Published in final edited form as:
J Immunol. 2010 August 15; 185(4): 1993–1998. doi:10.4049/jimmunol.1000108.

Much Ado about Adenosine: Adenosine Synthesis and Function in Regulatory T Cell Biology

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Abstract

Recent studies have reported that adenosine is a significant mediator of regulatory T cell (Treg) function. Indeed, activation of the adenosine receptor subtypes expressed by a broad range of immune and inflammatory cells attenuates inflammation in several disease models. This anti-inflammatory response is associated with an increase in intracellular cAMP that inhibits cytokine responses of many immune/inflammatory cells, including T cells and APCs. Thus, adenosine produced by Tregs can provide a paracrine feedback that shapes the host response following an immunologic provocation. This review discusses the evidence that adenosine is an integral part of Treg biology and presents some of the mechanisms that may account for its contribution to the resolution of inflammation and the regulation of the immune/inflammatory cell phenotype.

Immune-inflammatory cells respond to local mediators, accessory molecules, and microbial products to assume a phenotype suitable for the tissue and physiologic stimulus. This plasticity allows the host to maintain the appropriate expression of genes so that immunity is optimized without excessive inflammation. $CD4^+$ regulatory T cells (Tregs) produce mediators that contribute to this homeostasis by controlling gene expression in immune–inflammatory cells. One such anti-inflammatory mediator is adenosine, and the studies identifying its many anti-inflammatory effects are reviewed in detail elsewhere (1–3). However, like another mediator of Treg—that is, TGF-β1—adenosine has multiple effects. These mediators should not be considered as uniformly anti-inflammatory, but as part of a network of factors that, through their pleiotropic effects, shape the responses of target cells. Thus, the potential anti-inflammatory benefits of adenosine that can be exploited therapeutically have to be balanced against their possible detrimental effects.

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Disclosures

P.B.E. is the principal investigator on a grant that has a subcontract with PGXhealth, which makes adenosine analogues.

Adenosine synthesis and the control of inflammation

Adenosine accumulates in response to inflammation and ischemia. There are several means by which adenosine is produced (4), beginning with the metabolism of intracellular ATP, ADP, and AMP via the action of cytoplasmic 5′-nucleotidases. Second, it can be generated by *S*-adenosylhomocysteine hydrolase. Finally, adenosine can be generated extracellularly from ATP and ADP via the combined action of ecto-nucleoside triphosphate diphosphohydrolase (ecto-NTPDase-1 [CD39])—or possibly other NTDPases—and ecto-5′-nucleotidase (CD73). Adenosine concentrations are also influenced by adenosine deaminase (ADA), a purine salvage pathway enzyme that degrades adenosine to inosine and adenosine kinase, which phosphorylates adenosine to AMP. The role of adenosine in immune regulation can be appreciated when its concentration is modulated by a deficiency of ADA, CD39, or CD73 expression. Loss of ADA elevates adenosine (5), whereas deletion of CD39 (6) or CD73 (7) results in less extracellular adenosine and impaired control of inflammation. For example, *cd73*$^{-/-}$ mice exhibit defects in kidney function (8), thromboregulation (9), physiologic responses to hypoxia (7), and enhanced responses to some infections (10).

Evidence for the generation of adenosine by Tregs

The first suggestion that adenosine played a role in Treg biology emerged from an in vivo study in mice in which the adoptive transfer of Tregs prevents the induction of colitis by effector Th cells (Teffs) (11). As elaborated below, Tregs failed to control colitis by Teffs that lacked the adenosine A_{2A} receptor (A_{2A}AR), suggesting that adenosine contributed to the anti-inflammatory role conferred by Tregs. Shortly thereafter, murine Tregs were shown to express CD73, which converts 5AMP into adenosine (12). However, CD73 is expressed by most activated Th cells, and its expression is not enriched on Tregs. In two subsequent studies, Tregs were shown to express CD73 and CD39, thus possessing the full synthetic pathway enabling Tregs to synthesize adenosine from ATP (6,13). Importantly, these Tregs have been shown to regulate inflammation in an adenosine-dependent manner (6,13), and translational studies report that CD39 is expressed selectively with CD73 on Tregs from different human tissues (10,13–16).

