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The A_{2B} Adenosine Receptor Promotes Th17 Differentiation via Stimulation of Dendritic Cell IL-6

Jeffrey M. Wilson,* Courtney C. Kurtz,* Steven G. Black,* William G. Ross,* Mohammed S. Alam,* Joel Linden,† and Peter B. Ernst*

Adenosine is an endogenous metabolite produced during hypoxia or inflammation. Previously implicated as an anti-inflammatory mediator in $CD4^+$ T cell regulation, we report that adenosine acts via dendritic cell (DC) A_{2B} adenosine receptor ($A_{2B}AR$) to promote the development of Th17 cells. Mouse naive $CD4^+$ T cells cocultured with DCs in the presence of adenosine or the stable adenosine mimetic 5′-(*N*-ethylcarboximado) adenosine resulted in the differentiation of IL-17– and IL-22–secreting cells and elevation of mRNA that encode signature Th17-associated molecules, such as IL-23R and RORγt. The observed response was similar when DCs were generated from bone marrow or isolated from small intestine lamina propria. Experiments using adenosine receptor antagonists and cells from $A_{2B}AR^{-/-}$ or $A_{2A}AR^{-/-}/A_{2B}AR^{-/-}$ mice indicated that the DC $A_{2B}AR$ promoted the effect. IL-6, stimulated in a cAMP-independent manner, is an important mediator in this pathway. Hence, in addition to previously noted direct effects of adenosine receptors on regulatory T cell development and function, these data indicated that adenosine also acts indirectly to modulate $CD4^+$ T cell differentiation and suggested a mechanism for putative proinflammatory effects of $A_{2B}AR$. ***The Journal of Immunology*****, 2011, 186: 000–000.**

Discrete populations of $CD4^+$ T lymphocytes with unique cytokine-expression signatures are important for host health and immunity in the face of a diverse array of pathogenic and commensal microorganisms. Classically divided into just two subsets (1), more recent findings indicated a broader diversity in $CD4^+$ T cell heterogeneity. For instance, it is now well accepted that, in addition to Th1 and Th2 cells, regulatory T cells (Tregs) and Th17 cells represent functionally unique cells that develop from a common naive precursor (2, 3). Development into these subsets is controlled largely by cues derived from APCs during activation of Ag-inexperienced $CD4^+$ T cells. Elaborating the factors and processes that govern the differentiation of $CD4^+$ T cells remains a fundamental goal of cellular immunology.

Adenosine is a purine nucleoside with multitudinous physiological effects. Accumulating during hypoxia and inflammation, it is now clear that adenosine acts on both innate and adaptive immune cells, with the outcome depending on the combinatorial expression of four unique G protein-coupled receptors (reviewed in Refs. 4–6). Signaling via the G_s-coupled A_{2A} adenosine receptor ($A_{2A}AR$), adenosine regulates T cell activation directly (7–11) and has been implicated in the development and function of Tregs (12–14). Indeed, the protective effect of adenosine analogs in many inflammatory disease models has been largely attributed to anti-inflammatory actions on T cells (15, 16). Adenosine receptor (AR) expression in APCs is more diverse, although $A_{2A}AR$ and the G_s/G_q-coupled $A_{2B}AR$ predominate (17–19). Previous reports suggested that the effects of adenosine on APCs are also largely anti-inflammatory, as exemplified by attenuated TNF-α and augmented IL-10 (18, 20). Because Gs-coupled responses induce cAMP in both T cells and APCs, current hypotheses suggest that the accumulation of cAMP accounts for many of the inhibitory effects of adenosine (6, 21, 22). However, in some in vivo models, interference with the $A_{2B}AR$ protected animals from various forms of intestinal inflammation, suggesting that these receptors are proinflammatory (23, 24). These potential proinflammatory effects of adenosine are not well understood.

Because adenosine acts on both APCs and T cells and is abundant at inflammatory sites, it could represent a physiological mediator that influences $CD4^+$ T cell differentiation. Previous reports indicated that adenosine acts directly on T cells to inhibit Th1 cytokine responses (25) and promote Treg development (14). However, the role of adenosine in controlling $CD4^+$ T cell differentiation in the context of APCs has not been comprehensively examined, especially in light of the broader heterogeneity of $CD4^+$ T cell responses now appreciated. To determine the effects of adenosine on the fate of APC-primed naive $CD4^+$ T cells, highly purified FACS-sorted $CD4^+CD62L^{hi}CD44^{lo}CD25^{lo}$ T cells were cocultured with dendritic cells (DCs) in the presence of adenosine or a stable nonselective adenosine mimetic. In this article, we report that under these conditions, adenosine preferentially favored $CD4^+$ T cells with a Th17 cytokine and transcription factor signature. Induction of Th17 cells was predominantly controlled by DC $A_{2B}AR$ and was conserved, even in the presence of TGF-β1. IL-6 is an important mediator of the response, as revealed by neutralizing Ab to IL-6–gp130. $A_{2B}AR$ stimulation of DC IL-6 required the phospholipase C (PLC)β/protein kinase C (PKC) pathway, but cAMP was dispensable. These data collectively indicated that, under some circumstances, adenosine acts via $A_{2B}ARs$ expressed on DCs to alter the cytokine microenvironment and favor the development of Th17 cells. Thus, adenosine

