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Adenosine A_{2B} receptor antagonist suppresses differentiation to regulatory T cells without suppressing activation of T cells

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ABSTRACT

Extracellular adenosine activates P1 receptors (A_1, A_{2A}, A_{2B}, A_3) on cellular membranes. Here, we investigated the involvement of P1 receptor-mediated signaling in differentiation to regulatory T cells (Treg). Treg were induced *in vitro* by incubating isolated $CD4^+CD62L^+$ naïve murine T cells under Treg-skewing conditions. Antagonists of A_1 and A_{2B} receptors suppressed the expression of Foxp3, a specific marker of Treg, and the production of IL-10, suggesting the involvement of A_1 and A_{2B} receptors in differentiation to Treg. We also investigated the effect of these antagonists on T cell activation, which is essential for differentiation to Treg, and found that A_1 antagonist, but not A_{2B} antagonist, suppressed T cell activation. We conclude that A_1 and A_{2B} receptors are both involved in differentiation to Treg, but through different mechanisms. Since A_{2B} antagonist blocked differentiation to Treg without suppressing T cell activation, it is possible that blockade of A_{2B} receptor would facilitate tumor immunity.

1. Introduction

Nucleotides and nucleosides are physiologically released into extracellular space from various types of cells at sites of inflammation and are thought to modulate immune response [1–3]. Adenosine exerts its actions by activating P1 receptors, while nucleotides bind to $P2X_{1-7}$ and $P2Y_{1-14}$ receptors. As regards the process of T cell activation, it has been reported that ATP is released through a gap junction hemichannel, pannexin-1, and maxi-anion channel after stimulation of T cell receptor (TCR) [4,5]. We have recently shown that vesicular exocytosis of ATP and activation of $P2X_7$ and $P2Y_6$ receptors are involved in T cell activation via TCR [6,7]. Extracellular ATP is immediately degraded to adenosine by extracellular ectonucleotidases, including CD39 and CD73 [8], and activates P1 receptors [9,10]. Extracellular adenosine binds to P1 receptor, which belongs to the G protein-coupled receptor family. There are four different types of P1 receptors, i.e., adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors. Little is known about the functional significance of the A_{2B} receptor because of its lower affinity for the endogenous ligand, adenosine, and because no specific agonist for this receptor is available, but recent studies have revealed the importance of A_{2B} receptor in regulating immunity and inflammation [11]. However, the involvement of P1 receptors, especially A_{2B} receptors, in differentiation of T cells into T-cell subsets has not been reported.

Upon interaction with self- or cross-reactive antigen, naïve $CD4^+$ T helper cells are activated, then expand and differentiate into various effector T-cell subsets. Depending on the cytokines they produce, these T-cell subsets have very different properties. Thus, T helper cells include not only the well-defined effector subsets Th1 cells and Th2 cells [12], as well as the more recently described Th17 [13,14] and Th9 [15,16] cells, but also regulatory subsets such as induced regulatory T cells (Treg) [17,18] and Tr1 cells [19]. A $CD4^+$ T cell subset, IL-17-producing Th17 cells, has been described and shown to have a critical role in the induction of autoimmune diseases [13,14]. On the other hand, Treg plays a part in the maintenance of peripheral immune tolerance and prevention of immune-mediated diseases by suppressing immune responses [20]. Regulatory T cells express CD4, IL-2 receptor α-chain (CD25), and transcriptional factor Forkhead box P3 (Foxp3), which appears to function as a master regulator in the development and function of Treg [21]. Although the mechanisms of Treg action remain poorly understood and contentious, secretion of inhibitory cytokines TGF-β and IL-10 has been implicated in the effector function of Treg [22,23]. Both Th17 cells and Treg are derived from naïve $CD4^+$ T cells; the naïve cells differentiate to Th17 in the presence of TGF-β and IL-6 [9,10], and to Treg in the presence of TGF-β and IL-2 [24,25]. It has also been reported that extracellular ATP is involved in Th17 cell differentiation in lamina propria [26].

Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; Foxp3, Forkhead box P3; IL, interleukin; mAb, monoclonal antibody; TCR, T cell receptor; TGF-β, transforming growth factor-β; Treg, regulatory T cells.

