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

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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 adenylcyclase 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.

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 and protein kinase A. By contrast, A1R and A3R signal the Gi protein to trigger cAMP
57 degradation. In addition, A2bR also signals the Gq protein, which in turn activates phospholipase
58 C. In an immunological context, adenosine receptors are expressed by various immune cells,
59 including T cells and APCs (12). It is also suggested that adenosine stimulates neutrophil
60 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 adenylcyclase 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.

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
93 500 μL of DMEM medium containing 10% FCS, 100 U/ml penicillin, 100 μg/mL streptomycin,
94 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol (D10 medium) in 24
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 adenyl cyclase inhibitor (0–1 μM) (MDL-12330A,
100 Enzo Life Sciences, Farmingdale, NY, USA) or a protein kinase A inhibitor (0–1 μM) (H-89,
101 Tocris) plus adenosine (100 μM) to inhibit A2aR signaling; or in the presence of a CD39
102 inhibitor (0–1 μM) (ARL67156, Tocris) or a CD73 inhibitor (0–1 μM) (adenosine 5'-(α,

103 β-methylene) diphosphate (AMP-CP; Tocris) plus ATP (100 μM) (GE healthcare). After mixing,
104 the plates were incubated for 7 days at 37°C. The supernatants were collected for use in cytokine
105 ELISAs.
106
107 *Flow cytometry analysis*
108 The MLR was performed for 7 days in the presence or absence of adenosine (100 μM) (as
109 described above). After 7 days, cells were blocked with anti-mouse CD16/CD32 antibodies
110 (BioLegend, San Diego, CA, USA) and then stained for 30 min at 4°C with a fluorescein
111 isothiocyanate (FITC)-conjugated anti-mouse CD4 antibody (BioLegend). The cells were then
112 fixed, permeabilized, and stained with a phycoerythrin (PE)-conjugated anti-mouse IL-17A
113 antibody (BioLegend). Finally, the cells were washed and analyzed on a FACSCanto II flow
114 cytometer (BD Biosciences, Franklin Lakes, NJ) using FACSDiva acquisition software (BD
115 Biosciences).
116
117 *Isolation of $CD4^+$, $CD4^+CD62L^+$ T cells, and B cells*
118 $CD4^+$ T cells within the Balb/c and SJL/J splenocyte populations were isolated from the mixture
119 prepared previously after rupturing red blood cells (22). Cells were isolated by positive selection
120 of $CD4^+$ T cells using magnetic-activated cell sorting (MACS) (Miltenyi Biotec, Bergisch
121 Gladbach, Germany), according to the manufacturer's instructions. $CD4^+CD62L^+$ T cells were
122 isolated from Balb/c splenocytes by a combination of negative and positive selection by MACS.
123 During positive selection of $CD62L^+$ T cells, negatively isolated $CD4^+$ T cells were collected as
124 the flow through fraction. B cells were isolated from Balb/c splenocytes by positive selection
125 using MACS. Cells were resuspended in 1 mL of D10 medium and counted. The purity and

126 viability of $CD4^+$ T cells, $CD4^+CD62L^+$ T cells, and B cells were >90% (Sup. Fig. 1). The purity
127 and viability of cells in the flow through fraction collected during isolation of $CD4^+CD62L^+$ T
128 cells are shown in Supplementary Figure 1.
129
130 *Cell sorting*
131 Balb/c $CD4^+$ T cells (prepared as described above) were labeled for 30 min at 4°C with
132 PE-conjugated anti-mouse CCR3, CCR5, CCD6, CD25, or CD62L antibodies (BioLegend). The
133 cells were then washed and sorted using a FACS Aria II flow cytometer (BD Biosciences). The
134 purity and viability of the sorted cells are Supplementary Figure 1.
135
136 *CD3/CD28 stimulation*
137 $CD4^+$ T cells (1×10^6) were stimulated for 7 days at 37°C with anti-mouse CD3 (BioLegend) (1
138 μg/mL) and CD28 (BioLegend) (0.5 μg/mL) antibodies (CD3/CD28) in the presence of
139 adenosine (0–1 mM), each adenosine receptor agonist (0–10 μM) (A1R:
140 2-Chloro-N6-cyclopentyladenosine (CCPA), Tocris, Bristol, UK; A2aR: PSB0777, Tocris;
141 A2bR: BAY 60-6583, Tocris; A3R: HEMADO, Tocris), an A2aR antagonist (Istradefylline; 0–1
142 nM) plus adenosine (600 μM), or an adenyl cyclase inhibitor (0–1 μM) (MDL-12330A) or a
143 protein kinase A inhibitor (0–1 μM) (H-89) plus adenosine (600 μM) in 500 μL of D10 medium.
144 After cell sorting, cells (3×10^5) were plated in 24 well plates and stimulated for 7 days at 37°C
145 with anti-mouse CD3 (1 μg/mL) and CD28 (0.5 μg/mL) antibodies in the presence of adenosine
146 (600 μM) in 100 μL of D10 medium. After stimulation, the supernatants were collected for use
147 in cytokine ELISAs.
148

149 *Adenosine or ATP ELISAs*

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

162

163 *Differentiation of naïve $CD4^+$ T cells*

164 Naïve $CD4^+$ T cells (3×10^5) in 500 μL of D10 medium in 24 well plates were stimulated for 7
165 days at 37°C with anti-mouse CD3 (1 μg/mL) and CD28 (0.5 mg/mL) antibodies plus mouse
166 IL-6 (20 ng/mL) (Peprotech, Rocky Hill, NJ), and human TGF-β1 (2 ng/mL) (Peprotech) in the
167 presence of an A2aR antagonist (Istradefylline) (0–1 nM). After incubation, cells in 500 μL of
168 D10 medium were stimulated for another 7 days at 37°C with anti-mouse CD3 (1 μg/mL) and
169 CD28 (0.5 μg/mL) antibodies in the presence of Istradefylline (0–1 nM). Supernatants were
170 collected for use in cytokine ELISAs.

171

172 *EAE model*

173 EAE was induced as described previously (19). Briefly, SJL/J mice received a subcutaneous
174 inguinal injection (100 μg/mouse) of the proteolipid protein (PLP) peptide (PLP139-151, Tocris)
175 emulsified in complete Freund's adjuvant (CFA) containing mycobacterium tuberculosis H37Ra
176 (100 μg/mouse; Difco, Detroit, MI, USA). Mice also received oral PBS(-) or an A2aR antagonist
177 (Istradefylline) (6 μg/mouse) once every 2 days from Day –7 to Day +18 after immunization
178 with PLP peptide (Day 0). Mice were examined daily for signs of EAE, which were graded as
179 described (24).

180

181 *Peptide pulse assay*

182 At 7 days post-subcutaneous immunization with PLP peptide emulsified in CFA, splenocytes (2
183 $\times 10^6$ in 200 μL of D10 medium) were seeded in 96-well round plates and pulsed for 3 days at
184 37°C with PLP peptide (10 μM) in the presence of adenosine (600 μM) and an A2aR antagonist
185 (Istradefylline; 0–1 nM). Supernatants were collected for use in cytokine ELISAs.

186

187 *Cytokine ELISAs*

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

193

194 *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.

