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TLR ligand ligation switches adenosine receptor usage of BMDCs leading to augmented Th17 responses in experimental autoimmune uveitis

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ABSTRACT

The extracellular level of adenosine increases greatly during inflammation, which modulates immune responses. We have previously reported that adenosine enhances Th17 responses while it suppresses Th1 responses. This study examined whether response of DC to adenosine contributes to the biased effect of adenosine and determined whether adenosine and TLR ligands have counteractive or synergistic effects on DC function. Our results show that adenosine is actively involved in both in vitro and in vivo activation of pathogenic T cells by DCs; however, under adenosine effect DCs' capability of promoting Th1 versus Th17 responses are dissociated. Moreover, activation of A2ARs on DCs inhibits Th1 responses whereas activation of A2BRs on DC enhances Th17 responses. An intriguing observation was that TLR engagement switches the adenosine receptor from A2ARs to A2BRs usage of bone marrow-derived dendritic cells (BMDCs) and adenosine binding to BMDCs via A2BR converts adenosine's anti-to proinflammatory effect. The dual effects of adenosine and TLR ligand on BMDCs synergistically enhances the Th17 responses whereas the dual effect on Th1 responses is antagonistic. The results imply that Th17 responses will gain dominance when inflammatory environment accumulates both TLR ligands and adenosine and the underlying mechanisms include that TLR ligand exposure has a unique effect switching adenosine receptor usage of DCs from A2ARs to A2BRs, via which Th17 responses are promoted. Our observation should improve our understanding on the balance of Th1 and Th17 responses in the pathogenesis of autoimmune and other related diseases.

1. Introduction

Adenosine is produced in high concentrations at sites of injured tissues (Fredholm et al., 2001a; Haskó et al., 2008; Idzko et al., 2014; Linden, 2001). Studies have shown that adenosine play a critical role in the pathophysiological changes of disease, particularly inflammatory diseases (Fredholm et al., 2011; Haskó et al., 2008; Jacobson and Gao, 2006; Sauer et al., 2012). ATP leaked into extracellular compartment and acts like an endogenously generated TLR ligand and augments immune responses during inflammation (Beigi et al., 2003; Canaday et al., 2002; Hanley et al., 2004; Wilkin et al., 2001), the ATP metabolite adenosine is profoundly anti-inflammatory (Antonioli et al., 2013; Eltzschig and Carmeliet, 2011; Haskó et al., 2009; Naganuma et al., 2006; Ohta and Sitkovsky, 2001; Zarek et al., 2008). The discovery of the effect of adenosine on inflammation and immune responses has led to attempts to treat immune dysfunctions by targeting adenosine receptor (AR) signaling in treating cancer and neurological diseases (Cronstein et al., 1991; Jacobson and Gao, 2006; Ramlackhansingh et al., 2011).

The complex of adenosine effects under varying immune status has also be recognized. It was observed that adenosine was anti-inflammatory during the early onset of lung inflammation but turned to be proinflammatory in the chronic phases of the inflammation (Liang et al., 2014a; Zhou et al., 2009, 2011). We have studied the effect of adenosine deaminase (ADA) — an enzyme that degrades adenosine, in pathogenesis of autoimmune uveitis (EAU), our results showed that administration to EAU-inducing mice with ADA ameliorated EAU development (Liang et al., 2016b); however, such protective effect is timing restricted — ADA was only protective when administered at ongoing phase(s) of EAU but was ineffective if administered in quiescent disease stages (Liang et al., 2016b). Because of the finding that may immune cells are capable of release adenosine in inflammation and

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adenosine is one of the key regulators of DC function (Panther et al., 2001, 2003), we wished to determine the mechanisms by which adenosine exerts either anti- or pro-inflammatory effect and determine whether the "timing" effect of adenosine involved DC functional change.

Current scenario for understanding pro- and anti-inflammatory effect of adenosine including that myeloid cells express all four adenosine receptors (Haskó and Cronstein, 2004); ligation of the high affinity A2ARs is anti-inflammatory (Linden, 2001; Zarek et al., 2008), whereas the activation of the low affinity A2BRs often times caused pro-inflammatory effect (Kolachala et al., 2005, 2008b; Mustafa et al., 2007; Wei et al., 2013; Zhou et al., 2010). Such a prediction is supported by the observation that the low affinity A2BRs remain silent under normal physiological conditions, conceivably due to that A2BRs will only be activated by μM of adenosine, whereas in early phase of inflammation the adenosine levels remain in nM levels of adenosine (Fredholm et al., 2001b).

