plus +). Data are representative of at least three repeat experiments. *Second*
875 *row*: Isolated $CD4^+$ (by MACS), $CD4^+CCR3^{low\ (lo)}$ (by cell sorting), or $CD4^+CCR3^{high\ (hi)}$ (by cell
876 sorting) T cells were analyzed with a PE-conjugated anti-mouse CCR3 Ab (BioLegend) (x-axis)
877 and cell numbers were counted (y-axis). *Third row*: Isolated $CD4^+$ (by MACS), $CD4^+CCR5^{lo}$ (by
878 cell sorting), or $CD4^+CCR5^{hi}$ (by cell sorting) T cells were analyzed with a PE-conjugated
879 anti-mouse CCR5 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). *Fourth row*:
880 Isolated $CD4^+$ (by MACS), $CD4^+CCR6^{lo}$ (by cell sorting), or $CD4^+CCR6^{hi}$ (by cell sorting) T

881 cells were analyzed with a PE-conjugated anti-mouse CCR6 Ab (BioLegend) (x-axis) and cell
882 numbers were counted (y-axis). *Fifth row*: Isolated $CD4^+$ (by MACS), $CD4^+CD25^{lo}$ (by cell
883 sorting), or $CD4^+CD25^{hi}$ (by cell sorting) T cells were analyzed with a PE-conjugated anti-mouse
884 CD25 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). The number in each
885 panel represents the mean percentage ± SD of each immune subset (hi or lo) within the total cell
886 population (hi plus lo) (n = 3). **C**, After cell isolation, each immune cell type was mixed with
887 Trypan blue. Viability was calculated as the number of unstained cells/(stained cells + unstained
888 cells) × 100 (n = 3). The percentage represents the mean percentage ± SD.
889
890 **Sup. Fig. 2. Effect of an adenosine receptor agonist and antagonist on production of IL-17A**
891 **in an MLR and by CD3/CD28-stimulated $CD4^+$ T cells, and the effect of adenosine on**
892 **proliferation of Th cells. A**, An MLR was performed for 7 days in the presence of each
893 adenosine receptor agonist, or each adenosine receptor antagonist plus adenosine (100 μM) (n =
894 6–9). **B**, $CD4^+$ T cells were stimulated with an anti-CD3/CD28 antibody for 7 days in the
895 presence of each adenosine receptor agonist, or in the presence of each adenosine receptor
896 antagonist plus adenosine (600 μM) (n = 6–9). After 7 days, the supernatants were analyzed in
897 an IL-17A ELISA. *First row*: CCPA (an A1R agonist). *Second row*: PSB0777 (an A2aR agonist,
898 *left)* and Istradefylline (an A2aR antagonist, *right*). *Third row*: BAY 60-653 (an A2bR agonist).
899 *Forth row*: HEMADO (an A3R agonist). **C**, An MLR was performed in the presence or absence
900 of adenosine (100 μM). After 0, 3, and 7 days, splenocytes were stained with anti-CD4 (x-axis)
901 and CCR5 (a Th1 marker; top panels, y-axis), CCR3 (a Th2 marker; second panels, y-axis), or
902 CCR6 (a Th17 marker; bottom panels, y-axis) antibodies, followed by flow cytometry analysis
903 (n = 3). Values in the upper right of each panel denote the percentage of CCR-positive counts

904 within the CD4 positive counts. *P < 0.05 and **P < 0.01. Data are expressed as the mean ± SD
905 and are compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01,
906 compared with medium (**A**, *left*; **B**, *left*; and **C**), adenosine (100 μM) (**A**, *right*), CD3/28
907 stimulation plus adenosine (600 μM) (**B**, *right*).
908
909 **Sup. Fig. 3. Effect of adenosine on production of IFN-γ, IL-5, and IL-17A by**
910 **CD3/CD28-stimulated CD4$^+$ T cells.** CD4$^+$ T cells were stimulated for 1–7 days with
911 anti-CD3/CD28 antibodies in the presence or absence of adenosine (600 μM). After stimulation,
912 supernatants were analyzed in IFN-γ (*top row*), IL-5 (*second row*), and IL-17A (*bottom row*)
913 ELISAs (n = 4–6). Data are expressed as the mean ± SD and were compared using one-way
914 ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with CD3/CD28
915 stimulation.
916
917 **Sup. Fig. 4. Effect of adenosine on A2aR expression by CD4$^+$ T cells, and on expression of**
918 **MHC, costimulatory molecules, and A2aR and production of IL-6, IL-23, and TGF-β by**
919 **APCs. A**, CD4$^+$ T cells were stimulated for 0–7 days with anti-CD3/CD28 antibodies in the
920 presence of adenosine (0–1 mM). After stimulation, cells were stained with a FITC-conjugated
921 anti-mouse CD4 antibody (Ab) (BioLegend) (x-axis) and a PE-conjugated anti-A2aR Ab (Novus
922 Biologicals) (y-axis). *First row*: Splenocytes (*left*) or isolated CD4$^+$ T cells (*right*) were stained
923 with anti-CD4 (x-axis) and A2aR (y-axis) Abs. *Second row*: CD3/CD28-stimulated CD4$^+$ T cells
924 were stained with anti-CD4 (x-axis) and A2aR (y-axis) Abs**.** *Third row*: CD4$^+$ T cells were
925 stimulated with CD3/CD28 Abs in the presence of adenosine (0–1 mM) and then stained with
926 anti-CD4 (x-axis) and A2aR (y-axis) Abs. The number in each panel represents the percentage ±