200 ***Results***

201

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

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

216

217 ***Adenosine production in the MLR***

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

223 IL-17A production, inhibitors of CD39 and CD73 suppressed adenosine production at 24 h after
224 the start of the MLR (Fig. 2B and C). Since the MLR induces activation of $CD4^+$ T cells by
225 APCs (25), we also addressed adenosine production by activated $CD4^+$ T cells, B cells, and
226 BM-DCs (Fig. 2D–F). Production of both adenosine and ATP by activated $CD4^+$ T cells, B cells,
227 and BM-DCs was observed; inhibitors of CD39 and CD73 suppressed production by activated
228 $CD4^+$ T cells (Fig. 2D). This suggests that adenosine produced by $CD4^+$ T cells may induce
229 spontaneous IL-17A secretion by $CD4^+$ T cells in the MLR.
230
231 ***Th17 cells hypersecrete IL-17A in the presence of anti-CD3/CD28 antibodies and adenosine***
232 Next, we tried to identify the Th subset that generated IL-17A in the presence of adenosine. First,
233 we confirmed that $CD4^+$ T cells expressed the A2aR and secreted IL-17A by stimulating them
234 with agonistic anti-CD3/CD28 antibodies in the presence of adenosine (Fig. 3A). Time course
235 studies showed that adenosine-mediated IL-17A production was detected from 3 days
236 post-CD3/CD28 stimulation (Fig. 3B and Sup. Fig. 3). Administration of adenosine within 6 h of
237 antibody stimulation triggered IL-17A production; however, administration at 24 h
238 post-stimulation did not (Fig. 3C). As in the MLR, $CD4^+$ T cells also produced IL-17A after
239 stimulation with anti-CD3/CD28 antibodies in the presence of an A2aR agonist (Fig. 3D and Sup.
240 Fig. 4). Adenosine-mediated IL-17A production by CD3/CD28-stimulated $CD4^+$ T cells was
241 suppressed by an A2aR antagonist (Fig. 3D and Sup. Fig. 4). This was supported by data
242 showing that inhibitors of signaling molecules downstream of the A2R also suppressed IL-17A
243 production (Fig. 3E). Production of other Th17-related cytokines was also induced by adenosine
244 through the A2aR (Fig. 4). This again suggests that activated $CD4^+$ T cells produce IL-17A upon
245 A2aR activation. Furthermore, we noticed that $CD4^+CD62L^-$, but not $CD4^+CD62L^+$, cells

246 produced IL-17A after CD3/CD28 stimulation in the presence of adenosine, suggesting that
247 adenosine induces IL-17A production by effector Th cells (Fig. 3F). Therefore, we performed
248 immune subset studies after separating $CD4^+$ T cells using anti-chemokine receptor (CCR)
249 antibodies. As shown in Figure 3G, $CD4^+CCR6^{hi}$ T cells produced IL-17A upon stimulation of
250 CD3/CD28, and production was strongly up-regulated in the presence of adenosine. Since CCR6
251 is a typical marker of Th17 cells (28,29), this suggests that activated Th17 cells hypersecrete
252 IL-17A in the presence of adenosine.
253
254 ***An adenosine A2aR antagonist ameliorates IL-17A-related autoimmune EAE responses***
255 The above results raise the possibility that adenosine-mediated hypersecretion of IL-17A by
256 Th17 cells contributes to Th17-related autoimmune diseases. This hypothesis is supported by a
257 report showing that CD73 knockout mice are resistant to EAE (30), a Th17-mediated
258 autoimmune disease (20). We expected, therefore, that A2aR antagonist-mediated suppression of
259 Th17 responses should improve EAE. To address this, we examined the efficacy of an A2aR
260 antagonist in EAE model SJL/J mice (19). EAE was induced by immunization of mice with an
261 $I\text{-}A^s$ restricted helper peptide derived from a myelin PLP peptide comprising amino acids
262 139–151 (HSLGKWLGHPDKF). The peptide was emulsified in CFA. First, we confirmed that
263 the A2aR antagonist suppressed adenosine-mediated IL-17A production by
264 CD3/CD28-stimulated $CD4^+$ T cells from SJL/J strain mice (Fig. 5A). The A2aR antagonist also
265 significantly suppressed adenosine-mediated IL-17A production after, but not during,
266 differentiation of Th17 cells from naïve $CD4^+$ T cells (Fig. 5B). By contrast, and in agreement
267 with Figure 3G, adenosine administration did not induce IL-17A production during and after
268 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.

282 **Discussion**

283

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

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

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

305 different from those of previous reports (39,40); here, we observed that adenosine up-regulated
306 IFN-γ (a Th1-related cytokine) secretion at 5 and 7 days and had no significant effect on IL-5 (a
307 Th2-related cytokine) secretion by $CD4^+$ T cells after CD3/CD28 stimulation with 600 μM of
308 adenosine, although IL-17A production was significant (Sup. Fig. 3). This suggests that
309 adenosine induces hypersecretion of IL-17A by Th17 cell but does not suppress Th1 and Th2
310 activity. However, previous studies report that adenosine-mediated suppression of IFN-γ and
311 IL-5 was observed 1 day after T cell receptor-mediated stimulation of $CD4^+$ T cells (39,40). This
312 may indicate that in the short term adenosine prioritizes stimulation of Th17 cell activity rather
313 than that of Th1 and Th2 cells, and that it does not suppress effector Th activity in the long term.
314 It is also suggested that the A2aR agonist, CGS21680, suppresses Th17 differentiation
315 (15-17). This result is opposite to ours; one reason for this may be differences in the source of
316 the A2aR agonist. The A2aR agonist CGS 21680 is much less selective than the A2aR agonist
317 we used this study (PSB0777); this is because CGS21680 not only binds to the A2aR but also to
318 A1R and A3R, which are associated with the Gi protein (which has opposite effects to the Gs
319 protein) (41,42). Therefore, it is probable that CGS21680 may cancel out any agonist effects by
320 activating A1R and A3R. Also, it is suggested that the A2aR antagonist, SCH58261,
321 up-regulates Th17 differentiation in mice (17). We hypothesized that up-regulation of Th17
322 differentiation by SCH58261 may be induced through relative downregulation of A2aR activity
323 compared with that of A2bR; this relative increase in A2bR activity induces Th17 differentiation
324 in mice (43,44). Also, it is probable that SCH58261 induces relative increase in activity of G
325 protein-coupled receptors other than adenosine receptors (e.g., dopamine receptors) to induce
326 Th17 differentiation in mice (45). We also hypothesize that although SCH58261 may induce
327 Th17 differentiation *in vivo*, it may not stimulate Th17 activity; this is because our data show

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

332 Our data suggest that production of IL-17A is relatively higher after exposure to an
333 A2aR agonist, PSB0777, than after exposure to an A2bR agonist, BAY 60-6583. PSB0777 is a
334 potent adenosine A2aR agonist (Ki = 44.4 nM for rat brain striatal A2aR) (42), and BAY
335 60-6583 is a potent adenosine A2bR agonist (Ki = 100 nM for rat A2bR) (46). By assuming that
336 the Ki values of PSB0777 and BAY 60-6583 are comparable, we thought that production of
337 IL-17A mediated by activation of the A2aR might be higher than that mediated by activation of
338 the A2bR. This hypothesis is supported by the notion that the A2aR is a high affinity receptor
339 with activity in the low to mid-nanomolar range, whereas the A2bR has a much lower affinity for
340 adenosine (micromolar) (5); this suggests that adenosine activates the A2aR rather than the
341 A2bR.

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

351 followed by neutrophilic inflammation, which promotes tumor metastasis.