In this study, we examined the effect of adenosine on BMDC for the Th1 and Th17 pathogenic T cell responses in EAU-induced animals and before or after BMDCs were exposed to TLR ligand. Our results show that TLR engagement switches the adenosine receptor usage of BMDCs. BMDCs preferentially bind adenosine via A2ARs; however, the A2BRs become the dominant adenosine-binding receptors after BMDCs are exposed to TLR ligand. Ligation of A2ARs and A2BRs has fundamentally distinct effect on DC function, activation of A2BRs augmented Th17-promoting effect of the DCs, whereas activation of A2ARs inhibited DCs' AP function. The observation should improve or understanding on the balance of two types of autoimmune responses – the Th1 and Th17 responses, in the pathogenesis of autoimmune and other related diseases.

2. Materials and methods

2.1. Animals and reagents

All animal studies conformed to the Association for Research in Vision and Ophthalmology statement on the use of animals in Ophthalmic and Vision Research. Institutional approval by Institutional Animal Care and Use Committee (IACUC) of Doheny Eye Institute, University of California Los Angeles was obtained, and institutional guidelines regarding animal experimentation were followed.

Approximately four to five female mice per group were used in this study and 8- to 16-week-old mice were used in all studies. C57BL/6 (B6) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). $A2AR^{-/-}$ mice (Chen et al., 1999) were a gift from Dr. Jiang-Fan Chen (Boston University School of Medicine, Boston, MA, USA). They were housed and maintained in the animal facilities of the University of California Los Angeles. All protocols in this study were approved by the Committee on the Ethics of Animal Experiments of University of California, Los Angeles (IACUC permit number: ARC#2014-029-03A), in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Recombinant murine IL-1β, IL-7, and IL-23 were purchased from R & D Systems (Minneapolis, MN, USA). Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or allophycocyanin-conjugated antibodies (Abs) against mouse CD3, CD4, αβ T cell receptor (TCR), CD11c, or γδ TCR (GL3) and their isotype control antibodies were purchased from Biolegend (San Diego, CA, USA). PE -conjugated anti-mouse IFN-γ (XMG1.2), IL-17 (TC11-18H10.1), and A2AR monoclonal antibody were was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). PE-conjugated A2BR antibody was purchased from Novus Biological). The non-selective AR agonist 5′-N-ethylcarboxamidoadenosine (NECA) (Mahamed et al., 2015), selective A2AR agonist 2-p-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS21680), selective A1R agonist (CCPA), A2BR agonist BAY60–6538, A2AR antagonist (SCH 58261), and erythro-9-(2-hydroxy-3-nonyl) (EHNA, an inhibitor of adenosine deaminase [ADA]) were purchased from Sigma-Aldrich and were dissolved as a 1 mM stock solution in DMSO and diluted 1/10000 in culture medium before use. Toll-like receptor ligands lipopolysaccharide (LPS) and Pam3csk4 (Pam3) and ADA polyclonal antibody were purchased from Invivogen (San Diego, CA, USA). ADA was a gift from Sigma-Tau Leadiant Biosciences (Gaithersburg, MD).

2.2. Immunization and EAU induction and treatment of immunized mice with ADA

EAU was induced in B6 mice by subcutaneous injection of 200 μl of emulsion containing 200 μg of human interphotoreceptor retinoid-binding protein $(IRBP)_{1\text{-}20}$ (Sigma-Aldrich) in complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA) at six spots at the tail base and on the flank and intraperitoneal injection with 300 ng of pertussis toxin, then randomly divided into two groups (n = 6), one of which received an i.p. injection of ADA (5U/mouse) at day 8 post-immunization and the other received vehicle. They were then examined three times a week until the end of the experiment (day 30 post-immunization). To examine mice for clinical signs of EAU by indirect fundoscopy, the pupils were dilated using 0.5% tropicamide and 1.25% phenylephrine hydrochloride ophthalmic solutions. Fundoscopic grading of disease was performed using the scoring system described previously (Thurau et al., 1997). For histology, whole eyes were collected at the end of the experiment and prepared for histopathological evaluation. The eyes were immersed for 1 h in 4% phosphate-buffered glutaraldehyde, then transferred to 10% phosphate-buffered formaldehyde until processed. Fixed and dehydrated tissues were embedded in methacrylate, and 5 μm sections were cut through the pupillary-optic nerve plane and stained with hematoxylin and eosin.