927 SD of A2aR^{high} cells within the CD4^{+} population (n = 3). Data were compared using one-way
928 ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with CD3/CD28
929 stimulation (second and third rows). **B**, B cells and BM-DCs were stimulated for 1 day with LPS
930 (upper panels) or anti-mouse CD40 Ab (CD40) (lower panels) in the presence of adenosine (0.1–
931 1 mM). After stimulation, the IL-6, IL-23, and TGF-β1 concentrations in the supernatants were
932 analyzed by ELISA (n = 3). Data are expressed as the mean ± SD and were compared using
933 one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with LPS or
934 anti-mouse CD40 Ab stimulation. **C**, B cells (upper panels) and BM-DCs (lower panels) were
935 stimulated for 1 day with LPS or anti-mouse CD40 Ab (CD40) in the presence of adenosine (100
936 or 600 μM). After stimulation, the cells were stained with a FITC-conjugated anti-mouse CD80
937 (BioLegend), CD86 (BioLegend), or CD40 (BioLegend) Ab, PE-conjugated anti-mouse H-2
938 (BioLegend) or I-A/I-E (BioLegend) Ab, or a PE-conjugated anti-A2aR (Novus Biologicals) Ab
939 (x-axis), and cell numbers were counted (y-axis) (n = 3). The number in each panel represents
940 the mean fluorescence intensity ± SD (n = 3). Data were compared using one-way ANOVA with
941 Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with LPS or anti-mouse CD40 Ab
942 stimulation.
943

Fig. 1

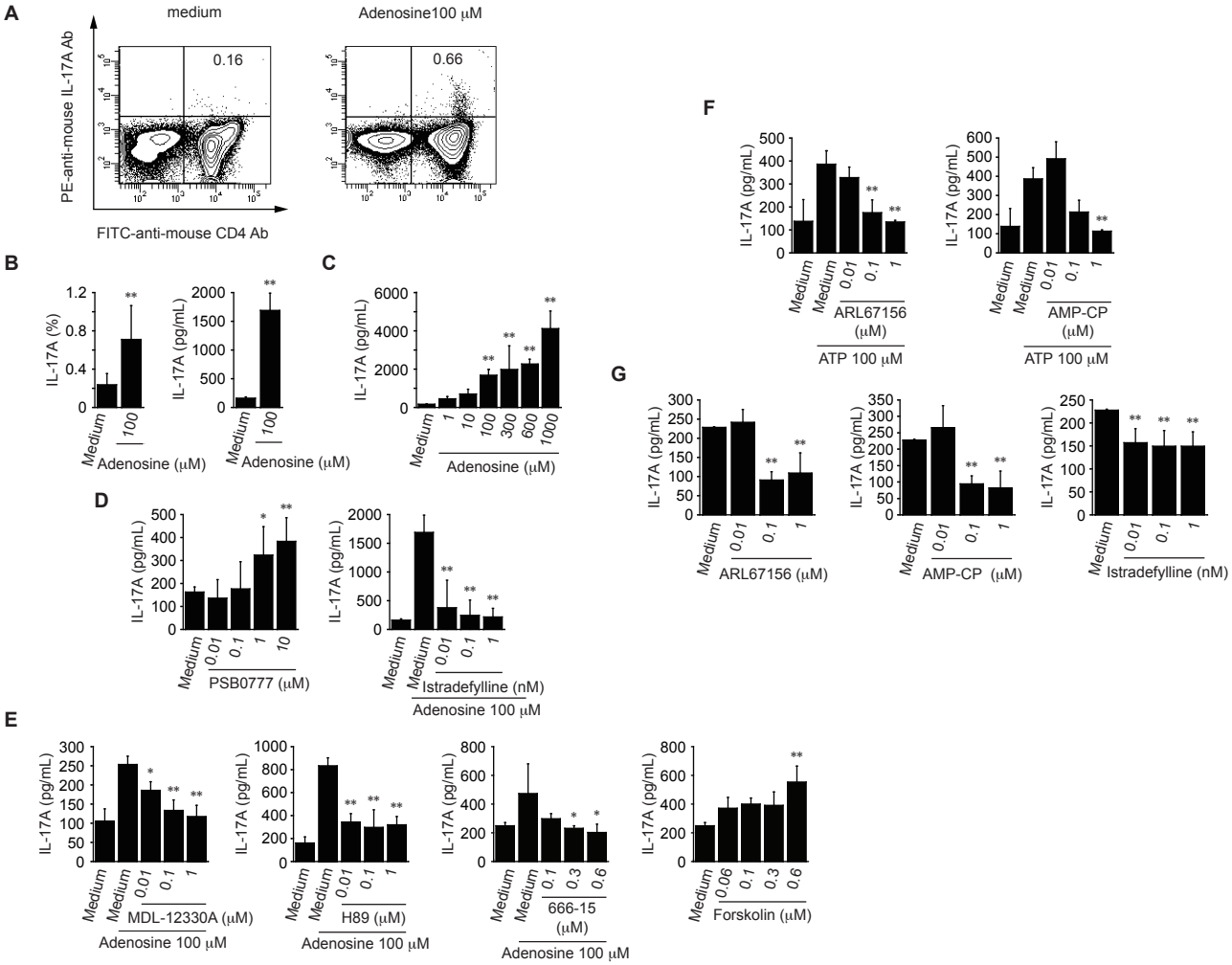

A
medium
Adenosine100 μM
0.16
0.66
PE-anti-mouse IL-17A Ab
FITC-anti-mouse CD4 Ab
B
IL-17A (%)
IL-17A (pg/mL)
Medium
100
Adenosine (μM)
C
IL-17A (pg/mL)
Medium
1
10
100
300
600
1000
Adenosine (μM)
D
IL-17A (pg/mL)
Medium
0.01
0.1
1
10
PSB0777 (μM)
Medium
Istradefylline (nM)
Adenosine 100 μM
E
IL-17A (pg/mL)
Medium
0.01
0.1
1
MDL-12330A (μM)
Adenosine 100 μM
H89 (μM)
Adenosine 100 μM
0.1
0.3
0.6
666-15 (μM)
Adenosine 100 μM
0.06
Forskolin (μM)
F
IL-17A (pg/mL)
ARL67156 (μM)
ATP 100 μM
AMP-CP (μM)
ATP 100 μM
G
IL-17A (pg/mL)
ARL67156 (μM)
AMP-CP (μM)
Istradefylline (nM)