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

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361

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540
541

542 **Abbreviations:** Th, T-helper; TGF, tumor growth factor; IL, interleukin; APCs, antigen
543 presenting cells; MLR, two-way mixed lymphocyte reaction; EAE, experimental autoimmune
544 encephalomyelitis; BM, bone marrow; BM-DC, bone-marrow-derived dendritic cell; CD3/CD28,
545 agonistic anti-CD3/CD28 antibodies; PLP, myelin proteolipid protein; PLP peptide, I-A^s
546 restricted helper peptide derived from the PLP; CFA, complete Freund's adjuvant; CCR,
547 chemokine receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; MACS,
548 magnetic-activated cell sorting; Ab, antibody; n, number of repeat experiments; SD, standard
549 deviation.
550

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559 **Author contributions**

560 M.T., R.T., S.M., and M.K., performed the experiments. M.T., S.M., and M.K., conceived and
561 designed the experiments. M.T., S.M., T.Y., and M.K., wrote the manuscript. All authors
562 discussed the results and commented on the manuscript.
563

564 **Conflicts of interest**

565 Sho Matsushita is an employee of iMmno, Inc.

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

567 **Figure legends**

568

569 **Fig. 1. Adenosine induces hypersecretion of IL-17A by CD4$^+$ T cells in an MLR. A–C**, An
570 MLR was performed for 7 days in the presence of adenosine (0–1 mM). After 7 days, cells were
571 stained with anti-CD4 (x-axis) and IL-17A (y-axis) antibodies (Abs), followed by flow
572 cytometry analysis (**A**, n (number of repeat experiments) = 4). The percentage of
573 IL-17A-producing CD4$^+$ T cells within the total CD4$^+$ T cell population is shown (**B**, n = 6–9).
574 Cells supernatants were analyzed in an IL-17A ELISA (**C**, n = 6–9). **D**, The effects of the A2aR
575 on IL-17A production in the presence of PSB0777 (an A2aR agonist) (*left*, n = 6–9),
576 Istradefylline (an A2aR antagonist) plus adenosine (100 μM) (*right*, n = 6–9). **E**, The effects of
577 A2aR signaling on IL-17A production in the presence of MDL-12330A (an adenyl cyclase
578 inhibitor) plus adenosine (100 μM) (*left*, n = 4–6), or H-89 (a protein kinase A inhibitor) plus
579 adenosine (100 μM) (*right*, n = 4–6) were analyzed in an IL-17A ELISA. **F**, The effects of
580 CD39/CD73 inhibitors on IL-17A production in the presence of ARL67156 (a CD39 inhibitor)
581 plus ATP (100 μM) (*left*, n = 4 – 6) or AMP-CP (a CD73 inhibitor) plus ATP (100 μM) (*right*, n
582 = 4–6) were analyzed in an IL-17A ELISA. **G**, The effects of an A2aR antagonist and
583 CD39/CD73 inhibitors on basal IL-17A production in the presence of Istradefylline (*left*, n =
584 4–7), ARL67156 (*center*, n = 4–7), or AMP-CP (*right*, n = 4–7) were analyzed in an IL-17A
585 ELISA. Data are expressed as the mean ± standard deviation (SD) and were compared using an
586 unpaired Student's t-test (**B**) or one-way ANOVA with Tukey's post-hoc test (**C–G**). *P < 0.05
587 and **P < 0.01, compared with medium.

588

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

590 supernatants were measured in an adenosine (*left*, n = 4–6) or ATP (*right*, n = 4–6) ELISA (0–24
591 h). **B** and **C**, The effects of CD39 (**B**, ARL67156, n = 4–6) and CD73 (**C**, AMP-CP, n = 4–6)
592 inhibitors on production of adenosine or ATP in the MLR were analyzed in an adenosine (*left*) or
593 ATP (*right*) ELISA. **D, E** and **F**, Levels of adenosine and ATP in the supernatants of
594 CD3/CD28-stimulated $CD4^+$ T cells (**D**, n = 4), LPS-stimulated B cells (**E**, n = 4), and
595 LPS-stimulated BM-DCs (**F**, n = 4) were analyzed in adenosine and ATP ELISAs at 24 h
596 post-stimulation in the presence of CD39 or CD73 inhibitors. Data are expressed as the mean ±
597 SD and were compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P <
598 0.01, compared with medium (**B** and **C**), CD3/CD28 stimulation (**D**), or LPS stimulation (**E** and
599 **F**).
600
601 **Fig. 3. Adenosine induces hypersecretion of IL-17A by Th17 cells. A** and **B**, $CD4^+$ T cells
602 were stimulated for 1–7 days with anti-CD3/CD28 antibodies in the presence of adenosine (0–1
603 mM). After stimulation, the supernatants were analyzed in an IL-17A ELISA (n = 4–6). **C**,
604 Adenosine (600 μM) was added at 0–3 days after CD3/CD28 stimulation. At 7 days
605 post-CD3/CD28 stimulation, supernatants were analyzed in an IL-17A ELISA (n = 4). **D**, Effects
606 of the A2aR on IL-17A production in the presence of PSB0777 (an A2aR agonist) (*left*, n = 4–6),
607 Istradefylline (an A2aR antagonist) plus adenosine (600 μM) (*right*, n = 4–6). **E**, Effects of
608 A2aR signaling on IL-17A production in the presence of MDL-12330A (an adenyl cyclase
609 inhibitor) plus adenosine (600 μM) (*left*, n = 4–6), or H-89 (a protein kinase A inhibitor) plus
610 adenosine (600 μM) (*right*, n = 4–6) were analyzed in an IL-17A ELISA. **F**, $CD4^+CD62L^+$ and
611 $CD4^+CD62L^+$FT cells were stimulated for 7 days with anti-CD3/CD28 antibodies in the
612 presence of adenosine (0–1 mM). After 7 days, supernatants were analyzed in an IL-17A ELISA

613 (n = 4). **G**, Subsets of $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28 antibodies in
614 the presence of adenosine (600 μM) after isolation of each CCR cell type (high (hi) and low (lo)
615 expression) (n = 4). Data are expressed as the mean ± SD and were compared using an unpaired
616 Student's t-test (**g**) or one-way ANOVA with Tukey's post-hoc test (**A**-**F**). *P < 0.05 and **P <
617 0.01, compared with CD3/CD28 stimulation (**A**–**C**, **D**, *left,* and **F**) or CD3/28 stimulation plus
618 adenosine (600 μM) (**D**, *right* and **E**).
619
620 **Fig. 4. Effect of adenosine and an A2aR antagonist on production of Th17-related cytokines.**
621 **A,** An MLR was performed for 7 days in the presence of adenosine (0–1 mM) (*top row*),
622 PSB0777 (an A2aR agonist) (*second row*), or Istradefylline (an A2aR antagonist) plus adenosine
623 (100 μM) (*bottom row*). At 7 days post-incubation, the supernatants were analyzed in an
624 IL-17A (*left*), IL-17F (*center*), or IL-22 (*right*) ELISA. All experiments were repeated six to nine
625 times. **B,** $CD4^+$ T cells were stimulated with an anti-CD3/CD28 antibody for 7 days in the
626 presence of adenosine (600 μM) (*top row*), PSB0777 (an A2aR agonist) (*second row*), or
627 Istradefylline (an A2aR antagonist) plus adenosine (600 μM) *(bottom row*). After stimulation,
628 supernatants were analyzed in IL-17A (*left*), IL-17F (*center*), and IL-22 (*right*) ELISAs. All
629 experiments were repeated four to six times. Data are expressed as the mean ± SD and were
630 compared using one-way ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01,
631 compared with CD3/CD28 stimulation.
632
633 **Fig. 5. Suppression of adenosine-mediated hypersecretion of IL-17A ameliorates EAE. A**
634 and **B**, SJL/J $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28 antibodies and an
635 A2aR antagonist (**A**, n = 4). Naïve $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28

