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Cutting Edge: Critical Role for A2A Adenosine Receptors in the T Cell-Mediated Regulation of Colitis

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A2A adenosine receptors (A2AAR) inhibit inflammation, although the mechanisms through which adenosine exerts its effects remain unclear. Although the transfer of regulatory Th cells blocks colitis induced by pathogenic CD45RB<sup>high</sup> Th cells, we show that CD45RB<sup>low</sup> or CD25<sup>+</sup> Th cells from A2AAR-deficient mice do not prevent disease. Moreover, CD45RB<sup>high</sup> Th cells from A2AAR-deficient mice were not suppressed by control CD45RB<sup>low</sup> Th cells. A2AAR agonists suppressed the production of proinflammatory cytokines by CD45RB<sup>high</sup> and CD45RB<sup>low</sup> T cells in association with a loss of mRNA stability. In contrast, anti-inflammatory cytokines, including IL-10 and TGF-β, were minimally affected. Oral administration of the A2AAR agonist ATL313 attenuated disease in mice receiving CD45RB<sup>high</sup> Th cells. These data suggest that A2AAR play a novel role in the control of T cell-mediated colitis by suppressing the expression of proinflammatory cytokines while sparing anti-inflammatory activity mediated by IL-10 and TGF-β. The Journal of Immunology, 2006, 177: 2765–2769.

Crohn’s disease and ulcerative colitis are chronic, relapsing inflammatory bowel diseases (IBD). Although the etiology of IBD remains unknown, recent studies suggest that disease results from an inappropriate regulated immune response to enteric Ags in a genetically susceptible host (1–4). In animal models of colitis, there is often a marked increase in Th1 cells (5, 6) and their ability to cause disease can be attenuated by subsets of Th cells that mediate some sort of regulatory activity (7, 8). Several mechanisms regulate the host response to luminal Ags, including oral tolerance, a state of hyporesponsiveness that controls the response to dietary or microbial Ags that persist in the lumen (2). Th cells resembling regulatory T cell (Treg) also respond to persistent infection (2, 4, 7, 9).

Adenosine is a signaling molecule released from inflamed or hypoxic tissues. The multiple physiological responses controlled by adenosine are mediated by four G protein-coupled receptors (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>), depending on cell type and species (10). The A<sub>2A</sub> adenosine receptors (A<sub>2A</sub>AR) are of interest because their activation on immune cells produces a response that, in general, can be categorized as anti-inflammatory (10–12). Recent studies (10) show that adenosine analogs limit collateral damage associated with severe inflammation, including colitis (13) and ileitis (14). In this study, these data suggest that A2AAR play a critical role in the T cell-mediated regulation of colitis due to their novel effects on T cell cytokine production.

Materials and Methods

Mice

C57BL/6 mice and SCID mice were purchased from The Jackson Laboratory, whereas A2AAR-deficient mice (A2AAR<sup>−/−</sup>) mice inbred onto the C57BL/6 background (15) were maintained in a conventional animal care facility at the University of Virginia (Charlottesville, VA). All procedures were approved by the animal care and use committee at the University of Virginia.

Purification of T cells

Splenocytes from 8- to 10-wk-old C57BL/6 or A2AAR<sup>−/−</sup> mice were enriched using CD4 microbeads (L3T4; Miltenyi Biotec) (16), while purity was confirmed by flow cytometry and ranged from 90 to 99%. The CD4<sup>+</sup> Th cells were then sorted into subsets (>98% purity) based on the expression of CD4<sup>+</sup>, CD45RB, and CD25 (16).

Adoptive transfer studies

CD45RB<sup>high</sup>, CD45RB<sup>low</sup>, or CD25<sup>+</sup> CD4<sup>+</sup> Th cells from control and A2AAR<sup>−/−</sup> mice were injected i.p. into SCID recipients at 5 × 10<sup>5</sup> cells (CD45RB<sup>high</sup> T cells) and 1 × 10<sup>6</sup> cells (CD45RB<sup>low</sup> or CD25<sup>+</sup> T cells). Recipient mice were weighed weekly. After 8 wk, the colons were collected, and H&E-stained sections were evaluated using a standardized histopathological scoring system (16). In some experiments, mice receiving CD45RB<sup>high</sup> T cells from control mice were fed chow containing 1.875 mg/kg of ATL313 (prepared by Dr. R. Figler, Adenosine Therapeutics, Charlottesville, VA).