Fig. 2

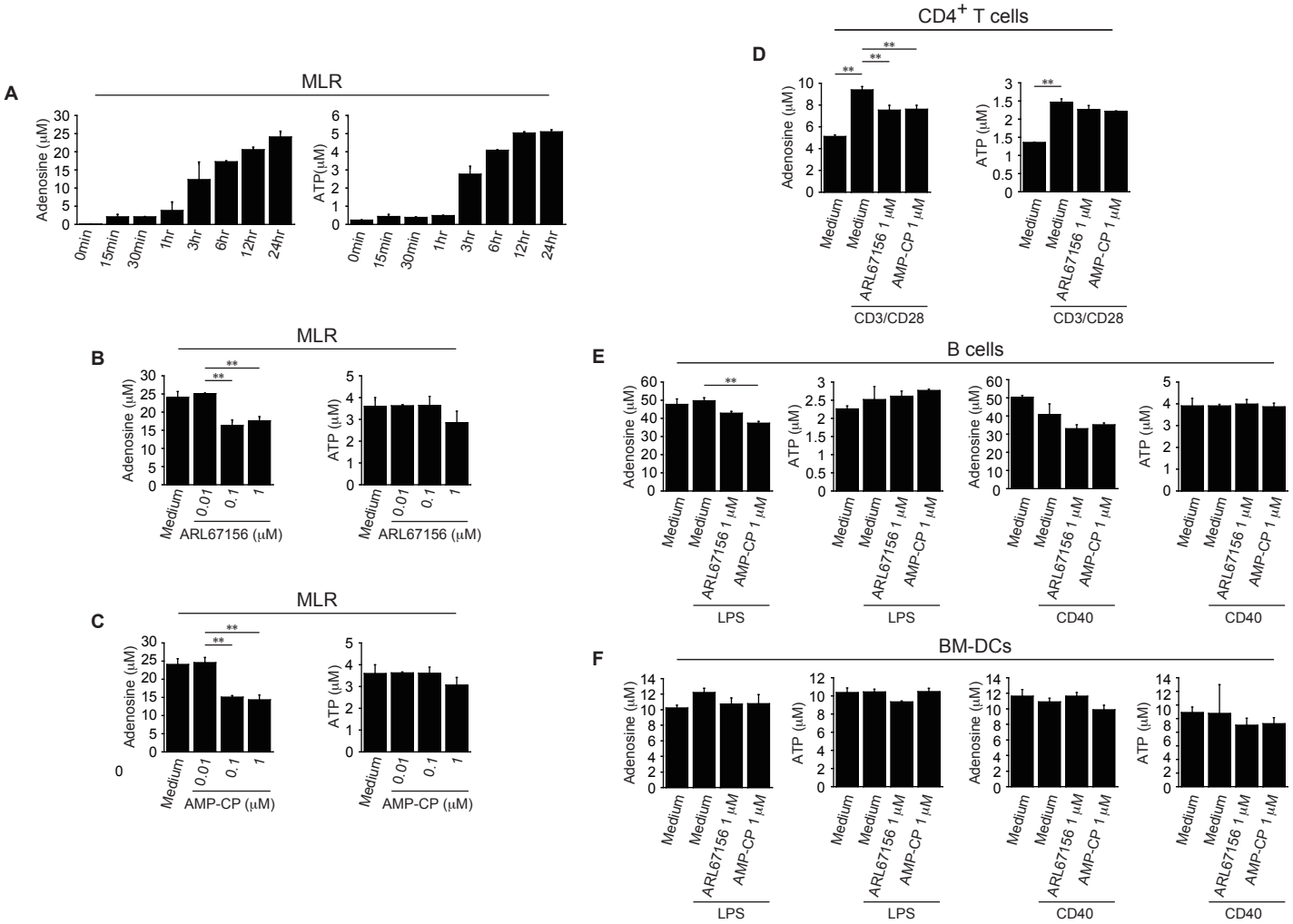

A
MLR
Adenosine (μM)
ATP(μM)
0min
15min
30min
1hr
3hr
6hr
12hr
24hr
B
MLR
Adenosine (μM)
ATP (μM)
Medium
0.01
0.1
1
ARL67156 (μM)
C
MLR
Adenosine (μM)
ATP (μM)
Medium
0.01
0.1
1
AMP-CP (μM)
D
CD4+ T cells
Adenosine (μM)
ATP (μM)
Medium
Medium
ARL67156 1 μM
AMP-CP 1 μM
CD3/CD28
E
B cells
Adenosine (μM)
ATP (μM)
Medium
Medium
ARL67156 1 μM
AMP-CP 1 μM
LPS
CD40
F
BM-DCs
Adenosine (μM)
ATP (μM)
Medium
Medium
ARL67156 1 μM
AMP-CP 1 μM
LPS
CD40