636 antibodies in the presence of IL-6, TGF-β1, and Istradefylline (an A2aR antagonist) (0–1 nM) (**B**,
637 *left*, n = 4). Alternatively, naïve $CD4^+$ T cells were stimulated for 7 days by anti-CD3/CD28
638 antibodies in the presence of IL-6 and TGF-β1, followed by another 7 day incubation with
639 anti-CD3/CD28 antibodies and Istradefylline (0–1 nM) (**B**, *right*, n = 4). After the stimulation,
640 supernatants were analyzed in an IL-17A ELISA. **C,** Mice were immunized subcutaneously with
641 PLP peptide emulsified in CFA (PLP peptide/CFA). At 7 days post-immunization, splenocytes
642 were incubated for 3 days with PLP peptide in the presence of Istradefylline and adenosine (600
643 μM). After the incubation, supernatants were analyzed in an IL-17A ELISA (**c**, n = 4). **D,** To
644 induce EAE, SJL/J mice were immunized with PLP peptide/CFA. Before and post-immunization
645 with the PLP peptide (Days 0 to 18), mice received oral Istradefylline (6 μg/mouse) or water
646 once every 2 days. Clinical scores were recorded every day during EAE induction (**D**, n = 15).
647 Data were obtained from five independent experiments (n = 3 mice/group). **(E)** At 18 days
648 post-immunization, spinal cord sections from mice administered oral Istradefylline (*right*) or
649 water (*left*) were stained with hematoxylin and eosin (*upper panels*) or with an anti-mouse CD3
650 antibody (*lower panels*) (Scale bar, 100 μm). Data are representative of five independent
651 experiments (all with similar results) and are expressed as the mean ± SD. Data were compared
652 using one-way ANOVA with Tukey's post-hoc test (**A**–**C**), or using a non-parametric
653 Mann-Whitney *U*-test (**D**). *P < 0.05 and **P < 0.01, compared with CD3/CD28 stimulation
654 plus adenosine (600 μM) (**A**), CD3/CD28 stimulation plus IL-6 and TGF-β1 (**B**), or PLP peptide
655 pulse (**C**).

656 **Legends for the supplementary figures**

657

658 **Sup. Fig. 1. Purity and viability of each immune cell subset. A**, *First row (left)*: Splenocytes
659 or isolated $CD4^+$ T cells were stained with a FITC-conjugated anti-mouse CD4 antibody (Ab)
660 (BioLegend) (x-axis) and a PE-conjugated anti-mouse CD3 Ab (BioLegend) (y-axis). *Second*
661 *row (left)*: Splenocytes or $CD4^+CD62L^+$ T cells isolated by MACS were stained with a
662 FITC-conjugated anti-mouse CD4 Ab (BioLegend) (x-axis) and a PE-conjugated anti-mouse
663 CD62L Ab (BioLegend) (y-axis). *First row (right)*: Splenocytes and B cells were stained with a
664 FITC-conjugated anti-mouse CD19 Ab (BioLegend) (x-axis) and cell numbers were counted
665 (y-axis). *Second row (right)*: Splenocytes, BM leukocytes, and BM-DCs were stained with a
666 FITC-conjugated anti-mouse CD11c Ab (BioLegend) (x-axis) and cell numbers were counted
667 (y-axis). The number in each panel represents the percentage of each immune subset within the
668 total cell population. **B**, *First row*: Isolated $CD4^+$ (by MACS)-, $CD4^+CD62L^-$ (by cell sorting)-,
669 or $CD4^+CD62L^+$ (by cell sorting) T cells were analyzed with a PE-conjugated anti-mouse
670 CD62L Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). The number in each
671 panel represents the percentage of each immune subset (- or +) within the total cell population (-
672 plus +). Data are representative of at least three repeat experiments. *Second row*: Isolated $CD4^+$
673 (by MACS), $CD4^+CCR3^{low\ (lo)}$ (by cell sorting), or $CD4^+CCR3^{high\ (hi)}$ (by cell sorting) T cells
674 were analyzed with a PE-conjugated anti-mouse CCR3 Ab (BioLegend) (x-axis) and cell
675 numbers were counted (y-axis). *Third row*: Isolated $CD4^+$ (by MACS), $CD4^+CCR5^{lo}$ (by cell
676 sorting), or $CD4^+CCR5^{hi}$ (by cell sorting) T cells were analyzed with a PE-conjugated
677 anti-mouse CCR5 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). *Fourth row*:
678 Isolated $CD4^+$ (by MACS), $CD4^+CCR6^{lo}$ (by cell sorting), or $CD4^+CCR6^{hi}$ (by cell sorting) T

679 cells were analyzed with a PE-conjugated anti-mouse CCR6 Ab (BioLegend) (x-axis) and cell
680 numbers were counted (y-axis). *Fifth row*: Isolated $CD4^+$ (by MACS), $CD4^+CD25^{lo}$ (by cell
681 sorting), or $CD4^+CD25^{hi}$ (by cell sorting) T cells were analyzed with a PE-conjugated anti-mouse
682 CD25 Ab (BioLegend) (x-axis) and cell numbers were counted (y-axis). The number in each
683 panel represents the mean percentage ± SD of each immune subset (hi or lo) within the total cell
684 population (hi plus lo). Data are representative of at least three repeat experiments. **C**, After cell
685 isolation, each immune cell type was mixed with Trypan blue. Viability was calculated as the
686 number of unstained cells/(stained cells + unstained cells) × 100. Each measurement was
687 performed at least three times. The percentage represents the mean percentage ± SD.
688
689 **Sup. Fig. 2. Effects of an adenosine receptor agonist and antagonist on production of**
690 **IL-17A in an MLR.** MLR was performed for 7 days in the presence of each adenosine receptor
691 agonist or each adenosine receptor antagonist plus adenosine (100 μM). After 7 days, the
692 supernatants were analyzed in an IL-17A ELISA. **A**: CCPA (an A1R agonist). **B**: PSB0777 (an
693 A2aR agonist, *left)* and Istradefylline (an A2aR antagonist, *right*). **C**: BAY 60-653 (an A2bR
694 agonist). **D**: HEMADO (an A3R agonist). All experiments were repeated six to nine times. Data
695 are expressed as the mean ± SD and were compared using one-way ANOVA with Tukey's
696 post-hoc test. *$P < 0.05$ and **$P < 0.01$, compared with medium.
697
698 **Sup. Fig. 3. Effect of adenosine and an A2aR antagonist on production of IFN-γ, IL-5, and**
699 **IL-17A by CD3/CD28-stimulated $CD4^+$ T cells.** $CD4^+$ T cells were stimulated for 1–7 days
700 with anti-CD3/CD28 antibodies in the presence or absence of adenosine (600 μM). After
701 stimulation, supernatants were analyzed in IFN-γ (*top row*), IL-5 (*second row*), and IL-17A

702 (*bottom row*) ELISAs. Data are expressed as the mean ± SD and were compared using one-way
703 ANOVA with Tukey's post-hoc test. *P < 0.05 and **P < 0.01, compared with CD3/CD28
704 stimulation. All experiments were repeated four to six times.
705
706 **Sup. Fig. 4. Effects of an adenosine receptor agonist and antagonist on production of**
707 **IL-17A by CD3/CD28-stimulated $CD4^+$ T cells.** $CD4^+$ T cells were stimulated with an
708 anti-CD3/CD28 antibody for 7 days in the presence of each adenosine receptor agonist, or in the
709 presence of each adenosine receptor antagonist plus adenosine (600 μM). After 7 days, the
710 supernatants were analyzed in an IL-17A ELISA. **A**: CCPA (an A1R agonist). **B**: PSB0777 (an
711 A2aR agonist, *left)* and Istradefylline (an A2aR antagonist, *right*). **C**: BAY 60-653 (an A2bR
712 agonist). **D**: HEMADO (an A3R agonist). All experiments were repeated six to nine times. Data
713 are expressed as the mean ± SD and were compared using one-way ANOVA with Tukey's
714 post-hoc test. *P < 0.05 and **P < 0.01, compared with medium.