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As mentioned above, ATP is released during T cell activation via TCR. Considering that extracellular ATP is immediately degraded to adenosine, we hypothesized that adenosine is involved in differentiation of naïve $CD4^+$ T cells into Th cell subsets, such as Treg. Since *in vitro* studies have shown that conversion of naïve peripheral $CD4^+$ T cells to functional $Foxp3^+$ Treg could be achieved through ligation of TCR in the presence of IL-2 and TGF-β [25], we utilized this Treg induction system to investigate the involvement of P1 receptors in Treg differentiation. Our data indicate that A_1 receptor is involved in both differentiation of Treg (expression of Foxp3 and production of IL-10) and TCR-dependent T cell activation (expression of CD25 and production of IL-2). On the other hand, A_{2B} receptor plays an important role in the process of differentiation to Treg, but not in T cell activation. Thus, we suggest that A_1 and A_{2B} receptors are both involved in the induction of Treg, but through different mechanisms.

2. Materials and methods

2.1. Animals

Male BALB/c mice were purchased from Sankyo Labo Service (Tokyo, Japan) and used at 6–8 weeks of age. They were housed in plastic cages with paper chip bedding and bred in rooms kept at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 10% under a 12 h light–dark cycle. They were allowed free access to tap water and normal diet, CE-2 (CLEA Co., Tokyo, Japan). The mice were treated and handled according to the Guiding Principles for the Care and Use of Laboratory Animals of the Japanese Pharmacological Society and with the approval of Tokyo University of Science's Institutional Animal Care and Use Committee.

2.2. Reagents and antibodies

CGS15943 (P1 receptor broad-spectrum antagonist), PSB36 (A_1 receptor antagonist), SCH442416 (A_{2A} receptor antagonist), PSB603 (A_{2B} receptor antagonist), and MRS3777 (A_3 receptor antagonist) were purchased from Tocris Biosciences (Ellisville, MO). Purified anti-mouse mAbs (IFN-γ: XMG1.2, IL-4: 11B11, and IL-2: JES6-1A12), biotin-conjugated anti-mouse IL-2 mAb (JES6-5H4), recombinant IL-2 and IL-10, FITC-conjugated anti-mouse mAbs (CD8α: 53-6.7, CD11b: M1/70), and PE-conjugated anti-mouse Foxp3 mAb (FJK-16s) were purchased from eBioscience, (San Diego, CA). FITC-conjugated anti-mouse CD62L mAb (MEL-14) was purchased from Beckman Coulter (Fullerton, CA). Soluble anti-mouse CD28 (37.51), PE-Cy5-conjugated anti-mouse CD4 mAb (RM4-5), and FITC-conjugated anti-mouse CD19 mAb (1D3) were purchased from BD Biosciences Pharmingen (San Diego, CA). FITC-conjugated anti-mouse CD25 mAb (PC61), purified anti-mouse IL-10 mAb (JES5-16E3), and biotin-conjugated anti-mouse IL-10 mAb (JES5-2A5) were purchased from BioLegend (San Diego, CA). Anti-mouse CD3ε mAb (145-2C11) (Lot No. AMQ07) and recombinant human TGF-β1 were purchased from R&D Systems (Minneapolis, MN).

2.3. Cell isolation

Naïve $CD4^+$ T cells were purified from murine spleen. The cells were first enriched by depletion using FITC-conjugated anti-CD8α, CD11b, CD19, and CD25 mAb and Dynabeads anti-rat IgG (Invitrogen, Carlsbad, CA), and then $CD4^+CD62L^+$ naïve $CD4^+$ T cells were separated on a FACSAriaII (BD Biosciences, San Jose, CA). The purity of naïve $CD4^+$ T cells thus obtained was >98%.

2.4. Differentiation and analysis of regulatory T cells

Purified naïve $CD4^+$ T cells (5×10^5 cells/well) were cultured in 48-well plates under Treg-skewing conditions: plate-bound anti-CD3ε mAb (5 μg/mL), anti-CD28 mAb (1 μg/mL), recombinant hTGF-β1 (5 ng/mL), recombinant murine IL-2 (8 ng/mL), anti-IFN-γ mAb (2 μg/mL), and anti-IL-4 mAb (2 μg/mL). P1 receptor antagonists were added and the plates were incubated for 96 h. After incubation, the cells were collected, stained with PE-Cy5-conjugated anti-CD4 mAb for 30 min at room temperature, washed with RPMI1640-based buffer [7], fixed with 4% paraformaldehyde for 10 min on ice, and then treated with 0.05% Triton X-100 for 5 min at 4 °C. After having been washed with RPMI1640-based buffer and blocked with 1% BSA/PBS for 30 min at room temperature, the samples were stained with PE-conjugated anti-Foxp3 mAb for 1 h at room temperature, and washed with RPMI1640-based buffer. Analysis was performed using a FACSCalibur with Cell Quest software (Becton–Dickinson, San Jose, CA).