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Abbreviations used in this article: $A_{2A}AR$, A_{2A} adenosine receptor; AR, adenosine receptor; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; EPAC, exchange proteins directly activated by cAMP; NECA, 5′-(*N*-ethylcarboximado) adenosine; PKC, protein kinase C; PLC, phospholipase C; SFB, segmented filamentous bacteria; Treg, regulatory T cell.

represents a biological mediator that controls CD4$^+$ T cell differentiation.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. $A_{2A}AR^{-/-}$, $A_{2B}AR^{-/-}$, and $A_{2A}AR^{-/-}/A_{2B}AR^{-/-}$ double knockout mice on a C57BL/6 background were bred and maintained at the University of Virginia (17). All mice were housed and handled in accordance with the Institutional Animal Care and Use Committee of the University of Virginia.

Reagents

Adenosine, the adenosine mimetic 5′-(*N*-ethylcarboximado) adenosine (NECA), the cAMP inhibitor protein kinase inhibitor, and the exchange proteins directly activated by cAMP (EPAC) agonist 8-pCPT-2′-*O*-Me-cAMP were purchased from Sigma. The AR antagonists ZM241385 and SCH58261 were purchased from Tocris, and ATL692 was kindly provided by PGxHealth, a division of Clinical Data (Charlottesville, VA). The following cell-signaling inhibitors were purchased from Santa Cruz Biotechnology: 2′, 5′-dideoxyadenosine, PD98059, SB203580, U-73122, LY294002, and bisindolylmaleimide I.

Cell purification

Naive CD4$^+$ T cells were isolated from spleen by physical disaggregation, followed by RBC lysis with ACK buffer and enrichment with CD4 microbeads and LS columns (Miltenyi Biotec), according to the manufacturer's instructions. Resultant cells were stained with anti-mouse CD4-FITC (BD Biosciences), CD44-PE (eBioscience), CD62L-PE/Cy5.5 (eBioscience), and CD25-allophycocyanin (BD Biosciences), and CD4$^+$ CD44^{lo}CD62L^{hi}CD25^{lo} cells were sorted with a BD FACSVantage cell sorter. In some cases, CD4$^+$ or CD4$^+$CD62L$^+$ T cells were enriched with CD4$^+$ or CD4$^+$CD62L$^+$ MACS kits (Miltenyi Biotec), according to the manufacturer's instructions.

Bone marrow-derived DCs (BMDCs) were generated as described previously (17). Briefly, bone marrow was flushed from femurs and tibias and RBCs were lysed with ACK buffer and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 μM 2-ME, 25 mM HEPES, 2 mM sodium pyruvate, and 20 ng/ml GM-CSF (PeproTech). After 6 d, nonadherent/semiadherent cells were harvested and enriched with CD11c microbeads and LS columns (Miltenyi Biotec). Cells were incubated overnight with 5 ng/ml GM-CSF and used the following day in coculture experiments.

APCs from small intestine lamina propria were isolated, as described previously (17, 26). Briefly, small intestines were removed from euthanized donor c57BL/6 mice, opened longitudinally to flush out feces, cut into 5-mm pieces, and incubated for 20 min at 37°C on a shaker in HBSS supplemented with 5% heat-inactivated FBS and 2 mM EDTA. After passing the preparation through a metal filter, intestinal fragments were collected, and the step was repeated. Then intestinal fragments were minced and incubated for 20 min at 37°C on a shaker in HBSS supplemented with 5% heat-inactivated FBS and 1 mg/ml type VIII collagenase (Sigma). After straining debris and washing once with complete DC media, cells were incubated with FcBlock, and APCs were enriched by a MACS kit using both CD11b and CD11c microbeads. Resultant cells were stained with CD11b (eBioscience) and CD11c (BD Biosciences) Ab, and APCs were sorted with a BD FACSVantage cell sorter.

CD4$^+$ T cell stimulation

DCs (1×10^4) and naive CD4$^+$ T cells (1×10^5) were cultured in 200 μl T cell media in 96-well plates in the presence of 1 μg/ml soluble anti-CD3ε. Cells were treated with 500 nM NECA or vehicle control and, in some cases, with 5 or 100 μM adenosine. Other experiments were conducted with TGF-β1 (R&D Systems), 5 μg/ml neutralizing Ab to IL-6–gp130 (R&D Systems), or 250 nM AR antagonists. Supernatant and cell lysates for real-time RT-PCR were collected at 72 h. Supernatant was assayed using commercial kits, according to manufacturer's instructions, for IL-17 (BioLegend), IFN-γ (BD Biosciences), IL-22 (R&D Systems), IL-10 (BD Biosciences), and IL-6 (BD Biosciences).

Intracellular cell staining and flow cytometry

For flow cytometric analysis of intracellular markers, cells were harvested on day 4 and washed three times. After resting overnight in complete T cell media, cells were stimulated with 50 ng/ml PMA, 750 ng/ml ionomycin, and GolgiStop (BD Biosciences) for 4–5 h. Cells were incubated for 15 min with FcBlock, stained with anti-CD4 (BD Biosciences), permeabilized, and stained with anti–IL-17, anti–IFN-γ, and anti-Foxp3 using a Cytofix/Cytoperm kit (BD Biosciences). Samples were assayed with a BD FACSCalibur flow cytometer, and data were analyzed with FlowJo (TreeStar) software.