Fig. 1

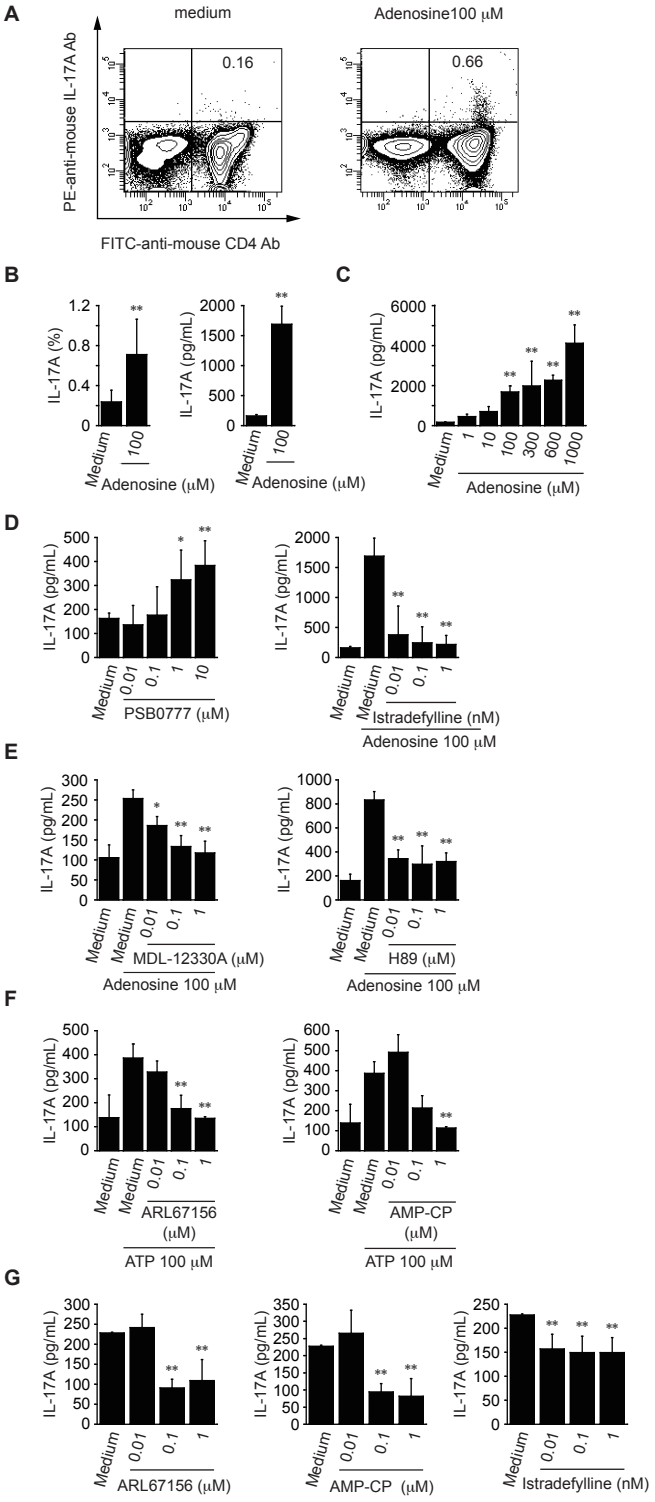

A
medium
Adenosine100 μM
0.16
0.66
PE-anti-mouse IL-17A Ab
FITC-anti-mouse CD4 Ab
B
IL-17A (%)
IL-17A (pg/mL)
Medium
100
Adenosine (μM)
C
IL-17A (pg/mL)
Medium
1
10
100
300
600
1000
Adenosine (μM)
D
IL-17A (pg/mL)
Medium
0.01
0.1
1
10
PSB0777 (μM)
Medium
Istradefylline (nM)
Adenosine 100 μM
E
IL-17A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

Fig. 2

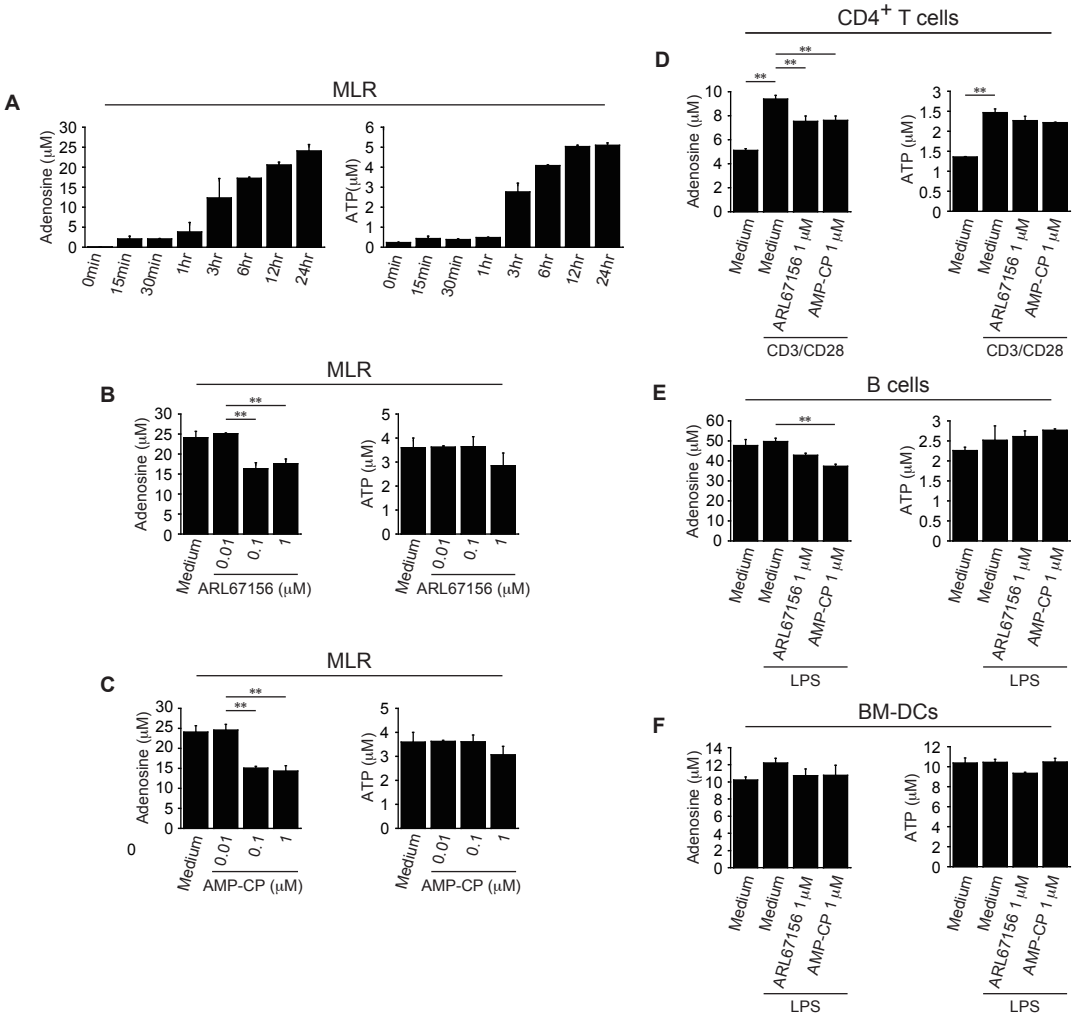

A
MLR
Adenosine (μM)
ATP(μM)
0min
15min
30min
1hr
3hr
6hr
12hr
24hr
B
MLR
Adenosine (μM)
ATP (μM)
Medium
0.01
0.1
1
ARL67156 (μM)
C
MLR
Adenosine (μM)
ATP (μM)
Medium
0.01
0.1
1
AMP-CP (μM)
D
CD4+ T cells
Adenosine (μM)
ATP (μM)
Medium
Medium
ARL67156 1 μM
AMP-CP 1 μM
CD3/CD28
E
B cells
Adenosine (μM)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