Fig. 3

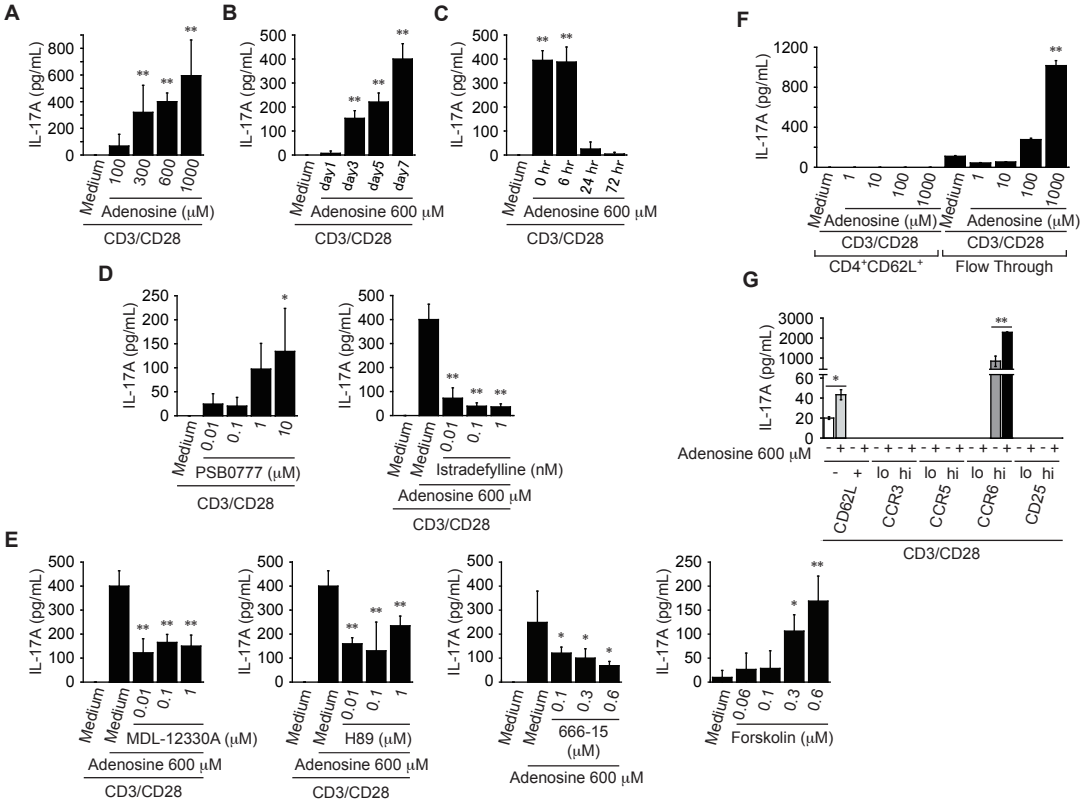

A
IL-17A (pg/mL)
Medium
100
300
600
1000
Adenosine (μM)
CD3/CD28
B
Medium
day1
day3
day5
day7
Adenosine 600 μM
CD3/CD28
C
Medium
0 hr
6 hr
24 hr
72 hr
Adenosine 600 μM
CD3/CD28
F
Medium
1
10
100
1000
Adenosine (μM)
CD3/CD28
CD4+CD62L+
Flow Through
D
PSB0777 (μM)
Istradefylline (nM)
Adenosine 600 μM
E
MDL-12330A (μM)
H89 (μM)
666-15 (μM)
Forskolin (μM)
G
Adenosine 600 μM
CD62L
CCR3
CCR5
CCR6
CD25
lo
hi