Semiquantitative real-time RT-PCR

Cells from coculture were collected and lysed, and total RNA was isolated with Qiagen mini-prep kits and reverse transcribed into cDNA with Superscript II (Invitrogen), according to the manufacturer's recommendations. Transcripts were measured by real-time RT-PCR with primer and dual-labeled probes (*Il4*, Mm00445259_m1; *Il6*, Mm00446190_m1; *Il10*, Mm00439616_m1; *Il17a*, Mm00439619_m1; *Il21*, Mm00517640_m1; *Il22*, Mm00444241_m1; *Il23r*, Mm00519943_m1; *Tnf*, Mm00443258_m1; *Ifng* Mm00801778_m1; *Rorc*, Mm01261022_m1; and *Foxp3*, Mm00475156_m1) (all from Applied Biosystems) detected in a Smart Cycler (Cepheid) and normalized against 18s rRNA. The fold change in the expression of a given gene of interest was determined using the $\Delta\Delta C_T$ method (27).

Statistical analysis

Data are reported as the mean ± SEM. Unless indicated, all data were pooled from three or more independent experiments. The two-tailed Student *t* test, assuming unequal variance, was performed using Microsoft Excel software. A p value < 0.05 was considered significant.

Results

Adenosine promotes differentiation of Th17 cells

To determine the role of ARs in controlling CD4$^+$ T cell differentiation, naive CD4$^+$ T cells were activated with DCs and anti-CD3, treated with a stable nonselective adenosine mimetic (NECA) or vehicle control, and assayed for factors associated with different Th lineages. Real-time RT-PCR indicated that NECA preferentially upregulated *Il17* compared with transcripts associated with other Th cell subsets (Fig. 1*A*). This IL-17 response was confirmed by ELISA analysis of 72-h supernatant and intracellular cytokine staining following restimulation (Fig. 1*B*, 1*C*). IFN-γ responses were modestly augmented by NECA, but to a lesser extent than IL-17. Importantly, adenosine itself also increased the frequency of IL-17–expressing CD4$^+$ T cells (Fig. 1*D*). Together, these results demonstrated that, in the presence of DCs, adenosine favors the development of IL-17–secreting CD4$^+$ T cells. Further studies were conducted to determine whether these cells had characteristics consistent with a bona fide Th17 cell. Indeed, IL-22 protein, as well as *Il21*, *Il22*, *Il23r*, and *Rorc* (RORγt), was significantly elevated (Fig. 2).

Published studies suggest that adenosine is an important molecule in Treg development and function (6). To assess whether ARs promote Tregs in the DC coculture model, we assessed Foxp3 transcript and intracellular protein in cells treated with NECA or vehicle control. NECA treatment had little effect on either *Foxp3* levels or intracellular Foxp3 protein in CD4$^+$ T cells (Figs. 1*A*, 3). To address the possibility that adenosine acts in concert with TGF-β1 to favor Tregs, some coculture experiments were conducted with exogenous TGF-β1. Although TGF-β1 alone is sufficient to promote the development of Foxp3-expressing cells, this response was impaired in the presence of NECA (Fig. 3). Moreover, IL-17–expressing CD4$^+$ T cells emerged concomitantly with the diminution of TGF-β1–induced Foxp3$^+$ Tregs.

Th17 response depends on DC A_{2B}AR

Adenosine signals via four G protein-coupled receptors, A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR, which are all expressed, albeit unequally, by different hematopoietic-derived cells. We conducted experiments using various pharmacological antagonists to determine which receptor(s) contributed to the observed Th17

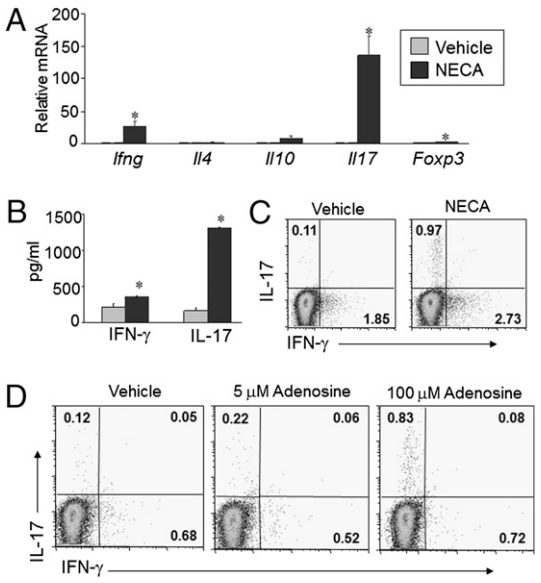

FIGURE 1. AR activation promotes differentiation of IL-17–secreting $CD4^+$ T cells. *A*, Naive $CD62L^{hi}CD44^{lo}CD25^{lo}CD4^+$ T cells were activated with BMDCs and anti-CD3 in the presence of 500 nM NECA or vehicle control. After 72 h, cells were harvested, and mRNA was assessed by real-time RT-PCR, or supernatant was collected and assessed by ELISA for IL-17 and IFN-γ (*B*). Data are pooled from three or more independent experiments and represent the mean ± SEM. $*p < 0.05$. *C*, Day 4 cells were harvested, washed, and replated in complete media overnight prior to restimulation with PMA/ionomycin. Dot plots show intracellular IFN-γ and IL-17 in $CD4^+$ T cells. *D*, Naive $CD4^+$ T cells were activated with BMDCs and anti-CD3 in the presence of vehicle control or 5 or 100 μM adenosine. Day 4 cells were harvested, washed, and replated in complete media overnight prior to restimulation with PMA/ionomycin. Dot plots show intracellular IFN-γ and IL-17 in $CD4^+$ T cells. Dot plots are representative of three or more independent experiments.