Fig. 3

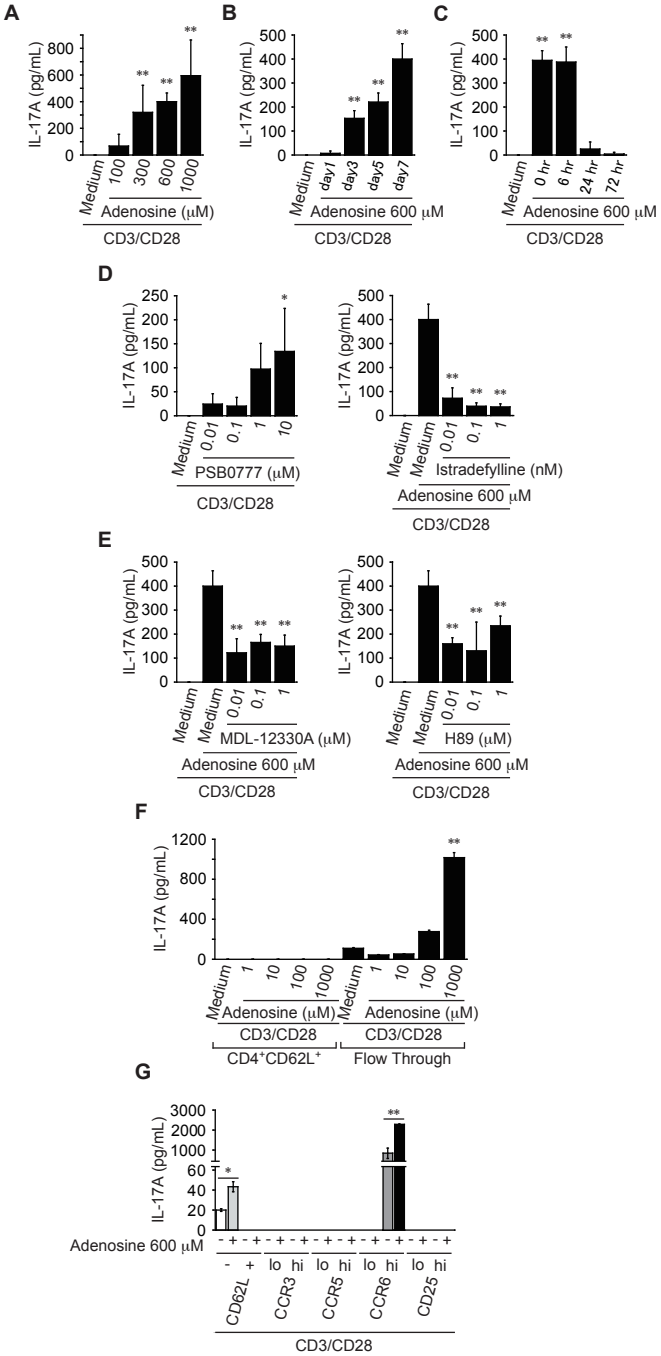

A
IL-17A (pg/mL)
Medium
100
300
600
1000
Adenosine (μM)
CD3/CD28
B
IL-17A (pg/mL)
Medium
day1
day3
day5
day7
Adenosine 600 μM
CD3/CD28
C
IL-17A (pg/mL)
Medium
0 hr
6 hr
24 hr
72 hr
Adenosine 600 μM
CD3/CD28
D
IL-17A (pg/mL)
Medium
0.01
0.1
1
10
PSB0777 (μM)
CD3/CD28
Medium
Medium
0.01
0.1
1
Istradefylline (nM)
Adenosine 600 μM
CD3/CD28
E
IL-17A (pg/mL)
Medium
Medium
0.01
0.1
1
MDL-12330A (μM)
Adenosine 600 μM
CD3/CD28
Medium
Medium
0.01
0.1
1
H89 (μM)
Adenosine 600 μM
CD3/CD28
F
IL-17A (pg/mL)
Medium
1
10
100
1000
Adenosine (μM)
CD3/CD28
CD4+CD62L+
Medium
1
10
100
1000
Adenosine (μM)
CD3/CD28
Flow Through
G
IL-17A (pg/mL)
Adenosine 600 μM
- + - + - + - + - + - + - + - + - + - +
- +
lo hi
lo hi
lo hi
lo hi
CD62L
CCR3
CCR5
CCR6
CD25
CD3/CD28

