



Research paper

Adenosine receptor activation in the Th17 autoimmune responses of experimental autoimmune uveitis

Li Xiaohua^a, Dongchun Liang^a, Hui Shao^b, Willi K. Born^c, Henry J. Kaplan^b, Deming Sun^{a,*}^a Doheny Eye Institute and Department of Ophthalmology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90033, USA^b Department of Ophthalmology and Visual Sciences, Kentucky Lions Eye Center, University of Louisville, Louisville, KY 40202, USA^c Department of Biomedical Research, National Jewish Health, Denver, CO 80206, USA

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ABSTRACT

Th17-type autoreactive T cells contribute to pathogenicity in autoimmune diseases, including autoimmune uveitis. However, the mechanisms of regulation of Th17 cell activities remain unsolved and are likely to be tissue- and disease specific. In this review, we have summarized our studies from the murine model of experimental autoimmune uveitis (EAU). The results demonstrate that $\gamma\delta$ T cells have a regulatory effect on Th17 response. The regulatory effects of $\gamma\delta$ T cells depend on their action state. Activated $\gamma\delta$ T cells express significantly high levels of adenosine receptor A2 (A2AR) but low CD73. Both molecules are crucially involved in adenosine generation, thus modifying T cell responses. While the increased expression of A2AR allows activated $\gamma\delta$ T cells to bind adenosine more effectively than other immune cells, the decreased CD73 restricts their ability to convert AMP to adenosine. Adenosine affects Th1 and Th17 autoimmune responses differently. Its activation of $\gamma\delta$ T cells shifts the Th1/Th17 balance towards the Th17 autoreactivity.

1. Extracellular ATP-adenosine metabolism and its effect on inflammation

In addition to its well-known effects of causing tissue injury and immune pathology, inflammation can be a protective response that removes injurious stimuli and initiates the tissue healing process [1]. Extracellular adenosine, a degradation product of adenosine triphosphate (ATP), is a key regulator that controls the inflammatory responses [2]. Under physiological conditions, ATP is predominantly an intracellular molecule. During stress and tissue injury, ATP is released from the intracellular compartment into the extracellular space, where it is degraded to adenosine through a cascade of enzymatic reactions catalyzed by ectonucleotidases including CD39 (nucleoside triphosphate diphosphohydrolase-1) and CD73 (5'-ectonucleotidase) [3–5]. Binding of adenosine to its receptors and the downstream signaling pathways modulate various pathophysiological processes including the immune responses [6–10].

Cells of the immune system, such as neutrophils, mast cells, endothelial cells, regulatory T cells and platelets, are all rich sources of extracellular adenosine [11]. Adenosine has strong effects on the functions of the immune cells [9]. Extracellular adenosine behaves as

an 'alarm' or danger signal. It activates adenosine receptors (AR), which are G protein-coupled receptors that generate various cellular responses aimed at restoring tissue homeostasis [9,12]. The discovery of the regulatory effect of adenosine and its receptors on inflammation and immune responses has led to attempts of treating immune dysfunctions by targeting AR-mediated signaling pathways [8,9,13,14]. Successful application of such treatment, however, has been hindered by an incomplete understanding of the variable purinergic signaling events that occur under different pathophysiological conditions [9,15].

Adenosine has four well-characterized receptors - A1R, A2AR, A2BR, and A3R [16,17]. The A2AR is typically associated with anti-inflammatory effects [18], whereas the A1-, A2B-, and A3-receptors are mainly implicated in the pro-inflammatory effects of adenosine [19,20]. T cells, including $\gamma\delta$ T cells [21], predominantly express A2AR [22–26]; whereas macrophages and neutrophils can express all four adenosine receptors, depending on their activation and differentiation states [27–31]. A1-, A2A- and A3-receptors are high-affinity receptors that can be activated by physiological concentrations of adenosine in the sub-micromolar range. The A2B-receptors are activated only by micromolar levels of adenosine. Balanced production and clearance of extracellular adenosine play a role in turning off the local inflammatory

Abbreviations: ADA, adenosine deaminase; AR, adenosine receptor; ATP, adenosine triphosphate; A2AR, adenosine 2A receptor; APCP, α,β -methylene ADP; EAU, experimental autoimmune uveitis; eATP, extra-cellular ATP; IRBP, interphotoreceptor retinoid-binding protein; NECA, 5'-N-ethylcarboxamidoadenosine

* Corresponding author.