Additional evidence associating adenosine production with Tregs comes from experiments in which the presence of Foxp3 is positively associated with CD39 expression (13,17). The documented ability of Tregs to convert ATP to adenosine provides direct evidence of the functional capacity of these enzymes (6,13) and supports the notion that adenosine is a mediator of Treg function (Fig. 1).

Tregs can be defined by the differential expression of surface markers, and subsets of Tregs appear to control different diseases (18). This diversity may be linked to the ability to synthesize adenosine, because Tregs that express CD39 preferentially control Th17 responses (19). More direct evidence in support of adenosine as a mediator of Tregs includes studies showing that Tregs from *cd39*$^{-/-}$ mice are less efficient at suppressing proliferation of Teffs from A_{2A}AR-deficient mice (6). However, it is impossible to ascertain from this study whether the lack of CD39 on the Treg or the lack of A_{2A}AR on the Teff targets was the most important factor. It is worth noting that the Teffs lacking the A_{2A}AR, which could not be controlled in the colitis model in vivo, were inhibited in the proliferation inhibition assay (20). These studies suggest that Tregs use different mediators, including adenosine, that have varied effects on Teff function in distinct assays.

Adenosine as an anti-inflammatory mediator in disease models

The digestive tract is ideal for addressing the biologic relevance of adenosine-mediated immune regulation in vivo, because adaptive Tregs control responses to the shifting

repertoire of luminal Ags in this organ. For example, Tregs regulate the magnitude of gastritis after infection with *Helicobacter* spp. (21–23). Consistent with a role for adenosine in the control of inflammation, gastritis was more severe when mice lacking CD73 or the A_{2A}AR were infected with *H. pylori* or *H. felis* (10,24). More direct evidence for adenosine production by Tregs emerged from the adoptive transfer of Tregs from *cd73*$^{-/-}$ mice, which failed to prevent gastritis as efficiently as Tregs from wild-type mice (10). Furthermore, the reduction in inflammation by adenosine in these models favored the persistence of these infections (10,24). CD39^{+} and CD73^{+} Tregs are also found in human gastric mucosa (10), and these responses are associated with the lifelong infection by *H. pylori* (25).

Adenosine production by Tregs also contributes to immune regulation in other regions of the digestive tract. Tregs prevent the wasting and colitis that follows the adoptive transfer of wild-type pathogenic CD4^{+} CD45RB^{hi} Th cells into *scid* mice (20). In this same model, A_{2A}AR agonists alone prevented colitis induced by pathogenic Th cells in the absence of Tregs (11). As mentioned above, wild-type Tregs failed to prevent colitis induced by the adoptive transfer of pathogenic Teffs from *A*$_{2A}$*AR*$^{-/-}$ mice, implicating an anti-inflammatory role for Treg-derived adenosine (11). A_{2A}AR on recipient myeloid cells is also required for optimal control of intestinal inflammation, because the cotransfer of Tregs and pathogenic T cells from wild-type mice into *rag/A*$_{2A}$*AR*$^{-/-}$ double knockout recipients still leads to colitis (C.C. Kurtz and P.B. Ernst, manuscript in preparation). The importance of adenosine in the control of intestinal inflammation is supported by the increased susceptibility of *cd39*$^{-/-}$ mice to colitis induced by dextran sodium sulfate and the association of mutations in the *cd39* gene with Crohn's disease in humans (26).

Based on these studies in the gastrointestinal tract, we propose that adaptive Tregs are important to cope with the complex microbiota and that the production of adenosine by Tregs is significant in this context. However, details of the distribution and expression of the molecules responsible for the synthesis and response to adenosine in the gut remain to be determined.

Mechanisms for adenosine-mediated Treg function

Adenosine binds to four distinct subtypes of adenosine receptors (ARs): A_1, A_{2A}, A_{2B}, and A_3 (1,4). Because of a lack of useful Abs for the assessment of cell surface AR expression, investigators must rely on multiple approaches to confirm the role of a specific AR subtype in each experimental model, including the use of cells or mice deficient in specific ARs and/or appropriate concentrations of highly selective agonists or antagonists. Using genetically engineered mice lacking the A_{2A}AR, adenosine has been implicated in the control of a wide range of inflammatory responses (27). It is now widely appreciated that Th cells express the A_{2A}AR predominantly (10,24,28–30), whereas APCs express the A_{2A}AR and/or A_{2B}AR (31–35). The A_{2A}AR is induced ~8-fold in naive Th cells upon activation (28,29), whereas induction in excess of 100-fold occurs in APCs in response to LPS (36). Because it has been known for many years that ARs can mediate anti-inflammatory effects, their induction upon immunologic provocation suggests that adenosine is a broadly relevant mediator providing a negative feedback to limit immune-mediated tissue damage. As such, ARs are an important target for Treg-derived adenosine.