Fig. 4

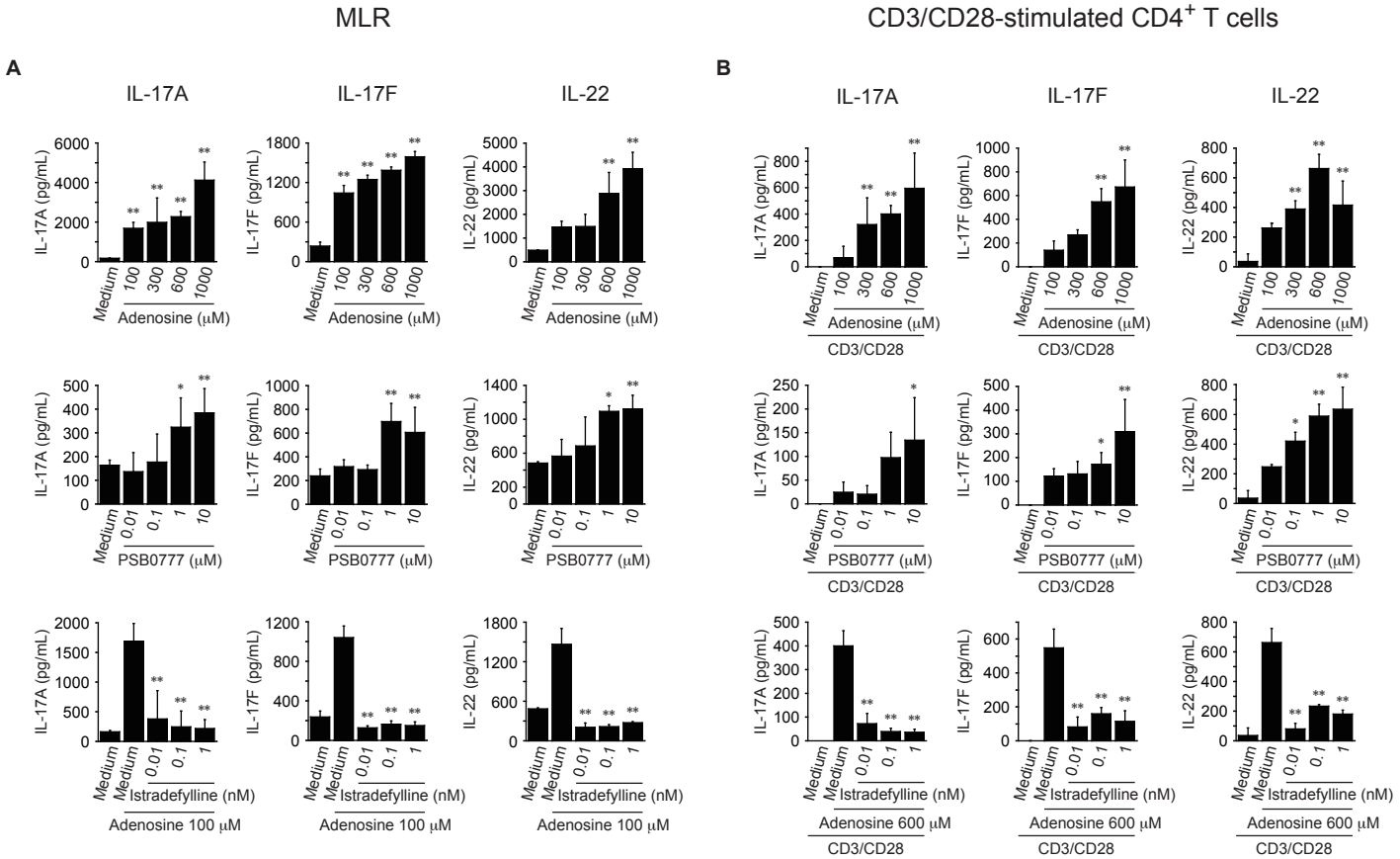
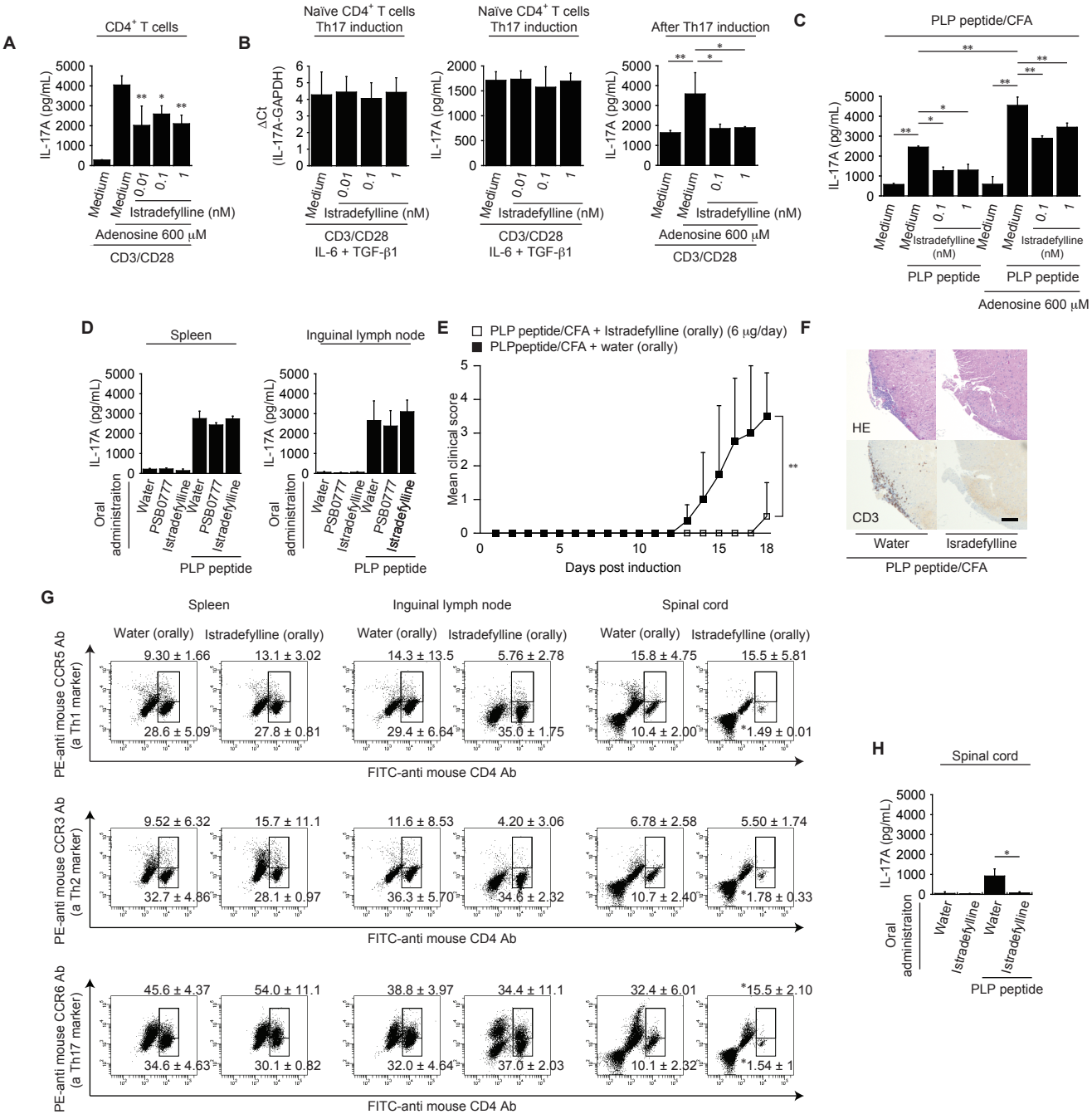
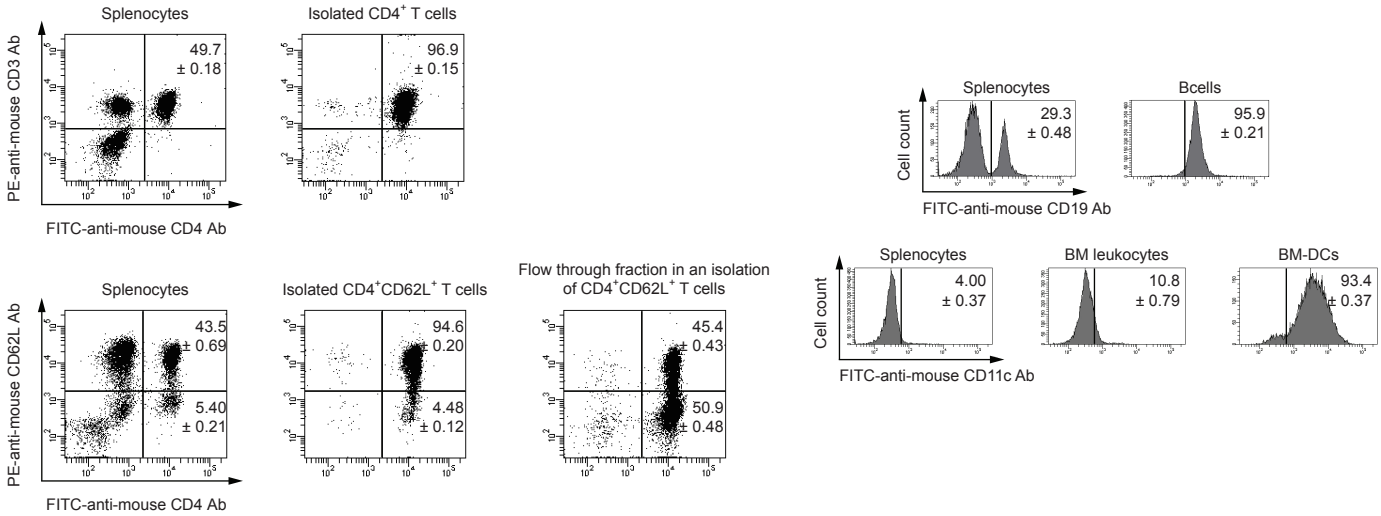