Stimulation with A<sub>2A</sub> Agonists

CD45RB<sup>high</sup>, CD45RB<sup>low</sup>, and unfractionated CD4<sup>+</sup> T cells (1 × 10<sup>6</sup> cells/ml) were stimulated with 10 μg/ml plate-bound anti-CD3 mAb (145–2C11), and 1 μg/ml anti-CD28 mAb (37.51) (16) in the presence or absence of A2AAR agonists.

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3 Abbreviations used in this paper: IBD, inflammatory bowel disease; Treg, regulatory T cell; A2AAR, adenosine A2A adenosine receptor; ARE, adenine- and uracil-rich element.
CD45RB<sup>low</sup> Th cells from A<sub>2A</sub>AR<sup>−/−</sup> mice failed to prevent wasting disease and colitis. A, SCID mice received a sham injection of PBS (n = 3), CD<sup>4</sup><sup>+</sup>CD45RB<sup>low</sup> cells from C57BL/6 mice (n = 6), CD<sup>4</sup><sup>+</sup>CD45RB<sup>high</sup> cells from A<sub>2A</sub>AR<sup>−/−</sup> mice (n = 7), CD<sup>4</sup><sup>+</sup>CD45RB<sup>high</sup> cells from C57BL/6 mice (n = 8), CD<sup>4</sup><sup>+</sup>CD45RB<sup>high</sup> cells from C57BL/6 mice, CD<sup>4</sup><sup>+</sup>CD45RB<sup>low</sup> cells from A<sub>2A</sub>AR<sup>−/−</sup> mice (n = 8), CD<sup>4</sup><sup>+</sup>CD45RB<sup>high</sup> cells from A<sub>2A</sub>AR<sup>−/−</sup> mice, CD<sup>4</sup><sup>+</sup>CD45RB<sup>low</sup> cells from C57BL/6 mice (n = 4), and CD45RB<sup>high</sup> and CD45RB<sup>low</sup> cells from A<sub>2A</sub>AR<sup>−/−</sup> mice (n = 4). The data are the mean value of all observations from two separate adoptive transfer studies. B, The histology of the colonies from recipient mice was examined 8 wk after transfer. Photomicrographs are all at the same magnification.

Cytokine assays

 Supernatants were collected for cytokine protein assays and cells were harvested as a source of mRNA (17). Cytokine levels were measured using a multiplex bead array (Upstate Biotechnology) and analyzed with the Bioplex workation and associated software (Bio-Rad). TNF-α, IFN-γ, and IL-2 mRNA was measured by real-time RT-PCR using primer and dual-labeled probes (Applied Biosystems) detected in a Smart Cycler (Cepheid) and normalized against 18S mRNA levels. Data for panels A and B are representative examples from two separate studies.

Statistics

Quantitative data were expressed as mean ± SEM and compared using paired Student t tests or rank-sum tests. Values of p < 0.05 were considered significant.

Results and Discussion

Treg from A<sub>2A</sub>AR<sup>−/−</sup> mice do not prevent wasting disease induced by pathogenic Th cells

C57BL/6 SCID mice injected with CD4<sup>+</sup>CD45RB<sup>high</sup> Th cells from control, C57BL/6, or A<sub>2A</sub>AR<sup>−/−</sup> mice resulted in weight loss (Fig. 1a) associated with diarrhea and rectal prolapse as described previously (6, 7). Colitis was characterized by mucosal thickening, crypt elongation, epithelial hyperplasia, goblet cell depletion, and a marked infiltration by inflammatory cells (Fig. 1b). Cotransfer of CD4<sup>+</sup>CD45RB<sup>low</sup> Th cells from control C57BL/6 mice prevented the disease induced by CD45RB<sup>high</sup> Th cells from the same strain, whereas A<sub>2A</sub>AR<sup>−/−</sup> CD45RB<sup>low</sup> cells did not. The lack of protection by A<sub>2A</sub>AR<sup>−/−</sup> CD45RB<sup>low</sup> Th cells was not due to their contamination with large numbers of pathogenic Th cells, because CD45RB<sup>low</sup> Th cells alone caused no disease. Moreover, disease could not be induced with the adoptive transfer of increasing numbers of CD45RB<sup>low</sup> Th cells to the immunodeficient recipients (data not shown). CD25<sup>+</sup> Th cells from A<sub>2A</sub>AR<sup>−/−</sup> mice also failed to prevent colitis (Fig. 2a). TNF-α mRNA levels in the colonic tissue of mice receiving CD45RB<sup>high</sup> and CD25<sup>+</sup> T cells from control mice was lower than the levels observed in recipients of pathogenic CD45RB<sup>high</sup> T cells alone or in recipients given control CD45RB<sup>high</sup> and CD25<sup>+</sup> T cells from A<sub>2A</sub>AR<sup>−/−</sup> mice.