E-mail address: Dsun@doheny.org (D. Sun).

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response, and thus protect against cellular damage [32]. On the other hand, abnormally high adenosine generation or deficiency in its clearance, as observed in CD73-deficient mice, exacerbates inflammation-associated pathology [33–38].

2. The roles of $\gamma\delta$ T cells in T cell mediated autoimmune uveitis

Over the past three decades, circumstantial evidence has supported the notion that a major subset of pathogenic T cells in autoimmune diseases produces IFN- γ and IL-2, and belong to the class of Th1-type of CD4⁺ T cells [39–46]. Meanwhile, a number of other studies collectively show that a major subset of pathogenic T cells produce interleukin (IL)-17, IL-21 and IL-22, and thus are designated as Th17 cells [47–49]. Both Th1 and Th17 autoreactive T cells have been found to be pathogenic in autoimmune uveitis in patients [48] and in animal models [47,49–51]. However, regulation of Th1 and Th17 auto-reactivities in uveitis remains largely unknown.

We have studied the role of $\gamma\delta$ T cells in mouse models of experimental autoimmune uveitis (EAU). In the induction of EAU by immunization with human interphotoreceptor retinoid-binding protein (IRBP) peptide 1–20 in complete Freund's adjuvant (CFA) [52,53], we have observed that this type of immunization procedure primarily activates V γ 4⁺ $\gamma\delta$ T cells whereas V γ 1⁺ $\gamma\delta$ T cells remain mostly unchanged [53].

Using TCR- $\delta^{-/-}$ mice to determine the effects of $\gamma\delta$ T cells on the overall T cell response in EAU, we have observed that IRBP-induced T cells from IRBP-immunized TCR- $\delta^{-/-}$ mice produce significantly lower amounts of IL-17 than did wild-type (wt) mice and have significantly decreased EAU-inducing ability. However, reconstitution of the TCR- $\delta^{-/-}$ mice with a small number of $\gamma\delta$ T cells that were isolated from IRBP-immunized wt-mice before immunization restored the disease-inducing capability of their IRBP-specific T cells and greatly enhance the generation of Th17 cells in the recipient mice [52]. In addition, the intensity of the $\alpha\beta$ TCR⁺IL-17⁺, but not the $\alpha\beta$ TCR⁺IFN- γ ⁺, IRBP-specific T-cell responses are correlated to the abundance and activation state of $\gamma\delta$ T cells in the responder T cells [54,55], indicating that $\gamma\delta$ T cells are important in the generation and activation of IL-17-producing autoreactive T cells and play a major role in the pathogenesis of autoimmune uveitis. Further investigations of the cellular and molecular events associated with $\gamma\delta$ T activation and regulation on Th17 cells have revealed that the functions of the $\gamma\delta$ T cells and the extracellular adenosine metabolism are intimately linked, and that the net effect determines susceptibility to this autoimmune disease [56–61].

3. Adenosine shapes the regulatory function of $\gamma\delta$ T cells in enhancing the Th17-reactivity but inhibiting the Th1-type T cell response