response. ZM241385, an antagonist to both A_{2A}AR and A_{2B}AR, effectively abolished the NECA-mediated IL-17 induction, thus indicating that some combination of the two receptors is likely involved (Fig. 4*A*). The response was similarly impaired by ATL692, an A_{2B}AR selective antagonist, whereas the A_{2A}AR selective inhibitor SCH58261 had little effect, showing that the Th17 response is predominantly mediated via A_{2B}AR. Because previous work demonstrated an important role for A_{2B}AR in the control of DC function, coculture experiments were conducted with DCs derived from $A_{2B}AR^{-/-}$ mice or mice lacking both A_{2B}AR and A_{2A}AR. NECA induction of IL-17 was minimal in

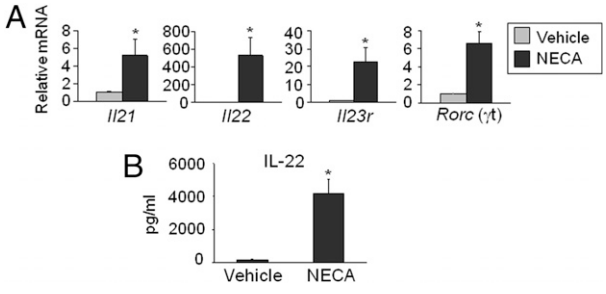

FIGURE 2. An adenosine mimetic promotes development of bona fide Th17 cells. Naive $CD4^+$ T cells were activated with BMDCs and anti-CD3 in the presence of 500 nM NECA or vehicle control. *A*, After 72 h, cells were harvested, and mRNA was assessed by real-time RT-PCR, or supernatant was collected and assessed by ELISA for IL-22 (*B*). Data were pooled from three or more independent experiments and represent the mean ± SEM. $*p < 0.05$.

these cocultures, suggesting that Th17 differentiation in this model requires A_{2B}AR expressed by the DCs (Fig. 4*B*). In contrast, NECA still promoted a significant IL-17 response in cocultures of wild-type DCs with $CD4^+$ T cells lacking A_{2A}AR or both A_{2B}AR and A_{2A}AR. Further reinforcing the importance of DCs in this system, NECA did not affect IL-17 when naive $CD4^+$ T cells were stimulated with anti-CD3/28 in the absence of APCs (data not shown).

A_{2B}AR promotes IL-6 to favor Th17, and IL-6 response is cAMP independent

The development of Th17 cells is controlled largely via factors derived from innate immune cells, such as IL-6 and TGF-β1. Previous reports showed that adenosine could promote IL-6 in a variety of cell types, including DCs (28, 29). To assess the role of IL-6 in the A_{2B}AR-mediated Th17 response, cocultures were conducted in the presence of a neutralizing Ab to the IL-6–gp130 receptor. NECA induction of IL-17 was abrogated in the presence of the IL-6–gp130 inhibitor, thus demonstrating that IL-6 is indeed an important mediator of the Th17 response (Fig. 5*A*, 5*B*). Although NECA decreased the number of cells expressing Foxp3 as Th17 cells increased, the decrease in Foxp3 did not occur when IL-6-gp130 was blocked. These findings suggested that IL-6 concentrations are a key determinant of the effects of adenosine in the reciprocal control of Th17 and Treg development (Fig. 5*B*). Experiments with a neutralizing Ab to TGF-β1 also impaired Th17 development, thus suggesting that TGF-β1 is also important in this system (data not shown).

To test whether IL-6 in this system is derived from DC A_{2B}AR, we conducted experiments with DCs generated from wild-type, $A_{2A}AR^{-/-}$, or $A_{2B}AR^{-/-}$ mice. $A_{2B}AR^{-/-}$ DCs produced little IL-6 upon NECA treatment, whereas wild-type and $A_{2A}AR^{-/-}$ DCs produced significant amounts of IL-6 in response to NECA (Fig. 5*C*). Importantly, $A_{2B}AR^{-/-}$ DCs did not have an intrinsic defect in IL-6 production, because LPS provoked abundant IL-6 (data not shown). Pharmacologic blockade with AR-selective antagonists corroborated that the IL-6 response depended on A_{2B}AR (data not shown). A role for A_{2B}AR in TGF-β1 induction was also assessed, but NECA treatment of DCs did not elevate TGF-β1 protein or mRNA, as assayed by ELISA or real-time RT-PCR (data not shown). This suggested that low levels of TGF-β1 are constitutively produced by DCs or, alternatively, that TGF-β1 is residually present in the FBS used in cocultures.