2.5. Measurement of cytokines

The culture supernatants were harvested and the concentrations of IL-2 and IL-10 were determined by ELISA as described below. A 96-well plate was coated with purified anti-mouse IL-2 mAb (1:500) or IL-10 mAb (1:500), and incubated overnight at 4 °C. Nonspecific binding was blocked with PBS containing 1% BSA. Culture supernatant was added for 2 h at room temperature. Biotin-conjugated anti-mouse IL-2 mAb (1:1000) or IL-10 mAb (1:500) was added for 1 h at room temperature, then avidin-horseradish peroxidase (Sigma, Saint Louis, USA) was added. The plate was incubated for 30 min at room temperature, then washed, and 3,3′,5,5′-tetramethylbenzidine was added for 10–30 min. The reaction was stopped by adding 5 N H_2SO_4, and the absorbance at 450 nm was measured with an ImmunoReader NJ-2000 (Nihon InterMed, Tokyo, Japan). Standard curves were established with recombinant mouse IL-2 and IL-10, and the concentrations of IL-2 and IL-10 were estimated from the standard curves.

2.6. Activation of T cells

Splenocytes were pre-incubated with P1 receptor antagonists at appropriate concentrations for 30 min at 37 °C. The cells (6×10^5

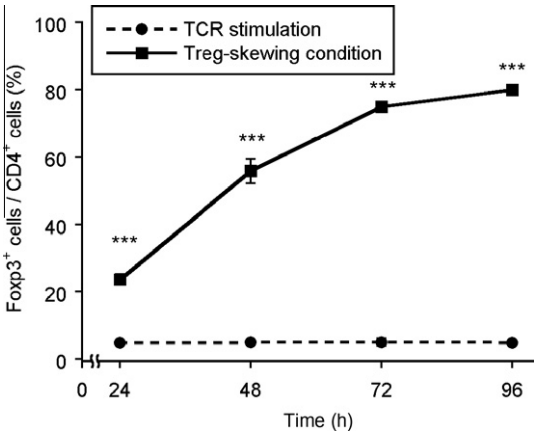

Fig. 1. Expression of Foxp3 after *in vitro* Treg induction. Isolated naïve $CD4^+$ T cells were cultured under Treg-skewing conditions: plate-bound anti-CD3ε mAb (5 μg/mL), anti-CD28 mAb (1 μg/mL), recombinant hTGF-β1 (5 ng/mL), recombinant murine IL-2 (8 ng/mL), anti-IFN-γ mAb (2 μg/mL), and anti-IL-4 mAb (2 μg/mL). After incubation for 24–96 h, expression of Foxp3 was measured. Data represent mean ± SE (n = 3–12). Significant differences of Treg cells vs. TCR-stimulated cells are indicated with $^{***}p < 0.001$.

cells/well) were incubated with plate-bound anti-CD3ε mAb (5 μg/mL) and anti-CD28 mAb (1 μg/mL) in a 96-well plate in RPMI 1640 medium containing 10% FBS in an atmosphere of 5% CO_2/95% air at 37 °C. After incubation for 24 h, the cells and the supernatant were collected for measurement of CD25 expression and IL-2 production, respectively. The cells were stained with PE-Cy5-conjugated anti-CD4 and FITC-conjugated anti-CD25 mAb for 30 min at room temperature, then washed with RPMI1640-based buffer, and analyzed on a FACSCalibur with Cell Quest software (Becton–Dickinson, San Jose, CA). The concentration of IL-2 in the culture medium was determined by ELISA.