A<sub>2A</sub>AR<sup>−/−</sup> mice used as the source of the donor T cells lacked any signs of disease, including colitis. Moreover, T cell expression of CD4, CD25, CD69, CD103, and glucocorticoid-related receptor and their in vitro Treg function was comparable in C57BL/6 or A<sub>2A</sub>AR<sup>−/−</sup> mice (data not shown). These observations suggest that the lack of protection in mice receiving Th cell subsets from A<sub>2A</sub>AR<sup>−/−</sup> mice was not attributable to the presence of activated, pathogenic T cells in the CD25 or CD45RB<sup>low</sup> preparations. Rather, these data suggest that A<sub>2A</sub>AR played an important role in controlling the inflammatory response in mice receiving CD45RB<sup>high</sup> and CD25<sup>+</sup> T cells from A<sub>2A</sub>AR<sup>−/−</sup> mice.
response of T cells to the antigenic challenges in the gut that arise in the CD45RB transfer model.

A2AAR agonists regulate cytokine production by Th cells

A2AAR could modulate Th cell phenotype at several levels, including activation, selective cell death, trafficking, and by altering effector mechanisms. Based on initial studies, A2AAR have modest effects on activation and do not lead to T cell death (data not shown). The differentiation of Th cells in A2AAR−/− mice occurs in the presence of APC that also lack A2AAR and thus, indirect effects may account for the aberrant Th cell phenotype. Although A2AAR could modulate T cell trafficking that perturbs T cell regulation in this transfer model, previous studies have shown that Treg do not have to home to the gut to attenuate pathogenic T cells (18). Because both CD45RBhigh T cells and Treg from A2AAR−/− mice were aberrant, we speculated that A2AAR could differentially control the expression of pro- and anti-inflammatory cytokines thereby affecting their behavior in the CD45RB transfer model.

As shown in Fig. 3a, the selective A2AAR agonist ATL202 impaired the production of IFN-γ, TNF-α, IL-2, and IL-4 in CD45RBhigh-, or CD45RBlow-activated Th cells, whereas IL-10 was partially inhibited in CD45RBlow Th cells. The receptor specificity of the inhibition was examined by comparing three different A2AAR agonists for their effects using Th cells isolated from control or A2AAR−/− mice. Although A2AAR agonists suppressed Th cells cytokines in control mice, no significant effect was observed using T cells from A2AAR−/− mice (Fig. 3b). In contrast, IL-10 levels were decreased but persisted, and the expression of TGF-β was not inhibited in a statistically significant manner. The specificity of the response was confirmed when the reduction of pro-inflammatory cytokines by A2AAR agonists was attenuated by the A2AAR antagonist ZM241385 (Fig. 3c), whereas the A2BAR antagonist ATL801 had no effect.

The changes in cytokine responses mediated by A2AAR agonists reflected a hierarchy with IL-2 and IFN-γ being impaired the most, whereas TNF-α was affected to an intermediate degree, and the production of IL-4, IL-10 and TGF-β changed the least. Kinetic studies showed that IL-2 IFN-γ and TNF-α expression was inhibited as early as 12 h posttreatment, whereas IL-4 was detected between 36 and 72 h (data not shown). The net effect of the treatment with A2AAR agonists was to shift the balance of pro- and anti-inflammatory cytokines as reflected, for example, in a decrease in the ratio of IFN-γ to IL-10 from 14.5 to 1.61; the ratio of IL-2 to IL-10 from 3.09 to 0.82, and the ratio of IFN-γ to TGF-β from 38.6 to 3.21. One might predict that the administration of an A2AAR agonist would ameliorate disease induced by the transfer of CD45RBhigh Th cells.