As introduced previously, the anti-inflammatory effects of adenosine are often attributed to signaling pathways involving A_{2A}AR and the intracellular mediator cAMP. This is consistent with the general anti-inflammatory effects of other cAMP-elevating agents, such as prostaglandin E_2 and vasoactive intestinal peptide (30). However, in our model, which incorporates both $CD4^+$ T cells and APCs, we describe a putative proinflammatory effect of adenosine on $CD4^+$ T cells that is A_{2B}AR dependent. A_{2B}AR differs from A_{2A}AR and the other ARs in that it can couple to both G_s and G_q, enabling it to activate both adenylyl cyclase and PLC, respectively (6). Thus, we sought to determine whether A_{2B}AR relied on cAMP or whether other signaling modalities were important.

To elucidate key pathways that control the NECA-mediated responses observed in our study, we treated DCs with NECA in the presence of various pharmacologic inhibitors known to inhibit G protein pathways and assessed IL-6 production. At the concentrations studied, the inhibitors did not impair DC viability, as assessed by trypan blue exclusion (data not shown). Inhibition of two distinct targets of the adenylyl cyclase/cAMP axis had no effect on NECA-mediated IL-6 induction, whereas inhibition of the PLC/

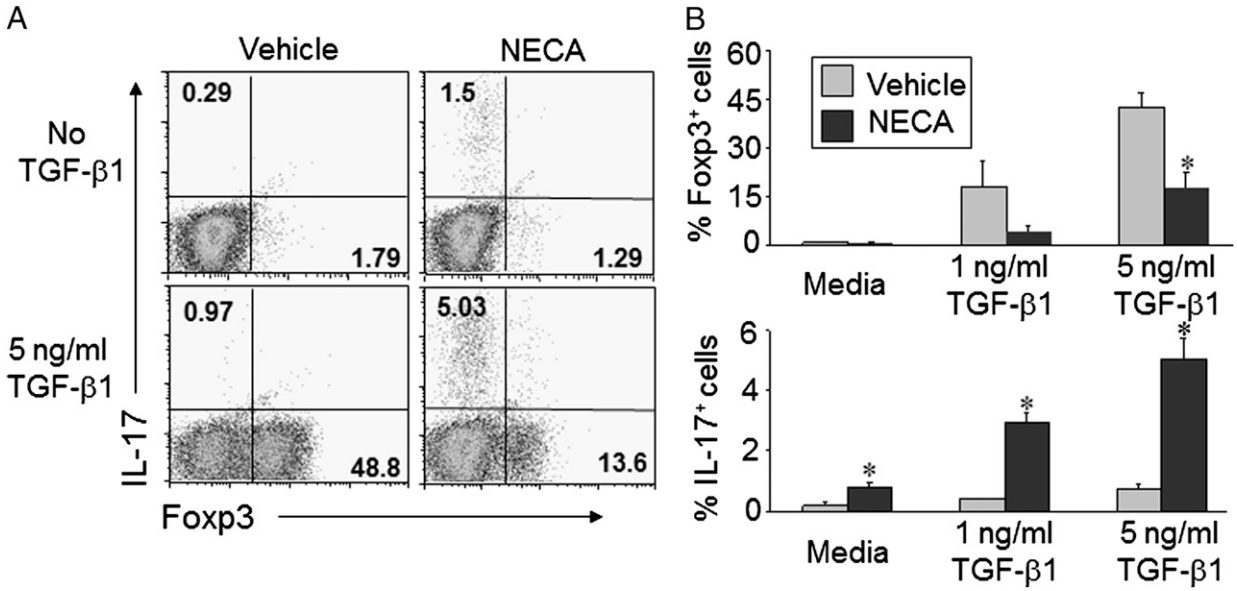

FIGURE 3. An adenosine mimetic favors Th17 cells in the presence of TGF-β1. Naive $CD4^+$ T cells were activated with BMDCs and anti-CD3 in the presence of TGF-β1 and 500 nM NECA or vehicle control. Day 4 cells were harvested, washed, and replated in complete media overnight prior to restimulation with PMA/ionomycin. Dot plots show intracellular IL-17 and Foxp3 in $CD4^+$ T cells. Dot plots (*A*) are representative of three or more independent experiments, and compiled data (*B*) were pooled from three or more independent experiments and represent the mean ± SEM. $*p < 0.05$.

PKC axis with either U-73122 or bisindolylmaleimide I impaired the IL-6 response significantly (Fig. 5*D*). Because MAPKs, including p38 and ERK, also play a role in mediating A_{2B}AR effects in some cells, additional inhibitors targeting other pathways were used. We found that the ERK inhibitor PD98509 and the PI3K inhibitor LY294002 both blocked NECA-mediated IL-6, but the p38 inhibitor SB203580 had little effect. Lastly, treating DCs with the cAMP-elevating agent forskolin or the EPAC agonist 8-pCPT-2′-*O*-Me-cAMP provoked little IL-6, suggesting that stimulating cAMP or EPAC pathways is not sufficient to drive DC production of IL-6 (data not shown). Taken together, this indicated that NECA promoted IL-6 in DCs independently of cAMP but that PLC/PKC, ERK, and PI3K are involved, and it explains why A_{2B}AR, but not A_{2A}AR, stimulates IL-6.