MLR
CD3/CD28-stimulated CD4+ T cells
A
B
IL-17A
IL-17F
IL-22
IL-17A (pg/mL)
IL-17F (pg/mL)
IL-22 (pg/mL)
Medium
Adenosine (μM)
PSB0777 (μM)
Istradefylline (nM)
Adenosine 100 μM
Adenosine 600 μM
CD3/CD28

Fig. 5

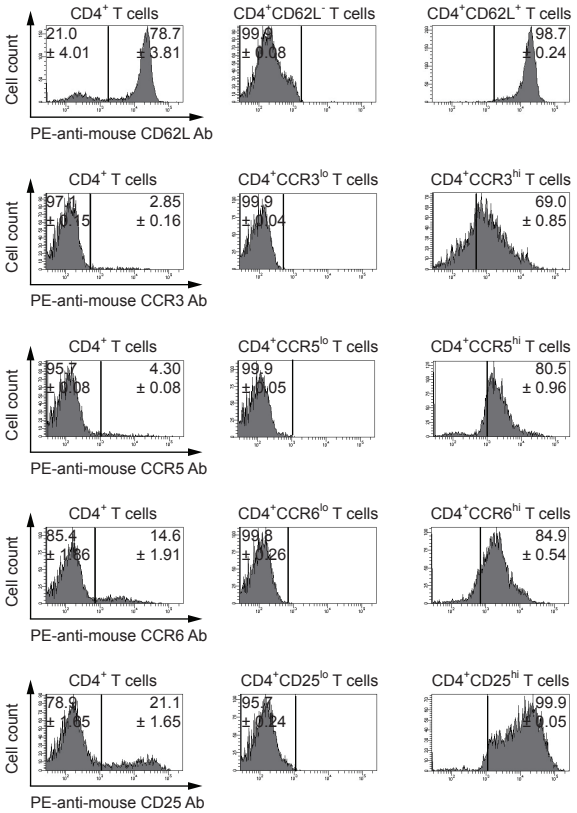

A
CD4+ T cells
IL-17A (pg/mL)
Medium
Medium
0.01
0.1
1
Istradefylline (nM)
Adenosine 600 μM
CD3/CD28
B
Naïve CD4+ T cells
Th17 induction
ΔCt
(IL-17A-GAPDH)
IL-17A (pg/mL)
CD3/CD28
IL-6 + TGF-β1
After Th17 induction
C
PLP peptide/CFA
PLP peptide
Adenosine 600 μM
D
Spleen
Inguinal lymph node
Oral administraiton
Water
PSB0777
Istradefylline
PLP peptide
E
PLP peptide/CFA + Istradefylline (orally) (6 μg/day)
PLPpeptide/CFA + water (orally)
Mean clinical score
Days post induction
F
HE
CD3
Water
Isradefylline
PLP peptide/CFA
G
Spleen
Inguinal lymph node
Spinal cord
Water (orally)
Istradefylline (orally)
PE-anti mouse CCR5 Ab
(a Th1 marker)
9.30 ± 1.66
13.1 ± 3.02
14.3 ± 13.5
5.76 ± 2.78
15.8 ± 4.75
15.5 ± 5.81
28.6 ± 5.09
27.8 ± 0.81
29.4 ± 6.64
35.0 ± 1.75
10.4 ± 2.00
*1.49 ± 0.01
FITC-anti mouse CD4 Ab
PE-anti mouse CCR3 Ab
(a Th2 marker)
9.52 ± 6.32
15.7 ± 11.1
11.6 ± 8.53
4.20 ± 3.06
6.78 ± 2.58
5.50 ± 1.74
32.7 ± 4.86
28.1 ± 0.97
36.3 ± 5.70
34.6 ± 2.32
10.7 ± 2.40
*1.78 ± 0.33
PE-anti mouse CCR6 Ab
(a Th17 marker)
45.6 ± 4.37
54.0 ± 11.1
38.8 ± 3.97
34.4 ± 11.1
32.4 ± 6.01
*15.5 ± 2.10
34.6 ± 4.63
30.1 ± 0.82
32.0 ± 4.64
37.0 ± 2.03
10.1 ± 2.32
*1.54 ± 1
H
Spinal cord
IL-17A (pg/mL)
Oral administraiton
Water
Istradefylline
PLP peptide