**FIGURE 3.** Suppression of cytokine production by CD4+ T cells is mediated through the A2AAR. A, Cytokines were measured in supernatants from CD45RBhigh and CD45RBlow T cells after stimulation for 72 h in the presence or absence of ATL202. Data reflect the mean ± SEM of at least three separate experiments. *, p < 0.05, compared with untreated controls. B, Cytokines were measured in the supernatants of CD4+ T cells from control, C57BL/6 mice (BL/6), or A2AAR−/− mice after stimulation for 48 h in the presence or absence of ATL202, ATL146e, or ATL313. Data are the mean ± SEM from four separate experiments. *, p < 0.05, compared with untreated controls. C, CD4+ T cells from C57BL/6 mice were stimulated in the presence or absence of 100 nM of ATL202, ATL146, or ATL313 with/without ZM24241385 (an A2AAR antagonist) or ATL801 (an A2BAR antagonist) for 48 h. Data reflect the mean ± SEM of four separate experiments. *, p < 0.05, compared with cells treated with the A2AAR antagonist. D, CD4+ CD45RBhigh T cells from C57BL/6 mice were injected into SCID mice that were fed either control (ATL− n = 8) or treated chow (ATL313, n = 12) on days 29–49 after reconstitution. The photomicrographs show the colons from control or treated mice. The inflammation was scored and summarized in the graph.
In fact, delivering ATL313 in the chow of mice after the adoptive transfer of CD45RB<sup>hi</sup> Th cells attenuated the colitis (Fig. 3d).

**A<sub>2</sub>AAR agonists decreased the stability of proinflammatory cytokine mRNA.**

To address the mechanism by which the A<sub>2</sub>AAR agonists suppressed the production of proinflammatory cytokines, the cytokine mRNA levels in T cells from control and A<sub>2</sub>AAR<sup>−/−</sup> mice were compared after activation in the presence or absence of the different A<sub>2</sub>AAR agonists. Proinflammatory cytokine mRNA levels in Th cells from control mice were significantly decreased by ATL202, ATL146e, and ATL313 (Fig. 4a), whereas IL-10 and TGF-β were not significantly affected (data not shown). The A<sub>2</sub>AAR agonists had no effect on cytokine mRNA levels when CD4<sup>+</sup> T cells from A<sub>2</sub>AAR<sup>−/−</sup> mice were used.

Other experiments showed that A<sub>2</sub>AAR agonists do not inhibit the activation of transcription factors that regulate the expression of IL-2 and IFN-γ (data not shown), leading to the possibility that they decreased levels of mRNA by affecting mRNA stability. Thus, Th cells were activated to induce cytokine mRNA expression before transcription was inhibited by the addition of actinomycin D in the presence or absence of ATL202. As predicted, when actinomycin D alone was added, cytokine mRNA began to decay spontaneously. However, the levels of mRNA for IL-2 and IFN-γ decreased more rapidly (p < 0.05) in the presence of both actinomycin D and ATL202 (Fig. 4b), leading to the conclusion that the inhibition of cytokine mRNA levels in Th cells is through an effect of A<sub>2</sub>AAR agonists on mRNA stability.

The current data are the first describing the ability of A<sub>2</sub>AAR agonists to destabilize mRNA. A recent study has implicated A<sub>2</sub>AAR in the stabilization of IL-10 mRNA due to protein interactions with the 3’ adenosine uracil-rich element (ARE) (19). The ARE are sites at which mRNA stability can be increased or decreased. One or more ARE are found in the 3’ untranslated region of IL-2, IL-4, IL-10, IFN-γ, and TNF-α. Of note, TGF-β expression was not affected by A<sub>2</sub>AAR agonists, which is consistent with the fact that ARE have not been described in the 3’ region of TGF-β mRNA. Experiments are in progress to determine whether the various AR subtypes can differentially affect the role of ARE in controlling the stability of mRNA for pro- or anti-inflammatory cytokines.

The novel results from this study suggest A<sub>2</sub>AAR play a critical role in controlling the function T cells that regulate colitis. This conclusion is based on five observations: 1) CD45RB<sub>low</sub> or CD25<sup>+</sup> Th cells lacking A<sub>2</sub>AAR failed to prevent disease caused by pathogenic CD45RB<sup>hi</sup> Th cells in an adoptive transfer model; 2) Pathogenic CD45RB<sup>hi</sup> Th from A<sub>2</sub>AAR-deficient mice could not be inhibited by Treg from control mice; 3) A<sub>2</sub>AAR agonists markedly changed the balance of proinflammatory and anti-inflammatory cytokines expressed by Th cells during their activation; 4) an A<sub>2</sub>AAR agonist attenuated disease in vivo and; and 5) A<sub>2</sub>AAR agonists preferentially destabilized the mRNA that encodes proinflammatory cytokines. Thus, A<sub>2</sub>AAR play an important and novel role in the control Th cell function and their impact on colitis in an animal model. Stimulation through the A<sub>2</sub>AAR may provide a novel strategy to manipulate the Th cell response to the therapeutic advantage of the host.

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**Disclosures**

Joel Linden own shares in Adenosine Therapeutics.

**References**