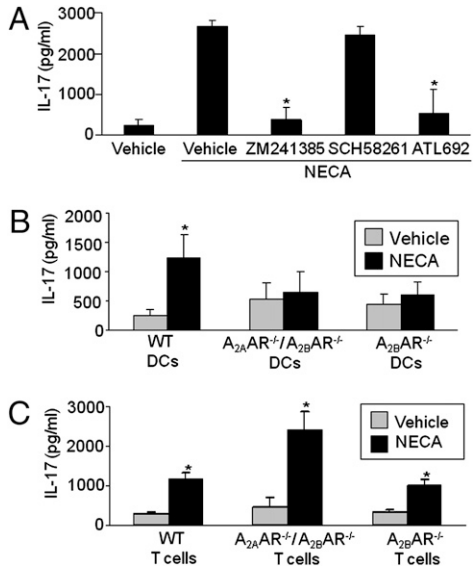

FIGURE 4. Th17 response depends on DC A_{2B}ARs. Naive $CD4^+$ T cells were activated with BMDCs and anti-CD3 in the presence of 500 nM NECA or vehicle control. Seventy-two–hour supernatant was collected and assessed by ELISA for IL-17. *A*, AR-selective antagonists or vehicle control was added to cultures at a concentration of 250 nM. Data were pooled from two independent experiments conducted in duplicate and represent the mean ± SEM. BMDCs (*B*) or $CD4^+$ T cells (*C*) were derived from $A_{2B}AR^{-/-}$ or $A_{2A}AR^{-/-}/A_{2B}AR^{-/-}$ mice, where indicated. Data were pooled from three or more independent experiments and represent the mean ± SEM. $*p < 0.05$.

Intestinal DCs support NECA-mediated Th17 response

Th17 cells are especially abundant at mucosal sites, such as the small intestine, where they are important in controlling neutrophil accumulation and epithelial barrier integrity (31). The intestine is subject to frequent hypoxia and, thus, is thought to be an adenosine-rich site (32). Therefore, to model the effects of adenosine on T cell activation in the gut, we isolated highly purified subsets of gut APCs and cultured them with naive $CD4^+$ T cells (Fig. 6). We found that the adenosine mimetic NECA significantly enhanced IL-17 in cocultures containing a population of $CD11c^{hi}$ $CD11b^+$ gut DCs, a subset previously associated with Th17 responses (26). Additionally *Il22* was significantly upregulated by NECA treatment with this DC subset. Conversely, NECA had no such effect in cultures with a population of $CD11c^{lo}CD11b^+$ macrophages, which is consistent with previous studies suggesting this population of APCs promotes Tregs (26). *Foxp3* was not altered in any of the NECA-treated groups.

Discussion

Previous work showed that direct engagement of A_{2A}AR on T cells inhibited proliferation, as well as Th1 and Th2 responses (9–11). A_{2A}AR also contributes to the generation and function of Tregs (10, 12–14, 33). However, T cell activation and differentiation do not occur in isolation but require APCs and a cadre of extracellular factors. Moreover, the accumulation of endogenous adenosine at inflammatory sites does not exclusively target A_{2A}AR; it stimulates other available AR subtypes. To more faithfully model how adenosine impacts $CD4^+$ T cell differentiation, we studied the effects of a nonselective adenosine mimetic in cocultures with DCs and highly purified naive $CD4^+$ T cells. In this study, we found that adenosine preferentially promoted de novo