Sup. Fig. 1

A



B

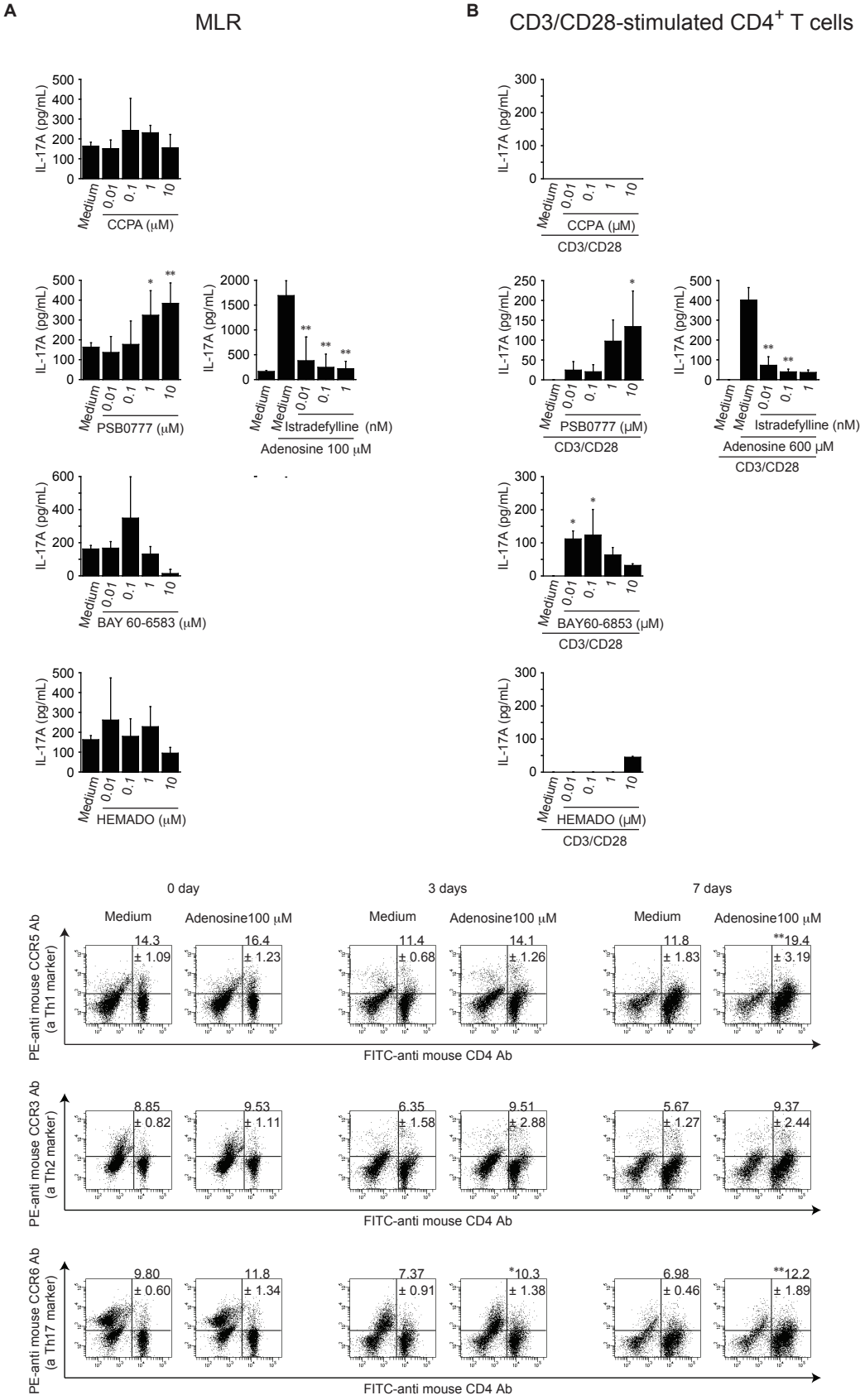


C

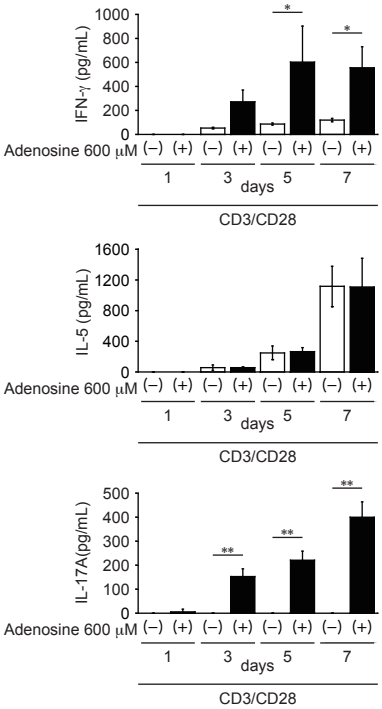
Cell type	Viability
Splenocytes	100 ± 0
$CD4^+$ T cells	100 ± 0
B cells	100 ± 0
BM-DCs	94.5 ± 1.67
Naive $CD4^+$ T cells	100 ± 0
Flow through of $CD4^+CD62L^+$ T cells	100 ± 0