2.3. T cell preparations

αβ T cells were purified from B6 mice immunized with the human $IRBP_{1\text{-}20}$ peptide, as described previously (Cui et al., 2009; Liang et al., 2013; Nian et al., 2010), while γδ T cells were purified from immunized and control (naïve) B6 mice. Nylon wool-enriched splenic T cells from naive or immunized mice were incubated sequentially for 10 min at 4 °C with FITC-conjugated anti-mouse γδ TCR or αβ TCR Abs and 15 min at 4 °C with anti-FITC Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), then the cells were separated into bound and non-bound fractions on an autoMACS™ separator column (Miltenyi Biotec GmbH). The purity of the isolated cells, determined by flow cytometric analysis using PE-conjugated Abs against αβ or γδ T cells, was >95%. Resting γδ T cells were prepared either by isolation from naïve mice or by incubating activated γδ T cells in cytokine-free medium for 5–7 days, at which time they show down-regulation of CD69 expression (Liang et al., 2013). Highly activated γδ T cells were prepared by incubating resting γδ T cells for 2 days with Abs against the γδ TCR (GL3) and CD28 (both 2 μg/ml, both from Bio-Legend, San Diego, CA), or cytokines combination (IL-1, IL-7 and IL-23).

2.4. Assessment of Th1 and Th17 polarized responses

Responder $CD3^+$ T cells (3×10^6) prepared from $IRBP_{1\text{-}20}$-immunized B6 mice were co-cultured for 48 h with $IRBP_{1\text{-}20}$ (10 μg/ml) and irradiated spleen cells (2×10^6/well) as antigen-presenting cells (APCs) in a 12-well plate under either Th17 polarized conditions (culture medium supplemented with 10 ng/ml of IL-23) or Th1 polarized conditions (culture medium supplemented with 10 ng/ml of IL-12). The Responder αβ T cells were collected from $IRBP_{1\text{-}20}$-immunized B6 mice, on day 13 post-immunization. To obtain a sufficient number of cells, we routinely pool the cells obtained from all six mice in the same group, before the T cells are further enriched using MACS column. Forty-eight hours after stimulation, IL-17 and IFN-γ levels in the culture medium were then measured using ELISA kits (R & D Systems) and the percentage of IFN-γ^+ and IL-17^+ T cells among the responder T cells was determined by

intracellular staining after 5 days of culture followed by FACS analysis, as described below (Liang et al., 2013; Peng et al., 2007a).

2.5. Generation of bone marrow dendritic cells (BMDCs)

Freshly isolated bone marrow cells from femur and tibia of B6 or immunized mice were cultured in RPMIs medium supplemented with 100 μg/mL penicillin and streptomycin mixture, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 10% heat-inactivated filtered fetal calf serum, and 10 ng/ml of recombinant murine GM-CSF and IL-4 (10 ng/ml) (R&D Systems) for 5 days at 37 °C in a 5% CO_2 incubator, as described previously (Inaba et al., 1992).

2.6. Cytokine assays by ELISA

Cytokine (IL-1, IL-6, L-12 and IL-23) levels in the culture medium were measured by ELISA. Purified αβ T cells (3×10^4 cells/well; 200 μl) from the draining lymph nodes and spleens of $IRBP_{1\text{-}20}$-immunized B6 mice were cultured in complete medium at 37 °C for 48 h in 96-well microtiter plates with irradiated syngeneic spleen APCs (1×10^5) in the presence of 10 μg/ml of $IRBP_{1\text{-}20}$, then a fraction of the culture supernatant was assayed for IL-17 and IFN-γ using ELISA kits (R & D).