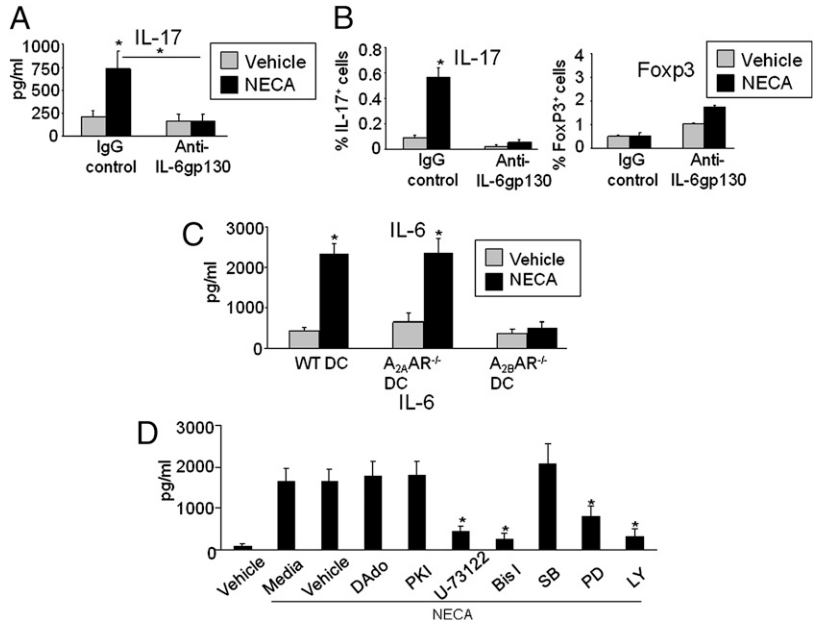

FIGURE 5. IL-6, stimulated in a cAMP-independent manner, is important in A_{2B}AR-mediated Th17 responses. *A*, Naive $CD4^+$ T cells were activated with BMDCs and anti-CD3 in the presence of a neutralizing Ab to IL-6–gp130 and 500 nM NECA or vehicle control. Day 4 cells were harvested and washed, and supernatants were collected and assayed for IL-17. *B*, Naive $CD4^+$ T cells were activated with BMDCs and anti-CD3 in the presence of a neutralizing Ab to IL-6–gp130 and 500 nM NECA or vehicle control. Day 4 cells were harvested, washed, replated in complete media overnight prior to restimulation with PMA/ionomycin, and assayed for the production of IL-17 or Foxp3 by flow cytometry. Data are representative of three or more independent experiments. *C*, BMDCs derived from wild-type, $A_{2A}AR^{-/-}$, and $A_{2B}AR^{-/-}$ mice were treated with 1 μM NECA or vehicle control, and 18-h supernatant was collected and assessed by ELISA for IL-6. *D*, BMDCs were treated with 1 μM NECA in the presence of 10 μM signaling inhibitors or vehicle control, and 18-h supernatant was collected and assessed by ELISA for IL-6. Bis I, bisindolylmaleimide I; DAdo, 2′5′ dideoxyadenosine; LY, LY294002; PD, PD98059; SB, SB203580. Data were pooled from three or more independent experiments and represent the mean ± SEM. $*p < 0.05$.

differentiation of $CD4^+$ T cells that express IL-17 and IL-22 and have a transcriptional profile consistent with Th17 cells (i.e., upregulation of genes encoding for IL-23R and the transcription factor RORγt) (34). Our data indicated that activation of A_{2B}AR on DCs stimulated IL-6 production in a cAMP-independent manner, and this IL-6 is an important intermediary that favors the Th17 response. Thus, adenosine represents a biological mediator that can enhance the development of Th17 cells.

In the murine system, TGF-β1 and proinflammatory cytokines, such as IL-6, are critical for Th17 differentiation, whereas a host of other cytokines, such as IL-2 and IFN-γ, are negative regulators (35). The data reported in this article suggest that adenosine alters the balance of these cytokines in a way that favors Th17 differentiation at the expense of Treg development. For instance, adenosine directly impairs IL-2 and IFN-γ production by Th cells (9–11, 14), and we previously showed that an adenosine mimetic impaired IL-12 by DCs (17). Conversely, adenosine was reported to promote or remain permissive for DC production of TGF-β1 and IL-6 (29). The importance of specific cytokines in controlling adenosine-mediated Th17 differentiation is evidenced by the fact that A_{2B}AR stimulates IL-6 and that neutralization of the IL-6–gp130 receptor abrogates the Th17 response. The necessary role for IL-6 in this system implies that Th17 cells are induced in a Stat3-dependent mechanism (36); however, that has yet to be

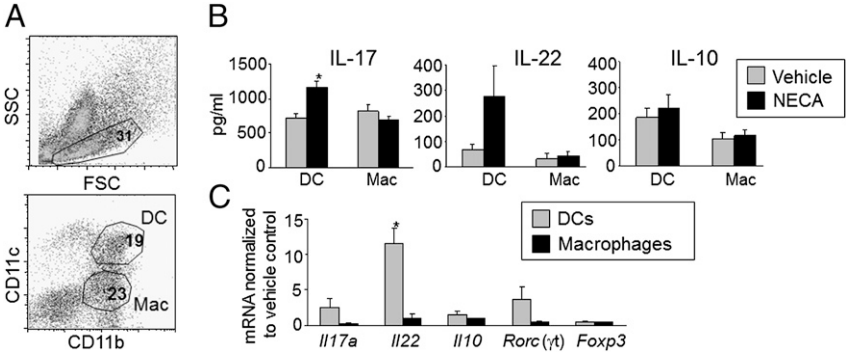

FIGURE 6. Gut $CD11c^+CD11b^+$ DCs, but not macrophages, support adenosine mimetic-mediated Th17 T cell response. *A*, Small intestinal lamina propria APCs were isolated by FACS based on forward light scatter/side scatter and CD11c/CD11b expression. *B*, MACS-naive CD4CD62L-enriched splenocytes were activated with $CD11c^{hi}CD11b^+$ DCs or $CD11c^{lo}CD11b^+$ macrophages and anti-CD3 in the presence of 500 nM NECA or vehicle control. After 72 h, lysates were collected and assessed by real-time RT-PCR. Data were pooled from three or more independent experiments and represent the mean ± SEM. *C*, MACS-naive CD4CD62L-enriched splenocytes were activated with $CD11c^{hi}CD11b^+$ DCs or $CD11c^{lo}CD11b^+$ macrophages and anti-CD3 in the presence of 500 nM NECA or vehicle control. After 72 h, supernatant was collected and assessed by ELISA for IL-17, IL-22, and IL-10. Data were pooled from three or more independent experiments and represent the mean ± SEM. $*p < 0.05$.

confirmed. The role of TGF-β1 in this system is less clear, because we did not find direct evidence that A_{2B}AR stimulation was sufficient to generate TGF-β1. However, the fact that neutralizing Ab to TGF-β1 diminished IL-17 production indicated that adenosine-mediated Th17 differentiation requires the presence of TGF-β1.