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

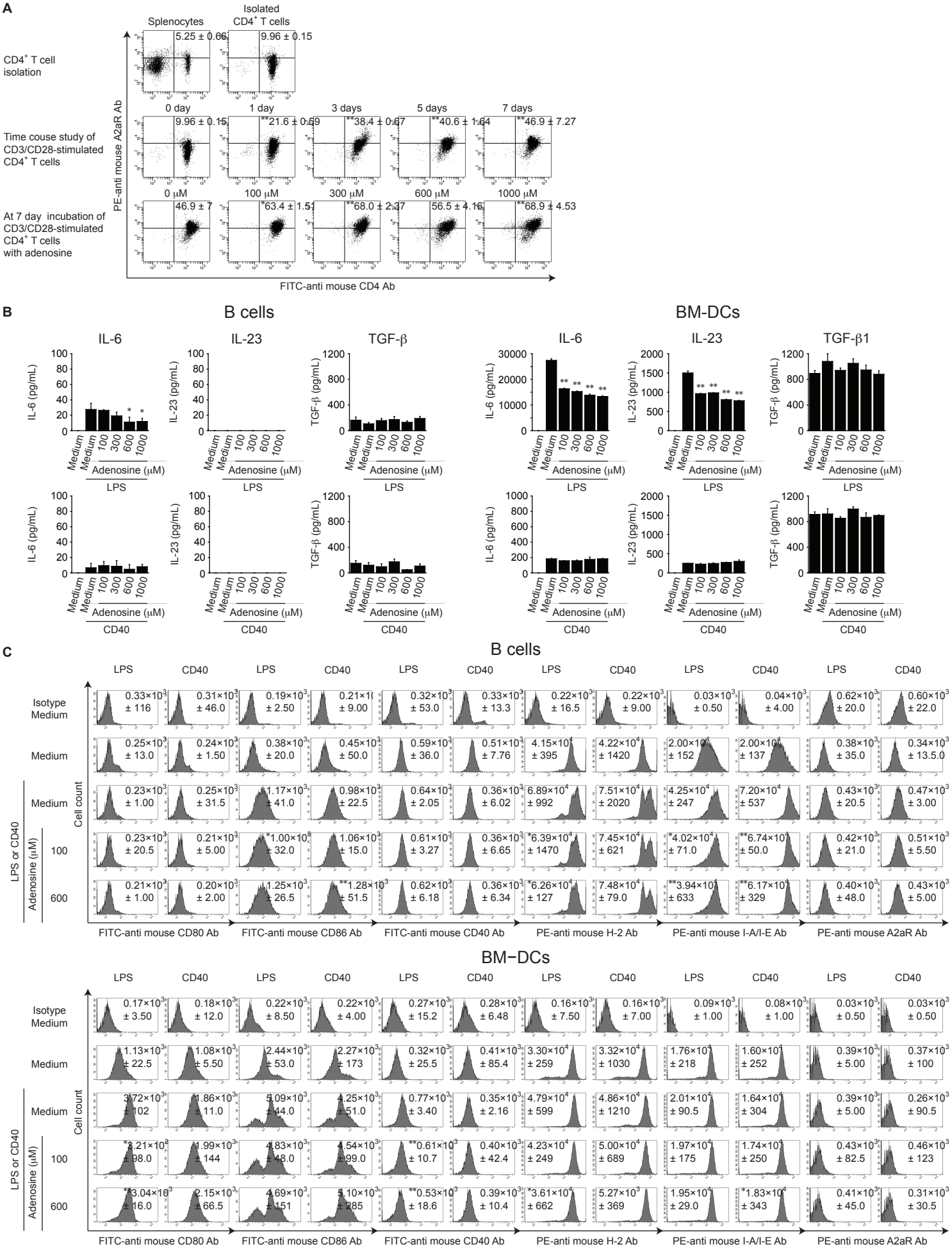
Sup. Fig. 2


A
MLR
B
CD3/CD28-stimulated CD4+ T cells
IL-17A (pg/mL)
Medium
0.01
0.1
1
10
CCPA (μM)
PSB0777 (μM)
Istradefylline (nM)
Adenosine 100 μM
Adenosine 600 μM
BAY 60-6583 (μM)
BAY60-6853 (μM)
HEMADO (μM)
CD3/CD28
C
0 day
3 days
7 days
Medium
Adenosine100 μM
PE-anti mouse CCR5 Ab (a Th1 marker)
PE-anti mouse CCR3 Ab (a Th2 marker)
PE-anti mouse CCR6 Ab (a Th17 marker)
FITC-anti mouse CD4 Ab
14.3 ± 1.09
16.4 ± 1.23
11.4 ± 0.68
14.1 ± 1.26
11.8 ± 1.83
19.4 ± 3.19
8.85 ± 0.82
9.53 ± 1.11
6.35 ± 1.58
9.51 ± 2.88
5.67 ± 1.27
9.37 ± 2.44
9.80 ± 0.60
11.8 ± 1.34
7.37 ± 0.91
10.3 ± 1.38
6.98 ± 0.46
12.2 ± 1.89

Sup. Fig. 3


IFN-γ (pg/mL)
1000
800
600
400
200
0
*
*
Adenosine 600 μM (–) (+) (–) (+) (–) (+) (–) (+)
1
3
5
7
days
CD3/CD28
IL-5 (pg/mL)
1600
1200
800
400
0
Adenosine 600 μM (–) (+) (–) (+) (–) (+) (–) (+)
1
3
5
7
days
CD3/CD28
IL-17A(pg/mL)
500
400
300
200
100
0
**
**
**
Adenosine 600 μM (–) (+) (–) (+) (–) (+) (–) (+)
1
3
5
7
days
CD3/CD28

Sup. Fig. 4


A
Splenocytes
Isolated CD4+ T cells
CD4+ T cell isolation
5.25 ± 0.66
9.96 ± 0.15
0 day
1 day
3 days
5 days
7 days
Time couse study of CD3/CD28-stimulated CD4+ T cells
9.96 ± 0.15
**21.6 ± 0.59
**38.4 ± 0.67
**40.6 ± 1.64
**46.9 ± 7.27
0 μM
100 μM
300 μM
600 μM
1000 μM
At 7 day incubation of CD3/CD28-stimulated CD4+ T cells with adenosine
46.9 ± 7
*63.4 ± 1.51
**68.0 ± 2.37
56.5 ± 4.16
**68.9 ± 4.53
PE-anti mouse A2aR Ab
FITC-anti mouse CD4 Ab
B
B cells
BM-DCs
IL-6
IL-23
TGF-β
TGF-β1
IL-6 (pg/mL)
IL-23 (pg/mL)
TGF-β (pg/mL)
Medium
100
300
600
1000
Adenosine (μM)
LPS
CD40
C
B cells
BM−DCs
LPS
CD40
Isotype Medium
Medium
LPS or CD40
Adenosine (μM)
Cell count
FITC-anti mouse CD80 Ab
FITC-anti mouse CD86 Ab
FITC-anti mouse CD40 Ab
PE-anti mouse H-2 Ab
PE-anti mouse I-A/I-E Ab
PE-anti mouse A2aR Ab