2.7. Immunocytochemical analyses

For immunofluorescence analyses, the cultured BMDCs were washed, fixed with 4% paraformaldehyde, and pre-incubated in 5% BSA and 0.1% Triton X-100 for 1 h at room temperature (RT). Cells were further incubated with the combination of PE-conjugated A2BR or A2AR and FITC-conjugated CD11c antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C and then mounted using Antifade reagent with 4′,6-diamino-2-phenylindole (Molecular Probes, Eugene, OR) followed by washing with phosphate-buffered saline (PBS). Negative, non-specific labeling was established with normal IgG isotypes. Cells were analyzed with Zeiss LSM 710NLO confocal microscope.

2.8. Adenosine assay

Adenosine in the medium of cultured cells was measured by an Adenosine Assay Kit (Fluorometric).from Biovision (CA). Breifly, 25 μl of cultured cell supernatant were mixed with assay buffer, adenosine convertor, adenosine detector, adenosine developer and adenosine probe from the kits to compose a100 μl reaction system. Kept in room temperature for 15 min and protected from the light. Fluorescence was read in a SpectraMax iD5 multi-mode microplate reader (Molecular Devices, LLC. USA) at Ex/Em = 535/587 nm.

2.9. Adenosine binding assay

BMDCs seeded in 96-well cell culture plates at a density of 1×10^5/ml in 500 μl of complete medium were incubated for 1 h with H^3-adenosine at final concentrations of 0–12,000 nM in triplicate, then cell-bound and free H^3-adenosine were separated by harvesting the cells on a cell harvester (PerkinElmer) and the cell-associated radioactivity measured by liquid scintillation. Scatchard plot analysis was then performed and the dissociation constant and maximum binding capacity calculated.

2.10. Statistical analysis

The results in the figures are representative of one experiment, which was repeated 3–5 times. The statistical significance of differences between groups in a single experimental was initially analyzed by used 2-way Students t-tests, and if statistical significance was detected the Student–Newman–Keuls post-hoc test was subsequently used. P values less than 0.05 was considered a statistically significant difference and marked with one *; when $P < 0.01$, two ** were used.

3. Results

3.1. Adenosine is critically involved in activation of autoreactive T cells

To determine the role of adenosine in T cell responses, we have assessed adenosine concentrations in the supernatants of cultured T cells and BMDCs. Cultured BMDCs were cultured in medium with or without LPS (100 ng/ml) and in the absence or presence with AMP (1 mM). Approximately 100 nM adenosine can be detected in the supernatants of LPS-treated BMDCs and in the presence of AMP, the adenosine levels increasing significantly (Fig. 1A), suggesting that LPS-treated BMDCs acquired increased ability to convert AMP to adenosine (Ko et al., 2020). High adenosine levels were also found in the culture supernatants of γδ T cells, which was also significantly higher in the supernatants of γδ T cells activated by anti-CD3 antibodies.

Adenosine is degraded by the enzyme designated as adenosine deaminase (ADA) which is expressed by immune cells and DCs have the strongest activity (Desrosiers et al., 2007; Mandapathil et al., 2010). To determine whether treatment of BMDCs with a reversible inhibitor of ADA (EHNA) [erythro-9-(2-hydroxy-3-nonyl)] [50, 51], which inhibits ADA activity and thus favor sustained accumulation of adenosine, will affect T cell responses, the antigen presenting (AP) function of BMDCs were assessed, in which responder T cells were co-cultured with the EHNA (10 μM)-treated or untreated BMDCs in the presence of immunizing antigen, at ratio of DC:T = 1:10 and the cytokine production of responder T cells were examined. The results show that exposure of BMDCs to EHNA significantly augmented Th17 responses whereas the Th1 responses were inhibited (Fig. 1B).

To further determined whether the in vivo DC function is also regulated by adenosine, the AP function of splenic DCs of mice, with or without a prior administration of ADA (Liang et al., 2016b) were compared. B6 mice immunized with $IRBP_{1\text{-}20}$ received a single injection of ADA on day 8 post-immunization (Liang et al., 2016b). Splenic $CD11c^+$ DCs cells were separated using auto-MACS column and the responder T cells were isolated from immunized B6 mice. After stimulation of responder T cells with splenic DCs under either Th17- or Th1-polarized conditions, the IL-17 and IFN-γ levels in the culture medium were measured by ELISA. The results show that DCs isolated from ADA administered immunized mice stimulated significantly decreased amounts of IL-17 production from the responder T cells whereas the IFN-γ production was not significantly affected (Fig. 1C).