2.7. Mobilization of intracellular Ca^{2+}

Splenocytes were suspended in Ca^{2+}-free RPMI1640-based buffer and stained with PE-Cy5-conjugated anti-CD4 mAb for 30 min at 4 °C. The cells were then washed twice and loaded with Ca^{2+}-sensitive fluorescent dye Fluo-4 AM (10 μM) (Invitrogen, Carlsbad, CA) in Ca^{2+}-free RPMI1640-based buffer for 30 min at 37 °C. After having been washed with Ca^{2+}-free RPMI1640-based buffer three times, the cells were re-suspended in Ca^{2+}-containing RPMI1640-based buffer. Before measurement of intracellular Ca^{2+} by flow cytometry, P1 receptor inhibitors were added and the cells were incubated for 5 min at 37 °C. After measurement of the basal Fluo-4 signal for 1 min, the cells were stimulated with anti-CD3ε mAb (10 μg/mL) and anti-CD28 mAb (0.5 μg/mL), and the fluorescence signal was analyzed for 5 min after the stimulation.

2.8. Statistical analysis

Values are given as mean ± SE. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni's

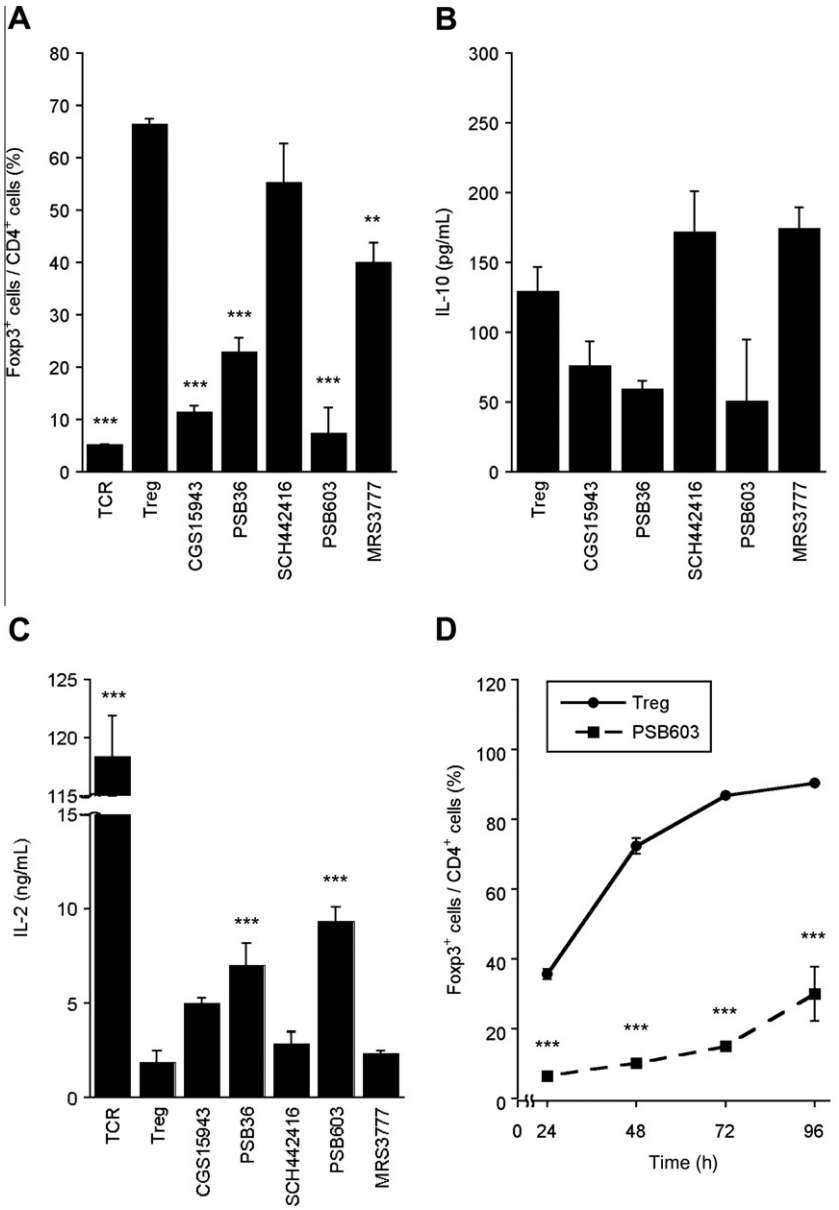

Fig. 2. Effects of adenosine receptor antagonists on Treg differentiation. Isolated naïve $CD4^+$ T cells were cultured with CGS15943 (10 μM), PSB36 (10 μM), SCH442416 (2 μM), PSB603 (10 μM), or MRS3777 (10 μM) under Treg-skewing conditions as described in Section 2. After incubation for 96 h, expression of Foxp3 (A) and concentration of IL-10 (B) and IL-2 (C) in the culture medium were measured. The time course changes in the effect of PSB603 on the expression of Foxp3 were measured (D). Data represent mean ± SE (n = 3–5). Significant differences from the Treg group are indicated with $^{**}p < 0.01$, and $^{***}p < 0.001$.

post hoc analysis. Significance was defined as $p < 0.05$ and calculations were done with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA).