Fig. 4

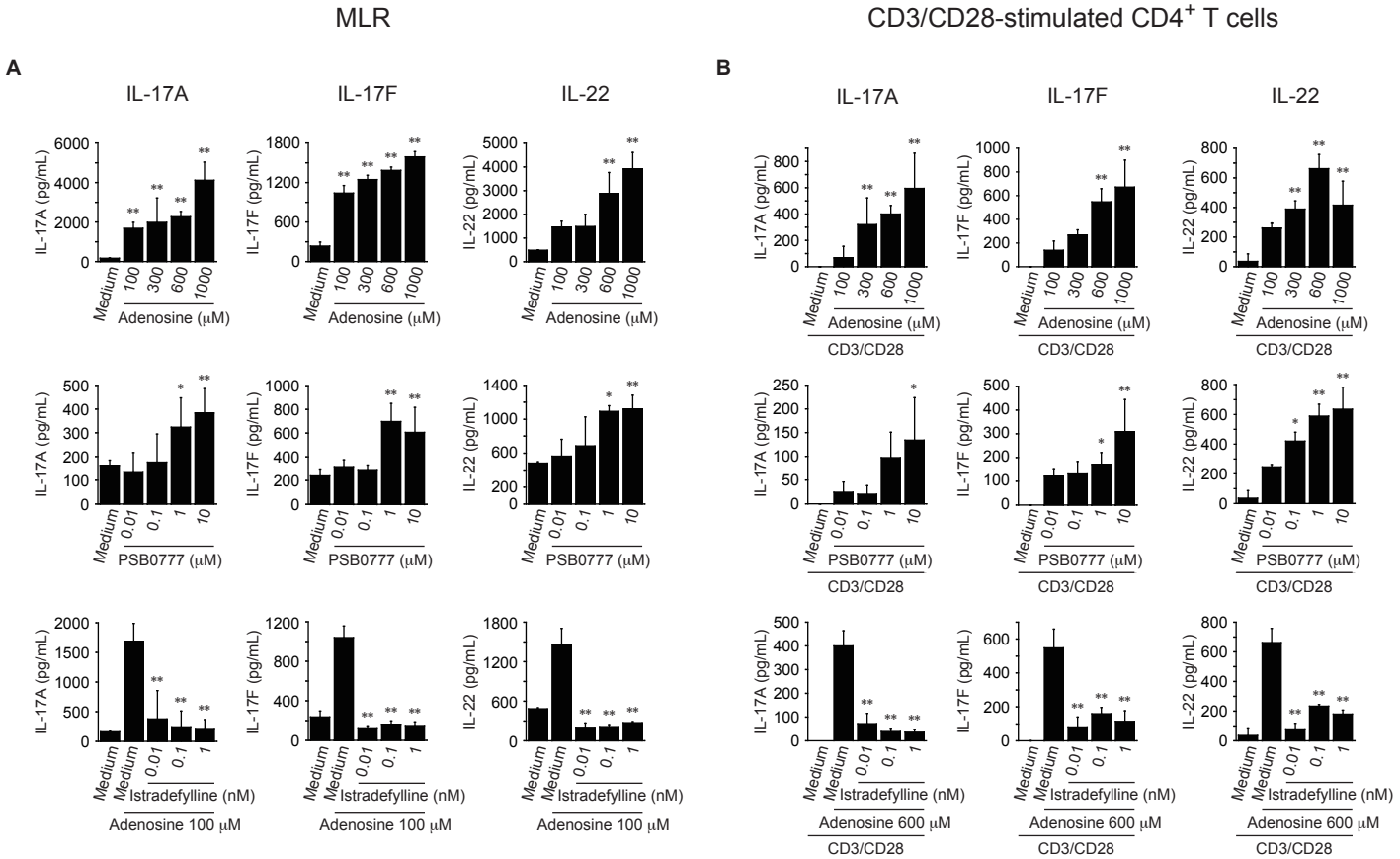
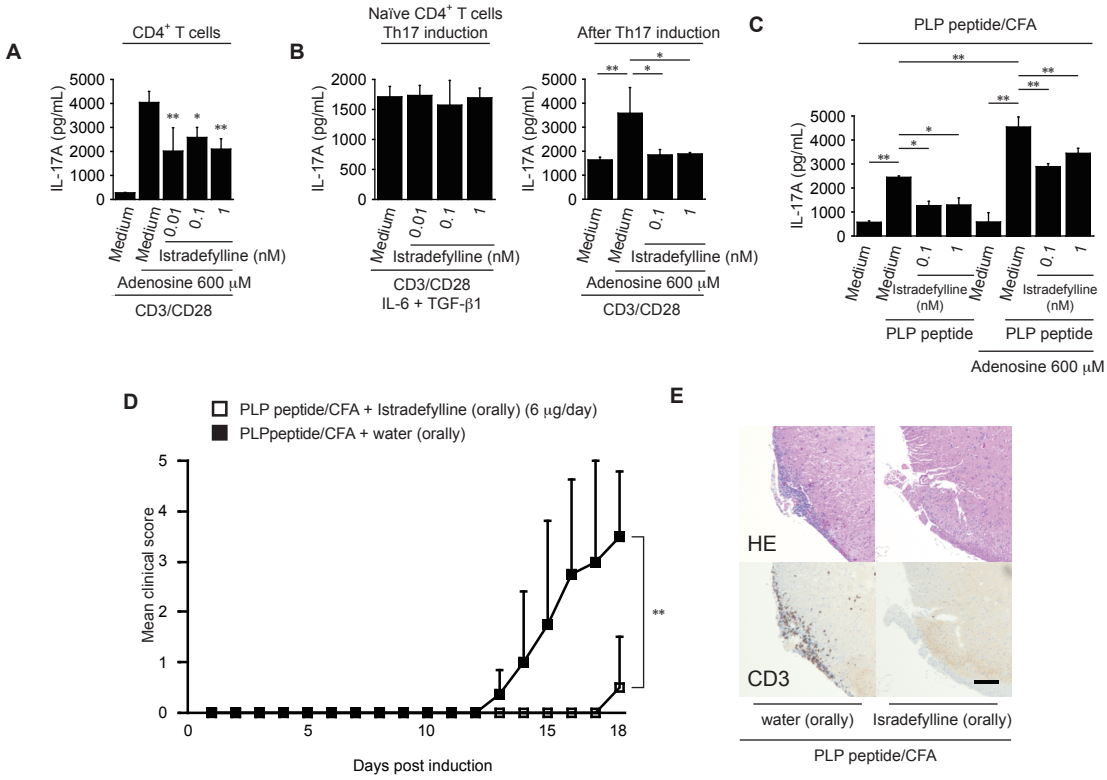
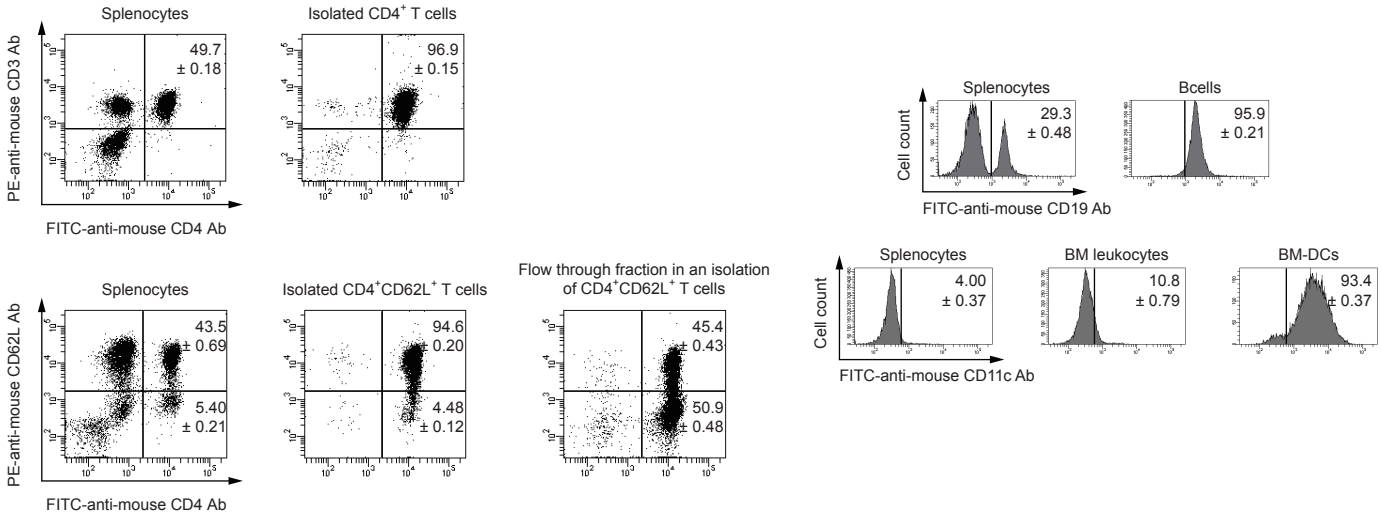

MLR
CD3/CD28-stimulated CD4+ T cells
A
B
IL-17A
IL-17F
IL-22
IL-17A (pg/mL)
IL-17F (pg/mL)
IL-22 (pg/mL)
Medium
Adenosine (μM)
PSB0777 (μM)
Istradefylline (nM)
Adenosine 100 μM
Adenosine 600 μM
CD3/CD28