3.2. BMDCs acquired increased sensitivity to adenosine after exposed to TLR ligands

We have examined the adenosine effect of on BMDC function, before and after BMDCs were treated with LPS (100 ng/ml). The result showed that before LPS treatment, adenosine or EHNA has little effect on BMDCs' AP function; after being treated with LPS BMDCs acquired increased stimulating effect on both Th1- and Th17 responses. More importantly, adenosine and/or EHNA significantly affected the AP function for both Th17 (Fig. 2A) and Th1 (Fig. 2B) responses of LPS-treated, but not LPS-untreated, BMDCs. Meanwhile, dual treated with adenosine and EHNA showed a synergistic effect in both inhibition of Th1 responses and enhancing Th17 responses, suggesting that adenosine effect on Th1 and Th17 responses is dissociated. The synergistic effect between adenosine and EHNA (Fig. 2A&B) suggests that both adenosines produced by DCs and γδ T cells and ADA expressed on DCs contributes to adenosine accumulation in the responding cultures. Comparison of IL-23 producing ability, the cytokine critically involved in Th17 responses, showed that treatment with adenosine analogue (NECA, 100 nM) and/or EHNA augmented IL-23 production of LPS-treated, but not untreated BMDCs (Fig. 2C), indicating that BMDCs acquired increased sensitivity to adenosine after exposed to TLR ligands.