Adenosine regulates T cell function indirectly by reducing the secretion of proinflammatory cytokines, including IL-12 and TNF-a, from stimulated APCs by as much as 80–90% (33,34,37). In the case of TNF-a, the impairment in expression is due to a decrease in mRNA stability (38). Adenosine also stimulates production of the anti-inflammatory cytokine IL-10 (31–34). In addition, the expression of CD86 and the immunogenicity of dendritic cells are impaired by adenosine acting through the A_{2B}AR (13,31,37,39),

mimicking some mechanisms of immune regulation conferred by Tregs (13,40,41). Thus, the marked induction of ARs after activation provides a feedback mechanism to control the host response to an infection or immunization.

A_{2A}AR signaling on activated Th cells directly inhibits the production of cytokines including IL-2, IL-4, IFN-γ, and TNF-α by as much as 90% (28,42), again by decreasing mRNA stability (11). Adenosine acts directly as an anti-inflammatory mediator, and the administration of adenosine analogs increases the number of Tregs (42). A role for adenosine in the optimal development of Treg responses could explain why Tregs from $A_{2A}AR^{-/-}$ mice failed to control disease caused by the adoptive transfer of wild-type pathogenic Th cells (11). The fact that Tregs produce and respond to adenosine implies that adenosine acts as an autocrine factor to optimize anti-inflammatory responses. This immunosuppressive action of adenosine may be mediated by increasing the concentration of intracellular cAMP. Indeed, Tregs can transfer intracellular cAMP to Teffs via gap junctions (43), and elevating cAMP in Tregs renders them more suppressive (44,45).

The transfer of cAMP to a target Teff would occur only when Tregs have direct contact with the target cell. This requirement for intimate cell–cell contact for the optimal expression and function of Treg mediators is shared by membrane-associated TGF-β1 (46) and IL-35 (47). Furthermore, inhibition of host responses mediated by interactions of LAG3 (48) and CTLA-4 (41) on Tregs with their respective receptors on target cells requires intimate contact (40). Because the $t_{1/2}$ of adenosine is measured in seconds, intimacy within the immunologic synapse shared by APCs, Tregs, and Teffs would be essential for any inhibitory role for adenosine (Fig. 2).

Coupling of ARs to the control of inflammatory responses

The A_{2A}AR and A_{2B}AR are distinguished by their ability to mediate increases in cAMP. The accumulation of cAMP is believed to responsible for the anti-inflammatory effects of adenosine delivered to T cells, APCs, or neutrophils (1,4,27). The A_{2A}ARs are coupled to members of the Gs α sub-family of G proteins, and their activation raises the level of cAMP (1,49) (Fig. 3). Similarly, the activation of phosphodiesterases (PDEs) that degrade cAMP enhances responses of activated Th cells (50,51). PDE activation is mediated in part through CD4. Mutation of class II MHC molecules on APCs so that they no longer bind CD4 prevents PDE activation in Th cells following stimulation and allows cAMP to accumulate (52). In association with this increase in cAMP, Th cells assume a phenotype resembling a Treg rather than an effector Th cell (53). Furthermore, the inflammatory potential of T cells is limited by blocking PDE activity pharmacologically (24,54) or by providing exogenous cAMP analogs (55). These results all indicate the important role for cAMP in the inhibition of immune–inflammatory responses.

The accumulation of cAMP leads to the activation of sensors, such as the cAMP-dependent protein kinase (PKA; Fig. 3). cAMP binds to the regulatory subunit of PKA, leading to the release of the active catalytic subunit and the phosphorylation of PKA substrates (56–58). Using PKA inhibitors such as H-89 suggested that activation of PKA is central to the ability of A_{2A}AR to inhibit the oxidative burst in neutrophils (59).