Although NECA favors Th17 cells and not Tregs, upon IL-6 neutralization, the opposite result was observed: Th17 expansion was abrogated as was the decrease in cells expressing Foxp3. These data suggested that IL-6 induced by NECA was predominantly, if not solely, responsible for the decrease in Foxp3-producing Th cells and that it is a key intermediate controlling the effects of adenosine on Th17 and Treg development. Stimulation of A_{2A}AR was implicated in the expansion of Tregs (14) when T cells were stimulated in the absence of APCs. However, A_{2A}AR activation did not inhibit Th17 cells when a source of APCs was provided (14).

The finding that A_{2B}AR acts on DCs to promote Th17 cell development conflicts with the conclusions of other investigators that adenosine signaling favors a Th2-biasing DC (37, 38). These groups studied human monocyte-derived DCs and restricted their investigation to Th1 and Th2 markers in MLR, specifically IFN-γ and IL-4 and/or IL-5 (37, 38). Thus, these investigators studied DCs with characteristics unique from BMDCs and did not assess markers associated with other Th phenotypes, such as IL-17 or IL-22. A potentially important distinction between the studies is the difference in the human and mouse systems. However, in addition to species variation, there are other factors that may be important. In the single study that compared the effects of adenosine, in the presence or absence of LPS, on DC-polarizing capacity, anti-Th1/pro-Th2 skewing was only observed in the group with LPS (37, 38). In our model system, no LPS was required to achieve the striking upregulation of IL-17 and IL-22.

The data obtained with BMDCs were consistent with observations made using DCs isolated from the intestinal lamina propria, a site rich in Th17 cells. When used in coculture experiments, the $CD11c^+CD11b^+$ DC subset preferentially increased IL-17 and IL-22 mRNA in the presence of the adenosine mimetic. These findings indicated that ARs modulate $CD11c^+CD11b^+$ lamina propria DCs and suggested that endogenous adenosine acts via lamina propria DCs to control intestinal Th responses, which agrees with other studies on intestinal DCs (26). The difference in A_{2B}AR-mediated effects between these two intestinal APC populations may reflect the fact that resting macrophages express fewer A_{2B}AR (17), and this receptor leads to the production of IL-6, whereas the A_{2A}AR does not. Although we showed that adenosine is one factor that can promote Th17 development, there are clearly redundant pathways that contribute to the in vivo response. For instance, $A_{2B}AR^{-/-}$ mice have numbers of Th17 cells in small intestine lamina propria comparable to wild-type controls; moreover, the abundance of Th17 cells in our $A_{2B}AR^{-/-}$ mice could be augmented by manipulating commensal flora (data not shown), presumably due to segmented filamentous bacteria (SFB), which persist in some, but not all, mouse facilities (39). Although ruling out a necessary role for A_{2B}AR in the SFB-mediated Th17 response, this does not discount the possibility that local accumulation of adenosine can act to promote Th17 cells.

The role of A_{2B}AR in the promotion of Th17 cells, and possibly inflammation, is somewhat surprising but not entirely unexpected. For example, some studies implicated A_{2B}AR in pulmonary inflammation (40). This is at least consistent with other evidence suggesting that intestinal inflammation is reduced in the absence of normal A_{2B}AR function (23, 24). However, it is important to note that another group investigating adenosine in the gut found the exact opposite (41). One interpretation of the conflicting results of A_{2B}AR regulation on intestinal inflammation is that the microbiome differs in the animal facilities of the respective investigators. Although the A_{2B}AR-dependent induction of Th17 could be consistent with these receptors promoting inflammation, emerging thought suggests that Th17 cells may confer protective/anti-inflammatory responses. For example, mice harboring SFB have a marked increase in intestinal Th17 cells but no disease (39). In addition, the coexpression of IL-22 by Th17 cells can be protective (42). Similarly, IL-17R knockout mice have worse intestinal disease in some models (43). Because Th1, Th2, and Th17 cells contribute to the protection of the host, which is why they exist, their contribution to the pathogenesis of disease may require the presence of other factors. For example, in Crohn's disease, Th17 responses are implicated in the pathogenesis but usually are the result of their heightened activation by accessory cytokines, such as IL-23 (44). Thus, the presence of IL-17–producing cells in the absence of other cues may not be sufficient for inflammation to ensue.

Factors that calibrate the magnitude and quality of $CD4^+$ T cell responses are critical for host health. Although previous work emphasized an inhibitory role for adenosine in controlling $CD4^+$ Th cells, the current study suggests that adenosine promotes Th17 cells. Thus, adenosine represents a biological mediator that regulates $CD4^+$ T cell development and function, albeit in a manner that was not predicted. Adenosine shares its pleiotropic functions with other factors, such as IL-2 and TGF-β1. For example, IL-2 drives the expansion of effector T cells, but it is required for the maintenance of Tregs (45). Similarly, TGF-β1 is important for the induction of Foxp3 and the accumulation of induced Tregs, and it contributes to Th17 cell development (46). The relative concentration of a nucleoside, the types of cells that it targets, and the specific ARs available for engagement may be important determinants of the final outcome. The current study is significant because it adds to the regulatory effects of adenosine and expands the current thinking on its role in the control of host responses.

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