3. Results and discussion

3.1. Differentiation of Foxp3$^+$ regulatory T cells

To confirm the *in vitro* induction of differentiation of regulatory T cells, we examined the expression of Foxp3, a specific marker of Treg, in splenic CD4$^+$CD62L$^+$ naïve T cells after incubation under Treg-skewing conditions, i.e., isolated naïve CD4$^+$CD62L$^+$ T cells were cultured with exogenous IL-2 and TGF-β under stimulation of TCR. The percentage of Foxp3-expressing CD4$^+$ T cells was increased within 24 h and reached a maximum at 96 h (Fig. 1). In subsequent pharmacological experiments, we analyzed the expression of Foxp3 in CD4$^+$ T cells after 96 h of culture.

3.2. Antagonists of adenosine A_1 and A_{2B} receptors suppressed the differentiation of Treg

We investigated the involvement of adenosine receptors in Treg differentiation. It has been shown that stimulation of TCR with anti-CD3 mAb and anti-CD28 mAb causes release of ATP from T cells through maxi-anion channel, hemichannel, and exocytosis [4,5,7,27]. Since extracellular ATP is rapidly degraded to adenosine, we examined whether adenosine receptors are involved in Treg differentiation. The effect of P1 receptor antagonists on Treg differentiation was examined in the *in vitro* induction system. As shown in Fig. 2A, the number of cells expressing Foxp3 reached approximately 70% during incubation under Treg-skewing conditions. On the other hand, incubation with CGS15943 (P1 receptor broad-spectrum antagonist), PSB36 (A_1 receptor antagonist), PSB603 (A_{2B} receptor antagonist), and MRS3777 (A_3 receptor antagonist) suppressed Foxp3 expression in CD4$^+$ T cells (11.52%, 22.98%, 7.48%, and 40.13%, respectively). The same tendency was observed in IL-10 production as in Foxp3 expression; the production of IL-10, an anti-inflammatory cytokine produced by Treg, was increased after 96 h incubation under the Treg-skewing conditions, and CGS15943, PSB36, and PSB603 suppressed the IL-10 production (Fig. 2B). The production of IL-2 was extremely high after incubation with plate-bound anti-CD3 mAb and anti-CD28 mAb, whereas it was suppressed after incubation under the Treg-skewing condition even though recombinant IL-2 was added in Treg-skewing condition (Fig. 2C). Since Treg is characterized by high expression of high-affinity IL-2 receptor and consumption of IL-2 is considered to be the one of the suppressive mechanisms of Treg [28,29], this result would be caused by IL-2 consumption by Treg and supports that induced Treg is functional at least in part. Blockade of A_1 or A_{2B} receptor with PSB36 or PSB603 resulted the higher production of IL-2 compared with the Treg group, implicating that these antagonists suppressed Treg differentiation. On the other hand, the production of IL-17 and IFN-γ were not detected in the media of Treg-skewing condition with/without P1 antagonists (data not shown). Moreover, the cytotoxicity of these P1 receptor antagonists was examined in splenocytes by measurement of propidium iodide uptake, and no cytotoxicity was observed (data not shown). These results indicate that adenosine receptors, especially A_1 and A_{2B} receptors, are involved in the differentiation of Treg.

Since PSB603 markedly suppressed Treg differentiation, we next further investigated the involvement of adenosine A_{2B} receptor in Treg differentiation. As shown in Fig. 2D, A_{2B} receptor antagonist PSB603 significantly suppressed the expression of Foxp3 at all time points up to 96 h at least from 24 h of incubation.