The cAMP response element binding proteins are some of the substrates for PKA. Members of this leucine zipper family of transcription factors include CREB, the cAMP response element modulator and activating transcription factor 1 (60). Following activation of PKA, the free catalytic subunit enters the nucleus and phosphorylates the kinase inducible domain of CREB on Ser133, which increases its transcriptional activity. These transcription factors play multiple and sometimes paradoxical roles in Th cell responses. For example, the proteins are subject to alternative splicing and a particular variant of the cAMP response

element modulator, termed *inducible cAMP early repressor*, attenuates cytokine induction after TCR stimulation (60). The expression of Foxp3, a major regulator of the development and function of Tregs is also regulated by CREB (61). Thus, the inhibition induced by adenosine from Tregs could be mediated, in part, through these pathways. However, raising the level of cAMP in T cells with forskolin followed by analysis with oligonucleotide arrays showed a significant induction of the genes for IL-2, IL-2Rα, and IFN-γ (62). This finding conflicts with evidence that phosphodiesterase inhibitors shift the T cell phenotype toward Th2 cells (63). The best explanation may be that the earliest stages of T cell activation include a role for cAMP while this reliance diminishes over time subsequent to the induction of PDE. This timing would match the induction of the A_{2A}AR on Th cells during activation and allow adenosine to induce cAMP as a negative feedback mechanism subsequent to the initiation of Th cell responses. However, the full complement of PKA substrates responsible for the effects of adenosine in immune/inflammatory cells are not known and need to be defined in detail.

Originally, cAMP was thought to activate PKA exclusively, but it is now apparent that it regulates other cAMP sensors including the exchange protein directly activated by cAMP (Epac) (58,64). Epac initiates a large number of cAMP-mediated events, including cell proliferation, cell survival, secretion, and Ca^{2+} metabolism (58,64). There are two iso-forms of Epac—Epac1 and Epac2, with Epac1 being more widely expressed and present in T cells (58,64). Epac1 is a guanine nucleotide exchange factor for the small GTP binding protein Rap (60), with major roles in integrin-mediated cell adhesion and the formation of cell–cell junctions (58,64). Epac1 can signal dependently or independently from PKA to inhibit proinflammatory responses, in part through its effects on suppressor of cytokine signaling proteins (SOCS) (65). SOCS1 and SOCS3 both inhibit responses in Th cells and APCs by multiple mechanisms (66). Moreover, adenosine stimulates the accumulation of IL-10 (32,34,37,67), which can act in an autocrine manner to induce SOCS3 (66).

A significant finding is that Epac1 and Rap activate a novel member of the phospholipase family—phospholipase (PLC) C-*ε* (64). PLC-*ε* is widely expressed, contains the requisite domains responsible for the phospholipase activity that hydrolyzes phosphatidylinositol bisphosphate to inositol trisphosphate (IP_3) and diacylglycerol (DAG), and is a CDC25 binding domain with guanine nucleotide exchange factor activity for small GTP-binding proteins in the Ras family (64). Thus, in addition to raising Ca^{2+} levels in cells, PLC-*ε*, Epac, and Rap initiate signaling cross-talk and potential links between cell membrane receptors and signaling pathways regulated by small GTP-binding proteins (64). This pathway of activation resembles that mediated through the Gq α subunit associated with the A_{2B}AR expressed by APCs (see below and Fig. 3). The roles of Epac1, Rap, and PLC-*ε* in the regulation of immune function by all of the immune/inflammatory cells are important subjects for future work.

The A_{2B}AR couples to two different G protein α subunits, the Gs α subunit, and the Gq α subunit (68). In cells where the A_{2B}AR is coupled to the Gs α subunit, activation of Gs increases the activity of adenylate cyclase, raises the level of cAMP, and can potentially activate PKA and Epac1 as well as their downstream targets. Although cAMP is associated with the inhibition of inflammatory responses, it should be remembered that cholera toxin, which stimulates large amounts of cAMP, is also one of the most potent adjuvants ever described (69). Thus, cAMP induction does not always equate with inhibition. Moreover, some proinflammatory cytokines, such as IL-6, are induced through the activation of the A_{2B}AR in dendritic cells (39), possibly by cAMP-sensitive promoters that can be activated by adenosine (70,71). It will be important to identify the targets for cAMP that confer inhibition of host responses.