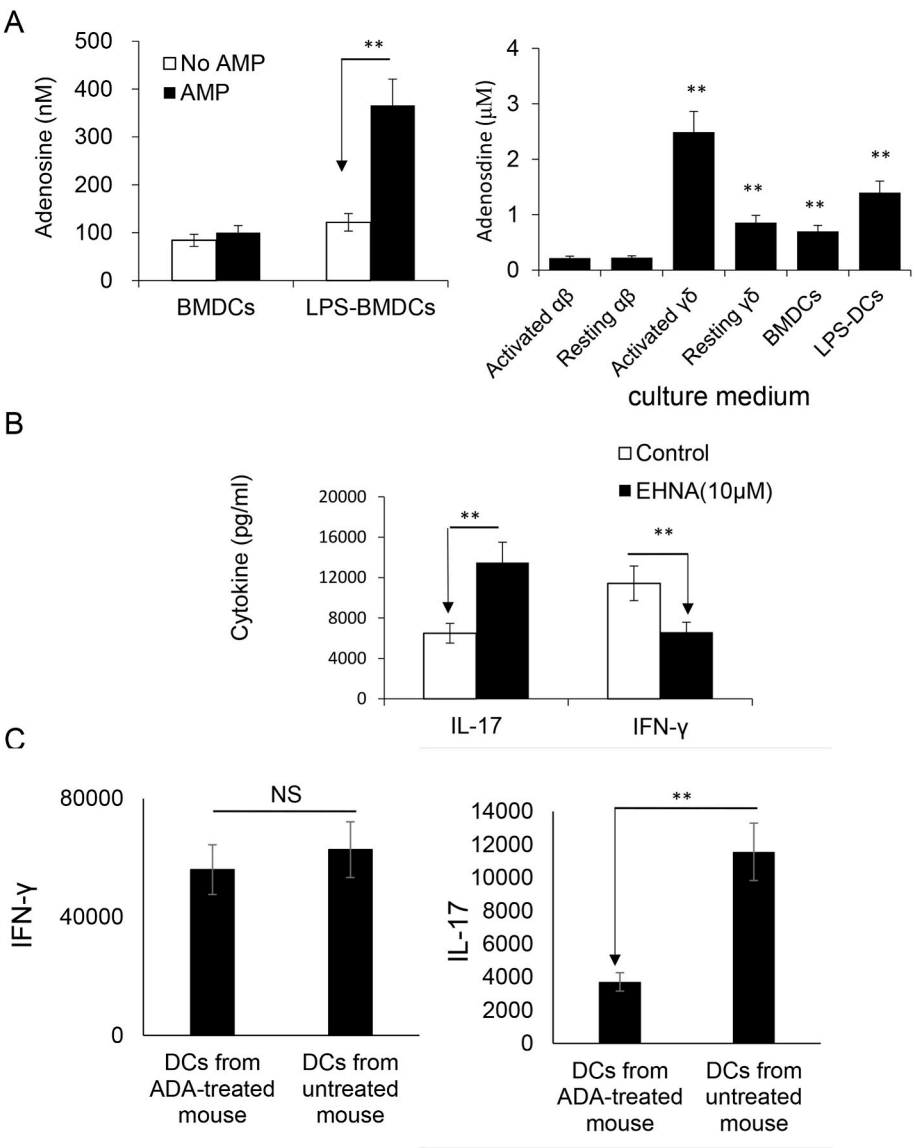

Fig. 1. Adenosine is critically involved in in vitro T cell responses. A) Detection of adenosine in supernatants of cultured BMDCs and γδ T cells. Purified αβ/γδ T cells and BMDCs were prepared as described in Materials and methods. In 24 well plate, 2 × 105/well BMDCs were cultured in the absence or presence of LPS (100 ng/ml) and AMP (10 μM) and 1 × 106/well of T cells cultured in the presence (activated) or absence (non-activated) of anti-CD3 antibodies and. After 24h, supernatants of the cultures were sampled for adenosine assay. **, $P < 0.01$; ns, not significant. B) EHNA-treated BMDCs demonstrated enhanced pro-Th17 effect but decreased pro-Th1 activity. The antigen presenting (AP) function of BMDCs were assessed with or without a pretreatment of BMDCs with a reversible inhibitor of ADA (EHNA, 10 μM). Responder $CD3^+$ cells were separated from spleen and draining lymph nodes cells of B6 mice that were immunized with IRBP1-20/CFA 13 days after immunization, using MACS column. T cells were co-cultured with BMDCs in the presence of immunizing antigen, at ratio of DC:T = 1:10 and the cytokine production of responder T cells were examined. **, $P < 0.01$. C) Splenic DCs isolated from ADA administered B6 mice have decreased Th17-stimulating activity. Splenic DCs were isolated from ADA-treated (see M&M) and untreated immunized B6 mice, using auto-MACS column. Responder $CD3^+$ cells were separated from spleen and draining lymph nodes cells of B6 mice that were immunized with IRBP1-20/CFA 13 days after immunization, using MACS column. After co-culture of responder T cells and DCs under Th1- of Th17-polarizing conditions for 2 days. The culture supernatants were assessed for IFN-γ and IL-17 production by ELISA. **, $P < 0.01$. The results are from a single experiment and are representative of three independent studies.

3.3. Activation of A2ARs on BMDCs inhibits Th1 responses, whereas activation of A2BRs enhances BMDCs' enhancing activity

Study of the AP function of BMDCs before and after LPS treatment showed that the LPS-treated BMDCs acquired greater stimulating activity for Th1 and Th17 responses (Fig. 3A). Since myeloid cells express both A2ARs and A2BRs (Haskó and Cronstein, 2004), we determined whether BMDCs are functionally different when A2ARs or A2BRs are activated. We have determined the effect of A2AR or A2BR agonists on LPS-untreated (Fig. 3B&C, left panels) and -treated (Fig. 3B&C, right panels) BMDCs to Th1 and Th17 response. The results show the A2BR activation has little, if any, effect on Th17 responses prompted by the LPS-untreated BMDCs (Fig. 3C, left panels); however, it significantly enhanced the Th17-promoting effect of the LPS-treated BMDCs (Fig. 3C, right panels). The effect of A2AR agonist is mainly inhibitory on Th1 response. However, the inhibitory effect of adenosine is limited in the Th1 responses induced by LPS-treated BMDCs (Fig. 3B, right panels), it is not appreciated in responses induced by LPS-untreated BMDCs (Fig. 3B, left panels).