$\gamma\delta$ T cells are readily activated by a number of proinflammatory cytokines. For example, a mixture of IL-1, IL-7, and IL-23 has a strong stimulatory effect on $\gamma\delta$ T cells [62]. When adenosine was added to the cytokine mixture, $\gamma\delta$ T cell-activation is significantly enhanced, even though adenosine does not activate $\gamma\delta$ T cells by itself [56]. Moreover, when the A2ARs on $\gamma\delta$ T cells are blocked by an A2AR antagonist, cytokine-induced $\gamma\delta$ T cell activation is greatly diminished [56]. The conclusion that $\gamma\delta$ T activation and regulation on Th17 cells are tightly linked with the extracellular adenosine metabolism is derived from following experimental results [57,61]. The mice treated with a non-selective AR agonist, 5'-N-ethylcarboxamidoadenosine (NECA), at an early stage after immunization have an inhibitory effect on both Th1 and Th17 responses, whereas injection of the same amount of NECA at a late stage (at 8–14 d post immunization) prior to the disease onset inhibits the Th1 response but enhances the Th17 response. The enhancing effect of NECA on Th17 responses is modulated by $\gamma\delta$ T cells, and that the response of $\gamma\delta$ T cells to NECA is determined by their activation status [57]. The similar results are also seen in the mice treated with adenosine deaminase (ADA), an enzyme that converts adenosine into

functionally inactive molecules. ADA treatment at 8–14 d post-immunization, shortly before EAU expression suppresses EAU. In contrast, ADA inhibitor (ENHA) treatment significantly increased EAU clinical score, serum IL-17 levels and a percentage of IL-17⁺ $\alpha\beta$ T cells, with little difference in the percentage of IFN- γ ⁺ T cells. The effect that ADA preferentially inhibited Th17 responses effect is also [57,61] $\gamma\delta$ T cell dependent [61]. The underlying molecular mechanisms that $\gamma\delta$ T cell activation is closely associated with augmented Th17 responses [55,60,62], and adenosine enhances the Th17 responses but suppresses Th1 responses [32,60,63–67] are unknown. Based on the limited literature and our own previous findings, we assume that the stimulatory effects of adenosine on Th17 but not on Th1 responses might be due to various AR receptor expression, their adenosine-binding affinities and/or downstream signaling pathways present on these two types of T cells.

4. Expression of functional CD73 molecules is crucial for inhibitory effect of $\gamma\delta$ T cell

CD73 is a glycosyl phosphatidylinositol-linked membrane protein that is pivotal in the conversion of immunostimulatory ATP into immunosuppressive adenosine [68,69]. CD39 and CD73 sequentially degrade ATP, ADP, and AMP to adenosine. CD39 converts ATP and ADP to AMP, and CD73 is the main enzyme responsible for the conversion of AMP into adenosine [68–72]. They have been viewed as immunological switches that shift ATP-driven proinflammatory activity toward an anti-inflammatory state mediated by adenosine [73,74,69,75]. AMP is one of the metabolites of ATP. Unexpectedly, AMP is unable to inhibit the proliferation of purified $\alpha\beta$ T cells, even though these cells express CD73. Only after a small percentage (2–5%) of $\gamma\delta$ T cells are added to the $\alpha\beta$ T cells, AMP can exert its inhibition on $\alpha\beta$ T cells. Such inhibitory effect was prevented by the CD73 inhibitor adenosine 5'-(α,β -methylene) diphosphate (APCP) [56]. The expression levels of CD73 on $\alpha\beta$ and $\gamma\delta$ T cells are comparable. These findings suggest that the CD73 molecules on $\gamma\delta$ T cells are much more potent in converting the non-suppressive AMP into suppressive adenosine. Indeed, when compared among all CD73⁺ immune cell-types, the $\gamma\delta$ T cells are found to be the most effective in converting AMP to adenosine, and that the CD73⁺ $\alpha\beta$ T cells are the least effective [60]. Furthermore, activation status of the $\alpha\beta$ T cells has limited influence on either the expression level of CD73 or its bio-activity of converting AMP to adenosine [57]. In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells express different levels of CD73 before and after their activation [60], and the level of CD73 expression correlated with the pro- and anti-inflammatory activities of $\gamma\delta$ T cells in the Th17 autoimmune responses of EAU [60]. In the mouse EAU model, clinical signs of eye inflammation usually develop at 18–20 days post-immunization. A kinetic study performed on B6 mice immunized with IRBP1-20 shows that at days 7–18 post-immunization, splenic $\gamma\delta$ T cells express significantly decreased levels of CD73 compared to $\gamma\delta$ T cells from naive mice. Almost 80% of splenic $\gamma\delta$ T cells expressed CD73 before immunization; this percentage dropped to 19% on day 13 and 17% by day 18 post-immunization. At day 23, after EAU is developed, CD73 expression returned to its baseline level. Parallel examination shows that CD73 expression on $\alpha\beta$ T cells remains unchanged throughout the test period. Reduced CD73 expression of activated $\gamma\delta$ T cell decreases their ability to convert AMP to adenosine, which would otherwise dampen immune/inflammatory responses of nearby effector T cells [76,77]. Consistently, injection of CD73^{+/+}, but not CD73^{-/-}, $\gamma\delta$ T cells before immunization of TCR- $\delta^{-/-}$ and TCR- δ /IFN- γ double-knockout recipient mice with the uveitogenic peptide IRBP1-20 developed less severe EAU [56,60]. The potential molecular mechanism of CD73 down regulation before EAU is unknown. Several candidates such as IFN- γ and hypoxia-inducible factor-1 have been reported to regulate CD73 expression on endothelium [78–80]. Since the regulation of CD73 expression and function is cell type-specific, the factors responsible for the regulation of CD73 on naive and activated $\gamma\delta$ T cells need to be identified.