Fig. 5

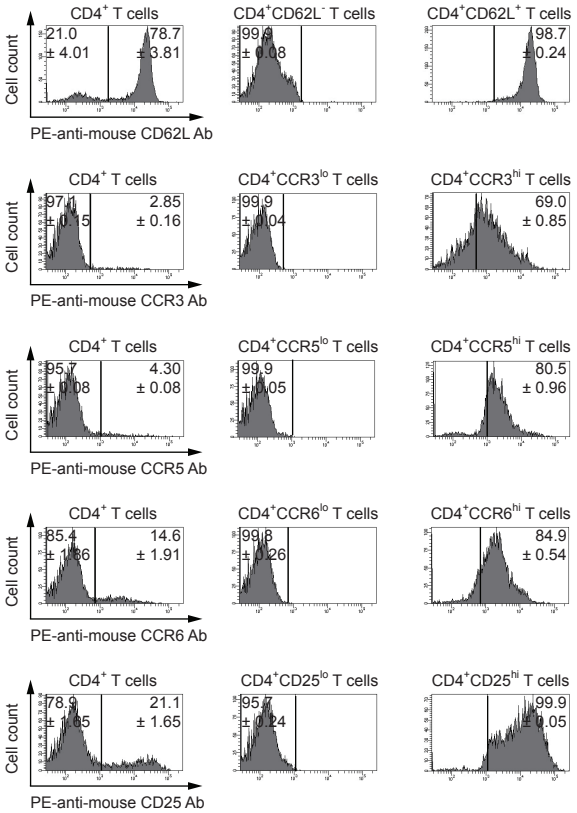

A
CD4+ T cells
IL-17A (pg/mL)
5000
4000
3000
2000
1000
0
Medium
Medium
0.01
0.1
1
Istradefylline (nM)
Adenosine 600 μM
CD3/CD28
B
Naïve CD4+ T cells
Th17 induction
IL-17A (pg/mL)
2000
1500
1000
500
0
Medium
0.01
0.1
1
Istradefylline (nM)
CD3/CD28
IL-6 + TGF-β1
After Th17 induction
IL-17A (pg/mL)
5000
4000
3000
2000
1000
0
Medium
Medium
0.1
1
Istradefylline (nM)
Adenosine 600 μM
CD3/CD28
C
PLP peptide/CFA
IL-17A (pg/mL)
5000
4000
3000
2000
1000
0
Medium
Medium
0.1
1
Istradefylline (nM)
Medium
Medium
0.1
1
Istradefylline (nM)
PLP peptide
PLP peptide
Adenosine 600 μM
D
PLP peptide/CFA + Istradefylline (orally) (6 μg/day)
PLPpeptide/CFA + water (orally)
Mean clinical score
5
4
3
2
1
0
0
5
10
15
18
Days post induction
E
HE
CD3
water (orally)
Isradefylline (orally)
PLP peptide/CFA

Sup. Fig. 1

A



B

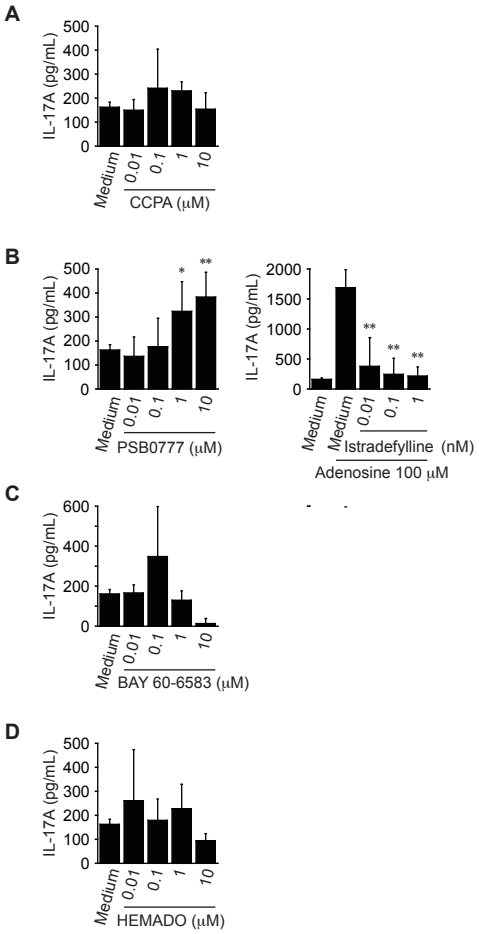


C

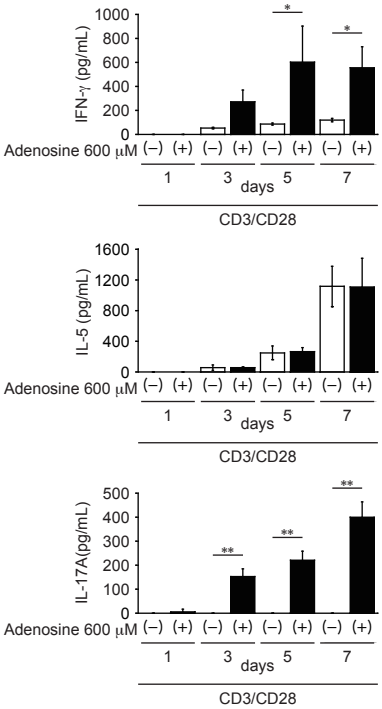
Cell type	Viability
Splenocytes	100 ± 0
$CD4^+$ T cells	100 ± 0
B cells	100 ± 0
BM-DCs	94.5 ± 1.67
Naive $CD4^+$ T cells	100 ± 0
Flow through of $CD4^+CD62L^+$ T cells	100 ± 0

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

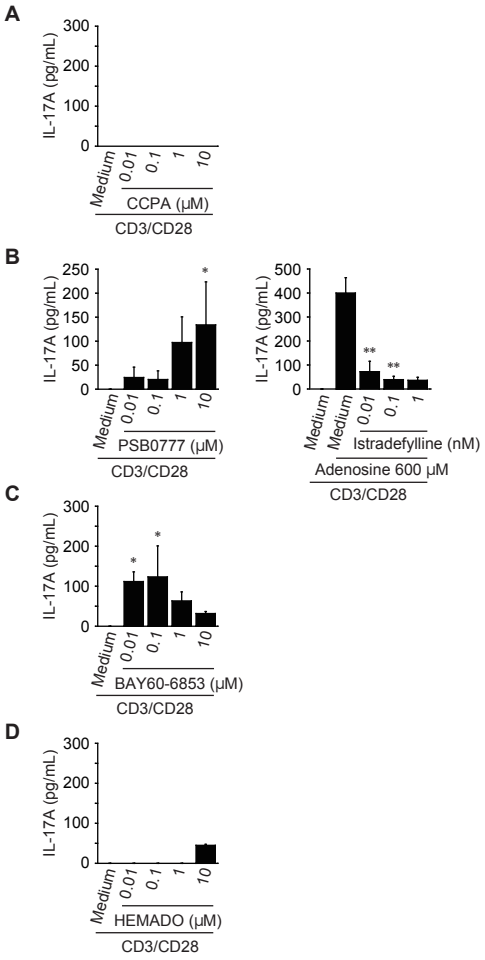
Sup. Fig. 2


A
IL-17A (pg/mL)
500
400
300
200
100
0
Medium
0.01
0.1
1
10
CCPA (μM)
B
IL-17A (pg/mL)
500
400
300
200
100
0
*
**
Medium
0.01
0.1
1
10
PSB0777 (μM)
IL-17A (pg/mL)
2000
1500
1000
500
0
**
**
**
Medium
Medium
0.01
0.1
1
Istradefylline (nM)
Adenosine 100 μM
C
IL-17A (pg/mL)
600
400
200
0
Medium
0.01
0.1
1
10
BAY 60-6583 (μM)
D
IL-17A (pg/mL)
500
400
300
200
100
0
Medium
0.01
0.1
1
10
HEMADO (μM)

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
IL-17A (pg/mL)
300
200
100
0
Medium
0.01
0.1
1
10
CCPA (μM)
CD3/CD28
B
IL-17A (pg/mL)
250
200
150
100
50
0
*
Medium
0.01
0.1
1
10
PSB0777 (μM)
CD3/CD28
IL-17A (pg/mL)
500
400
300
200
100
0
**
**
Medium
Medium
0.01
0.1
1
Istradefylline (nM)
Adenosine 600 μM
CD3/CD28
C
IL-17A (pg/mL)
300
200
100
0
*
*
Medium
0.01
0.1
1
10
BAY60-6853 (μM)
CD3/CD28
D
IL-17A (pg/mL)
300
200
100
0
Medium
0.01
0.1
1
10
HEMADO (μM)
CD3/CD28