To determine whether the biased pro-Th17 effect of A2BR activation on BMDCs was associated with altered cytokine production of BMDCs. IL-23 production of BMDCs were assessed under the effect of A2AR/A2BR agonists (Fig. 3D). The results showed that IL-23 production of BMDCs elicited by LPS was enhanced by A2BR agonist but not A2AR agonist (Fig. 3D). We further determined the effect of the A2AR or A2BR antagonist on a panel of cytokine production by BMDCs showed that the production of the pro-Th17 cytokines, including IL-23, IL-1β and IL-6, but not IL-12, was significantly blocked by A2BR antagonists (Fig. 3E).

3.4. TLR ligand exposure changes the adenosine receptor usage of BMDCs leading to enhanced Th17 response

To determine the possibility that different adenosine effect on LPS treated- and untreated BMDCs is caused by change of adenosine-binding, we compared the adenosine-binding activity of BMDCs before and after LPS treatment. BMDCs were pretreated with LPS (100 ng/ml) and/or Pam3 (100 ng/ml) for 48 h, followed by examination of A2AR- or A2BR-mediared binding of a ^{3}H-labeled adenosine. The results of binding assay (Fig. 4A), in which the binding of the ^{3}H-labeled adenosine BMDCs were tested after BMDCs were pre-incubated with the specific antagonists for A2ARs or A2BRs, showed that the total adenosine binding ability of BMDCs does not alter significantly after exposure to TLR ligand (Fig. 4A. Control group) and the pre-incubation of BMDCs to adenosine completely blocked the binding of ^{3}H-labled adenosine