In settings in which the A_{2B}AR is coupled to the Gq α subunit, it can increase the activity of PLC-β, causing the breakdown of phosphatidylinositol bisphosphate to IP_3 and DAG. This activity briefly elevates the IP_3/Ca^{2+} and DAG signals in the activated cells, thereby activating Ca^{2+}-calmodulin–dependent enzymes, especially the calmodulin-sensitive protein kinases, such as Ca^{2+}-calmodulin kinase II (72) and the serine-threonine kinase, protein kinase C. Because protein kinase C has been implicated in the activation of T cells and APCs (73), it is possible that adenosine signaling through the A_{2B}AR induces potentially proinflammatory effects.

Can there be too much of a good thing?

In some circumstances, adenosine can exacerbate immunopathology as illustrated in the murine model of multiple sclerosis—experimental autoimmune encephalomyelitis, (EAE). Disease in this model depends on the migration into the CNS of pathogenic T cells sensitized by prior immunization with a peptide derived from myelin oligodendrocyte protein. Contrary to expectations, *cd73*$^{-/-}$ mice were found to be highly resistant to EAE (74); this was not caused by a lack of T cell responsiveness to immunization with myelin oligodendrocyte protein, because T cells of *cd73*$^{-/-}$ mice were activated, secreted increased quantities of proinflammatory cytokines, and caused severe disease when adoptively transferred into *tcrα*$^{-/-}$ mice, which are *cd73*$^{+/+}$. These findings strongly suggested that CD73-derived adenosine and perhaps AR signaling were needed for T cell entry into the CNS. This notion was confirmed by the observation that an A_{2A}AR antagonist protected wild-type mice from EAE (74). Thus, this model demonstrates that the anti-inflammatory effects of adenosine can be offset by the effect of adenosine on the access of pathogenic T cells to the CNS.

Adenosine, like other Treg mediators, can provoke disparate responses. Forexample, TGF-β1 acting alone is anti-inflammatory and favors the emergence of adaptive Tregs (75); however, in combination with IL-4 or IL-6, it stimulates Th9 (76) and Th17 (77) cells, respectively. Similarly, the inhibition of IL-12 coupled with the concomitant induction of IL-6 in dendritic cells after exposure to adenosine analogs is sufficient to convert the anti-inflammatory potential of TGF-β1 into a proinflammatory milieu that induces Th17 cells (J.M. Wilson and P.B. Ernst, submitted for publication). We propose that adenosine complements TGF-β1 as a mediator of suppression and an inducer of Th17 cells.

Additional evidence suggests that adenosine exacerbates intestinal inflammation in response to bacteria or chemicals via its effects on the A_{2B}AR (78). However, other investigators have reported the opposite (79). In the lung, adenosine is believed to contribute to inflammation, largely through the A_{2B}AR (80). Other data indicate that adenosine contributes to fibrosis in the liver or lung through the A_{2A}AR (81) or A_{2B}AR (80). The stimulation of fibrosis may be an attempt to “wall off” inflammation, but the widespread fibrotic atrophy of functional tissue is not usually desirable.

Anti-inflammatory mediators from Tregs can also contribute to immunosuppression that limits the clearance of infections (10,82) or tumors (83). For example, the absence of the A_{2A}AR enhances immune reactivity, allowing the regression of tumors in most A_{2A}AR-deficient mice tested, whereas no tumors were resolved in wild-type mice (84). Similarly, knockdown of CD73 expression in ovarian or breast tumors or treatment of mice with a specific CD73 inhibitor or anti-CD73 Ab slowed tumor growth (85–87). Adenosine has also been suggested to exacerbate sepsis (88), although its anti-inflammatory effects complement antibiotic treatment to improve survival in other models (89). Undoubtedly, there are situations in which adenosine production provides a premature anti-inflammatory feedback that competes with the host's ability to mount a sufficient response.

Conclusions

Adenosine is one of several mediators that accumulate in the inflammatory milieu, conferring pleiotropic effects in a paracrine or autocrine manner that may be either beneficial or undesirable. These diverse outcomes reflect patterns of AR expression associated with distinct cellular lineages and differentiation states, as well as linkage to divergent G protein-coupled signaling pathways. Although the outcome of AR signaling is sometimes paradoxical, this inconsistency points to the complexity of biology rather than arguing against a role for adenosine in Treg function. Similar to other mediators (e.g., TGF-β1, IL-10) adenosine is not produced solely by Tregs, nor is it always beneficial. Clearly, a deeper understanding of the effects of adenosine and its interaction with other anti-inflammatory mediators will be required before we are able to translate current findings into novel anti-inflammatory therapies for the treatment of human disease.