5. High level-expression of A2AR by activated $\gamma\delta$ T cells promotes their enhancing activity

The importance of the A2AR in mediating immunoregulation has been demonstrated in A2AR-deficient (A2AR^{-/-}) mice, which are unable to control inflammation, leading to fatal tissue destruction [81,82]. Previous studies in mouse and human subjects using adenosine receptor subtype-selective agonists and antagonists have indicated that adenosine inhibits T-cell activation and effector activity, primarily via the A2AR. Indeed, A2ARs have been extensively investigated as targets for anti-inflammatory therapies to treat patients with asthma, arthritis and other acute and chronic inflammatory diseases [83]. Activation of A2AR on T cells inhibits specific and non-specific T-cell-mediated cytotoxicity, cytokine production [84] and T-cell proliferation [3,85] and promotes T-cell anergy [86]. Engagement of A2AR inhibits IL-12 production but increases IL-10 production by human monocytes [87] and dendritic cells (DC) [31]. A2AR activation also suppresses the production of both IFN- γ and IL-4 by naive CD4⁺ T cells [24,63]. In addition, A2AR-activation on regulatory T cells promotes their expansion, which in turn increases immune regulatory activities [88]. Interestingly, regulatory T cells can exert their suppressive action through the production of adenosine [3,4,89]. By signaling through the A2AR, adenosine also inhibits neutrophils [90] macrophages [91], lymphocytes [24,25] and platelets [92]. Conversely, several studies determined a pro-inflammatory role for A2BR in mouse models of chronic pulmonary inflammation and colitis [93–95] and this effect might depend on cell types and diseases. The anti-inflammatory effects of A2A R engagement by adenosine are mediated by increases in intracellular cAMP levels; the ability of the low affinity A2BR to mediate pro-inflammatory pathways may be attributed to their action as a Gq- protein coupled receptor [95].