3.3. Adenosine A_{2B} receptor antagonist did not suppress activation of T cells

Activation is required for naïve CD4$^+$ T cells to differentiate to Treg [30,31]. Therefore, it is necessary to determine whether A_1 and A_{2B} receptor antagonists inhibit the T cell activation process or inhibit the differentiation process to Treg. To investigate the effect of P1 antagonists on T cell activation, splenocytes were incubated under TCR stimulation with/without P1 receptor antagonists. Expression of CD25 in CD4$^+$ T cells and production of IL-2, which are activation markers of T cells, were measured (Fig. 3). Intriguingly, CGS15943, PSB36, and MRS3777 suppressed the expression of CD25, whereas PSB603 did not, suggesting that

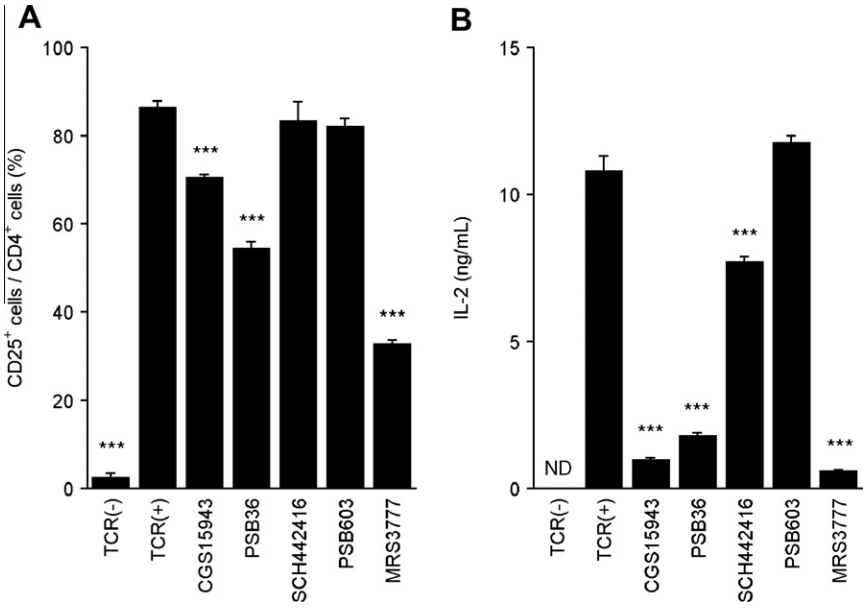

Fig. 3. Effects of adenosine receptor antagonists on T cell activation. Splenocytes were pretreated with CGS15943 (10 μM), PSB36 (10 μM), SCH442416 (2 μM), PSB603 (10 μM), and MRS3777 (10 μM) for 30 min. After incubation with plate-bound anti-CD3 mAb and anti-CD28 mAb for 24 h, expression of CD25 (A) and IL-2 concentration in the culture medium (B) were measured. Data represent mean ± SE ($n = 5$). Significant differences from the TCR (+) group are indicated with $^{***}p < 0.001$.