Acknowledgments

We thank Dr. Ken Tung and Jeff Wilson (University of Virginia) and Paul Kincade (Oklahoma Medical Research Foundation) for sharing constructive suggestions for the content of this review.

This work was supported by National Institutes of Health Grants AI 070491 and AI 079145 (to P.B.E.) and AI 18220 (to L.F.T.). L.F.T. holds the Putnam City Schools Distinguished Chair in Cancer Research.

Abbreviations used in this paper

A_{2A}AR	adenosine 2A adenosine receptor
A_{2B}AR	adenosine 2B adenosine receptor
ADA	adenosine deaminase
AR	adenosine receptor
DAG	diacylglycerol
ecto-NTPDase-1	ecto-nucleoside triphosphate diphosphohyrdolase
Epac	exchange protein directly activated by cAMP
IP_3	phosphatidylinositol bisphosphate
PDE	phosphodiesterase
PKA	cAMP-dependent protein kinase
PLC	phospholipase C
SOCS	suppressor of cytokine signaling
Teff	effector Th cell
Treg	regulatory T cell

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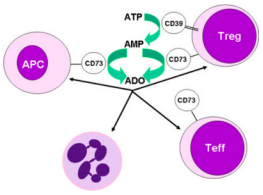

FIGURE 1.

CD39 and CD73 on Tregs generate extracellular adenosine, which can engage ARs on Teffs, APCs, and myeloid cells. This aspect of Treg function would be dependent on the presence of the substrates (local ATP, ADP, and/or AMP), which accumulate as a consequence of inflammation, hypoxia, metabolic stress, and/or cell death.

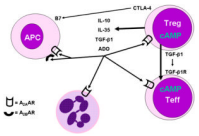

FIGURE 2.
Many molecules associated with Treg (CTLA-4, LAG3, IL-35, TGF-β1, adenosine, and cAMP) require cell–cell contact for their respective actions.

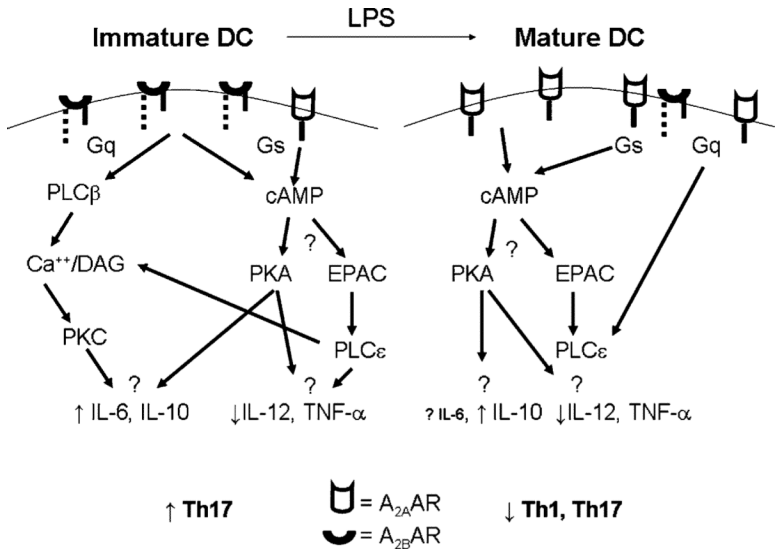

FIGURE 3.

The paracrine action of adenosine regulates the phenotype of adjacent cells, depending on the relative expression of AR subtypes or intracellular signaling molecules. Using APCs as an example, the resting cell expresses A_{2B}AR predominantly, which accounts for the ability of adenosine to induce IL-6 and IL-10 (39) and inhibit IL-12 and TNF-α (37). Because the Gs coupling to the A_{2A} and A_{2B}AR accounts for the increase in cAMP, one might predict that responses mediated selectively through the A_{2B}AR are attributable to Gq-activated pathways. A_{2A}AR are the major receptor on dendritic cells after activation with LPS, (37) and adenosine (e.g., from Tregs) could provide a negative feedback through the A_{2A}AR that impairs dendritic cell function and subsequently, a more general impairment in Th cell activation (35). Although many outcomes of adenosine on cytokine production have been reported, the linkages between specific signaling pathways and these eventual responses in the context of Treg biology remain to be defined more thoroughly.