We found that the stronger promotion of EAU development by activated $\gamma\delta$ T cells [55,56,60,62] is associated with expression of greatly increased amounts of A2ARs [56,60]. Real-time PCR showed that cytokine-activated $\gamma\delta$ T cells increase their expression of A2AR more than 1000x, in contrast to a merely 10x increase of the A2BR as compared to naive $\gamma\delta$ T cells [56]. Adenosine-binding tests revealed a hierarchical order of adenosine capture by immune cells with $\gamma\delta$ T cells \gg $\alpha\beta$ T cells \gg DCs. Importantly, the $\gamma\delta$ T cells showed much greater adenosine-binding than DCs or $\alpha\beta$ T cells after their activation [56]. In the inflamed tissue, this difference may lead to preferential binding of adenosine to $\gamma\delta$ T cells, thereby restricting the availability of adenosine to $\alpha\beta$ T cells and diminishing the suppressive effect of adenosine on $\alpha\beta$ T cells. In other words, activated $\gamma\delta$ T cells may function as a “sink” for adenosine. In contrast to the responses of $\alpha\beta$ T cells, the binding of adenosine to $\gamma\delta$ T cells enhances their activation [56]. Because activated $\gamma\delta$ T cells enhance the Th17 response [52,55,60,62], binding of adenosine to $\gamma\delta$ T cells promotes the Th17 reactivity [56].

To further test whether increased adenosine-binding activity of $\gamma\delta$ T cells accounts for the observed enhancing effect by removal of adenosine binding to responder $\alpha\beta$ T cells, we performed a competitive adenosine-binding test using radioisotope-labeled adenosine. NECA and the A2AR-specific agonist CGS21680 significantly amplified IL-17 production from A2AR^{+/+} $\gamma\delta$ T cells stimulated by cytokine mixture. No such synergistic effect could be found in the cytokine response of A2AR^{-/-} $\gamma\delta$ T cells. Moreover, when A2ARs were blocked by an A2AR antagonist, cytokine-induced CD25 expression on $\gamma\delta$ T cells was decreased, indicating that A2ARs on $\gamma\delta$ T cells are crucially involved in $\gamma\delta$ T cell activation. Thus, expression of a high density of A2AR is essential for $\gamma\delta$ T cells to exert their autoimmune-enhancing effect; and an augmented enhancement of autoimmune responses by $\gamma\delta$ T cells relies on expression of higher levels of A2ARs by $\gamma\delta$ T cells, which allows them to compete with the binding of adenosine by other immune cells. The results demonstrated that activated $\gamma\delta$ T cells express A2ARs at significantly higher levels, and that A2AR is a major functional molecule in the Th17-enhancing activity of $\gamma\delta$ T cells [96]. Our studies

revealed the complexity of the adenosine on activated $\gamma\delta$ T cells, and on Th1 and Th17 cells, which might be determined by multiple factors. One of them might be altered AR expression on these cells. ARs are coupled to inhibitory-or stimulatory-G-proteins (Gi/o or Gs). G-proteins are important signal transducers that can modulate for instance cAMP levels in the cell, interfering with many downstream pathways and respective cellular processes such as T cell proliferation, apoptosis and cytokine production.

6. Concluding marks

The regulatory effect of $\gamma\delta$ T cells on adaptive immunity has been observed [90–93]; but how these cells regulate the immune response is poorly understood, and it remains largely obscure how $\gamma\delta$ T cells enhance an immune response in some cases but inhibit it in others.

Adenosine has been recognized as a regulator of inflammatory responses. Although both anti- and pro-inflammatory effects of adenosine were observed, the cellular and molecular mechanisms by which adenosine regulates remained unclear. Using a well-established mouse model of autoimmune uveitis, we show that adenosine may either enhance or inhibit EAU pathogenesis, depending on which types of immune responses prevail. Our studies demonstrate that downregulated expression of CD73 renders activated $\gamma\delta$ T cells less capable of suppressing immune responses, leading to augmented immune responses. Activated $\gamma\delta$ T cells also express high levels of A2ARs, which are crucially important in their immune-enhancing role. Depending on their activation state, $\gamma\delta$ T cells exerts various immune modulatory functions in EAU [52,54,62,97].

Our observations present a good opportunity to develop novel pharmacological approaches to modulate CD73 and ARs for the treatment of patients with autoimmune diseases. However, there are still a long way to go, future studies aiming at the relative contribution of CD73 and ARs and pro- or anti-inflammatory adenosine signaling in specific immune cells in response to inflammatory insults are important and necessary.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2018.09.004>.

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