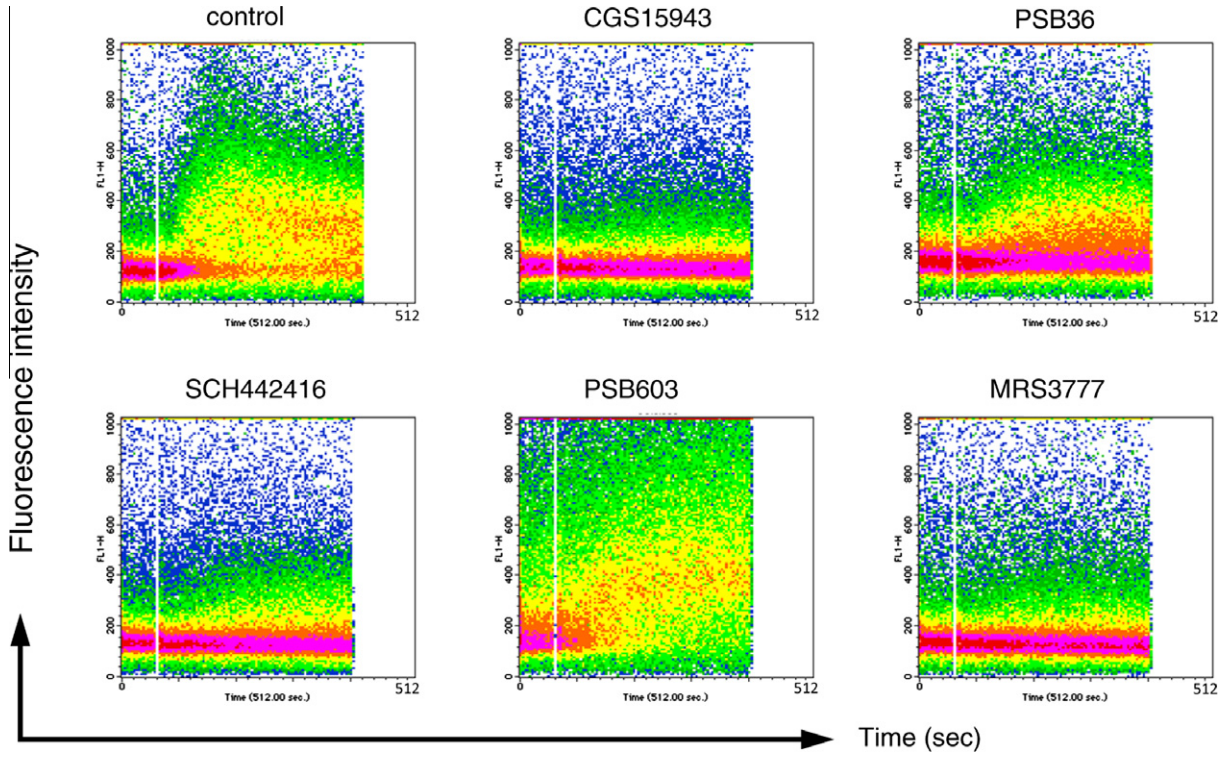

Fig. 4. Effect of adenosine receptor antagonists on TCR-dependent intracellular Ca^{2+} mobilization. Splenocytes loaded with Fluo-4 were preincubated with CGS15943 (10 μM), PSB36 (10 μM), SCH442416 (2 μM), PSB603 (10 μM), and MRS3777 (10 μM) for 5 min. After stimulation with anti-CD3 mAb and anti-CD28 mAb, the change in fluorescence was analyzed for 5 min.

A_1, A_{2A}, and A_3, but not A_{2B}, receptors contribute to T cell activation. These results also indicate that A_{2B} receptor antagonist suppresses the differentiation process of Treg (expression of Foxp3), but not the activation process of $CD4^+$ T cells (expression of CD25 and production of IL-2), suggesting that the adenosine A_{2B} receptor is involved in Treg differentiation.

Increase of intracellular Ca^{2+} ($[Ca^{2+}]_i$) plays an essential role in TCR-signaling-mediated activation of T cells [32]. We also examined the effect of P1 receptor antagonists on the elevation of $[Ca^{2+}]_i$ induced by stimulation of TCR. In accordance with CD25 expression and IL-2 production data, CGS15943, PSB36, and MRS3777 inhibited the elevation of $[Ca^{2+}]_i$, whereas PSB603 did not (Fig. 4). These results indicate that A_1, A_{2A}, and A_3 receptor, but not A_{2B} receptor, contribute to CD25 expression and IL-2 production during T cell activation, and also suggest A_{2B} receptor is involved in Foxp3 expression and IL-10 production during differentiation to Treg after T cell activation.

Our findings indicate that A_1 and A_{2B} receptors on $CD4^+$ T cells are key molecules for differentiation to Treg. Although A_{2B} receptor does not contribute to expression of CD25, production of IL-2, or elevation of $[Ca^{2+}]_i$ during TCR-dependent activation of $CD4^+$ T cells, this receptor does contribute to expression of Foxp3 and production of IL-10 during TGF-β- and IL-2-induced differentiation to Treg, suggesting that A_{2B} receptor plays a significant role in Treg differentiation via a mechanism other than TCR signaling. On the other hand, A_1 receptor is involved in both activation of T cells and differentiation to Treg.

Treg plays an important role in the maintenance of peripheral immune tolerance and prevention of immune-mediated diseases by suppressing immune responses. It is also known that a decrease of Treg causes enhancement of tumor immunity [33]. Since A_{2B} antagonist PSB603 suppressed differentiation of $CD4^+$ T cells to Treg without suppressing activation of T cells, probably including $CD8^+$ T cells, A_{2B} antagonists may facilitate tumor immunity and exhibit an anti-tumor effect. Our results may therefore provide the basis for a novel therapeutic approach for the treatment of cancer. We next plan to investigate the mechanisms through which Treg differentiation is regulated by A_1 and A_{2B} receptor signaling.

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