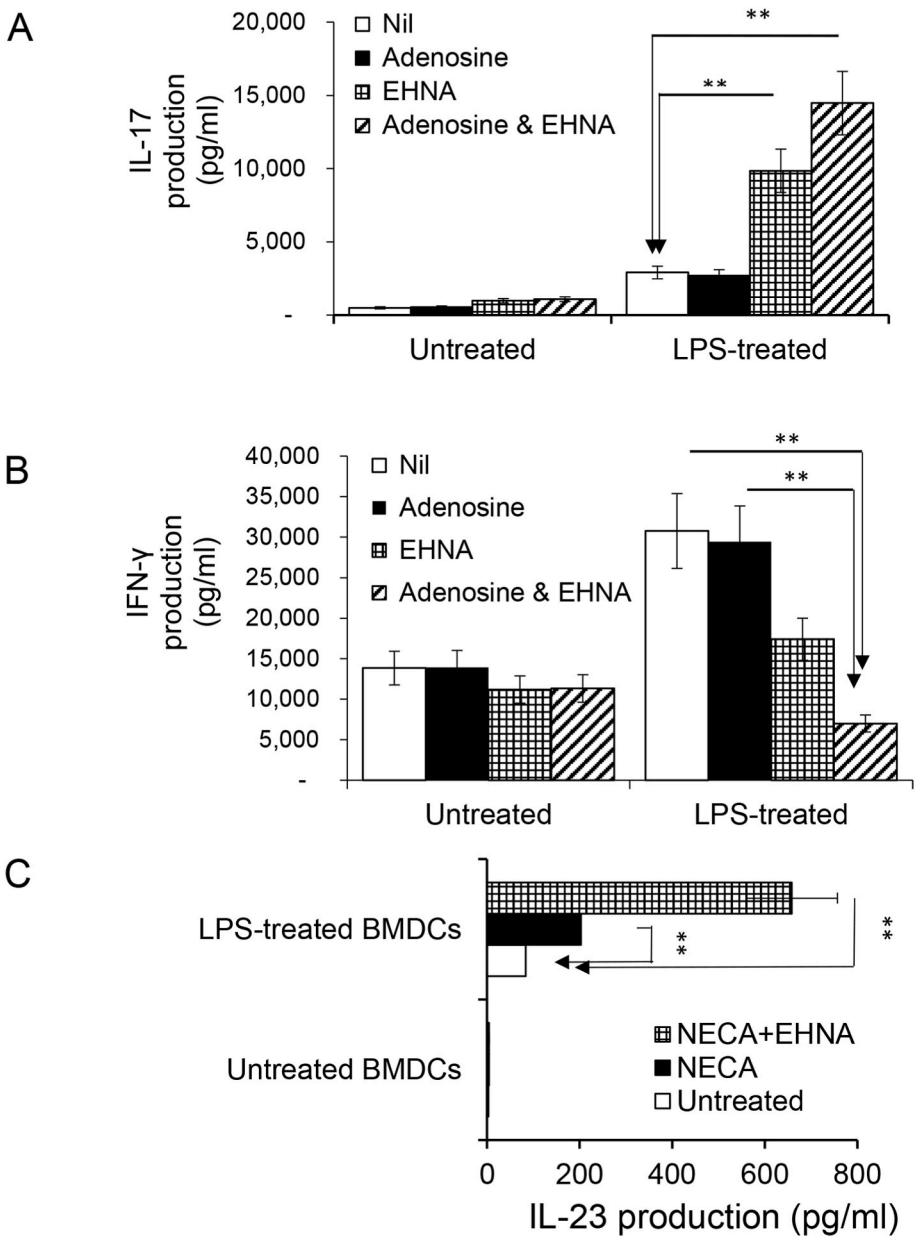

Fig. 2. BMDCs acquired increased sensitivity to adenosine after exposed to TLR ligands A&B) The AP function of LPS-treated, but not untreated, BMDCs is regulated by adenosine and EHNA. The effect of adenosine and EHNA on AP function of BMDCs were examined, before (Fig. 2A&B, left panels) and after (Fig. 2A&B, right panels) BMDCs were treated with LPS (100 ng/ml). The IL-17 assay (Fig. 2A) was performed after the responder T cells and BMDCs cocultured under Th17-polarized condition (culture medium containing IL-23) and the IFN-γ was assessed (Fig. 2B) after the responder T cells and BMDCs cocultured under Th1-polarized condition (culture medium containing IL-12). C) LPS-treated BMDCs acquired increased ability to produce IL-23. ELISA assay determining the IL-23 producing ability of LPS-treated (upper panels) and untreated (lower panels) BMDCs. The stimulators for cytokine production include the adenosine analogue (NECA, 100 nM) and/or EHNA (10 μM). The results are from a single experiment and are representative of three independent studies. **, $P < 0.01$.

(Fig. 4B. Adenosine group). Pre-incubation of BMDCs with A2AR antagonist, but not A2BR antagonist, significantly blocked the subsequent binding of ^{3}H-labeled adenosine to untreated BMDCs. However, for LPS/PAM3-exposed BMDCs the A2BR antagonist became effective to block the subsequent binding of ^{3}H-labeled adenosine, whereas the A2AR antagonist become ineffective. Given that A2BR ligation favor DCs' acquisition of Th17 promoting activity. Therefore, TLR ligands facilitates Th17 promoting activity of BMDC by switching from A2AR to A2BR.

TLR ligand exposure changes the adenosine receptor usage of BMDCs is also supported by the immunochemical study, in which expression of A2ARs and A2BRs by BMDCs before and after an exposure to TLR ligand was examined. The results show that BMDCs express both A2ARs and A2BRs. After exposure to LPS (TLR4 ligand), the A2AR expression on BMDCs was decreased whereas the A2BRs significantly increased (Fig. 4B).

3.5. BMDCs derived from A2AR disabled (A2AR$^{-/-}$) mice are hyperreactive to adenosine analogue (NECA) in IL-23 production

To support the prediction that activation of A2BR favors BMDCs to acquire enhanced Th17-stimulating activity, we also compared the cytokine-producing ability of A2AR$^{+/+}$ (isolated from B6 mouse) and A2AR$^{-/-}$ BMDCs (isolated from A2AR$^{-/-}$ mouse), in response to stimulation of LPS and LPS + NECA. The results show that the A2AR$^{+/+}$ and A2AR$^{-/-}$ BMDCs produced comparable amounts of IL-12 after exposure to LPS, which was dramatically decreased after co-stimulation with NECA (Fig. 5A). The IL-23 production of the A2AR$^{+/+}$ and A2AR$^{-/-}$ BMDCs differed greatly - the A2AR$^{-/-}$ BMDCs produce greatly increased amount of IL-23 compared to A2AR$^{+/+}$ BMDCs after NECA treatment, suggesting that's A2BR activation greatly augmented the IL-23 production of BMDCs as which is essential for pro-Th17 responses (Fig. 5B).

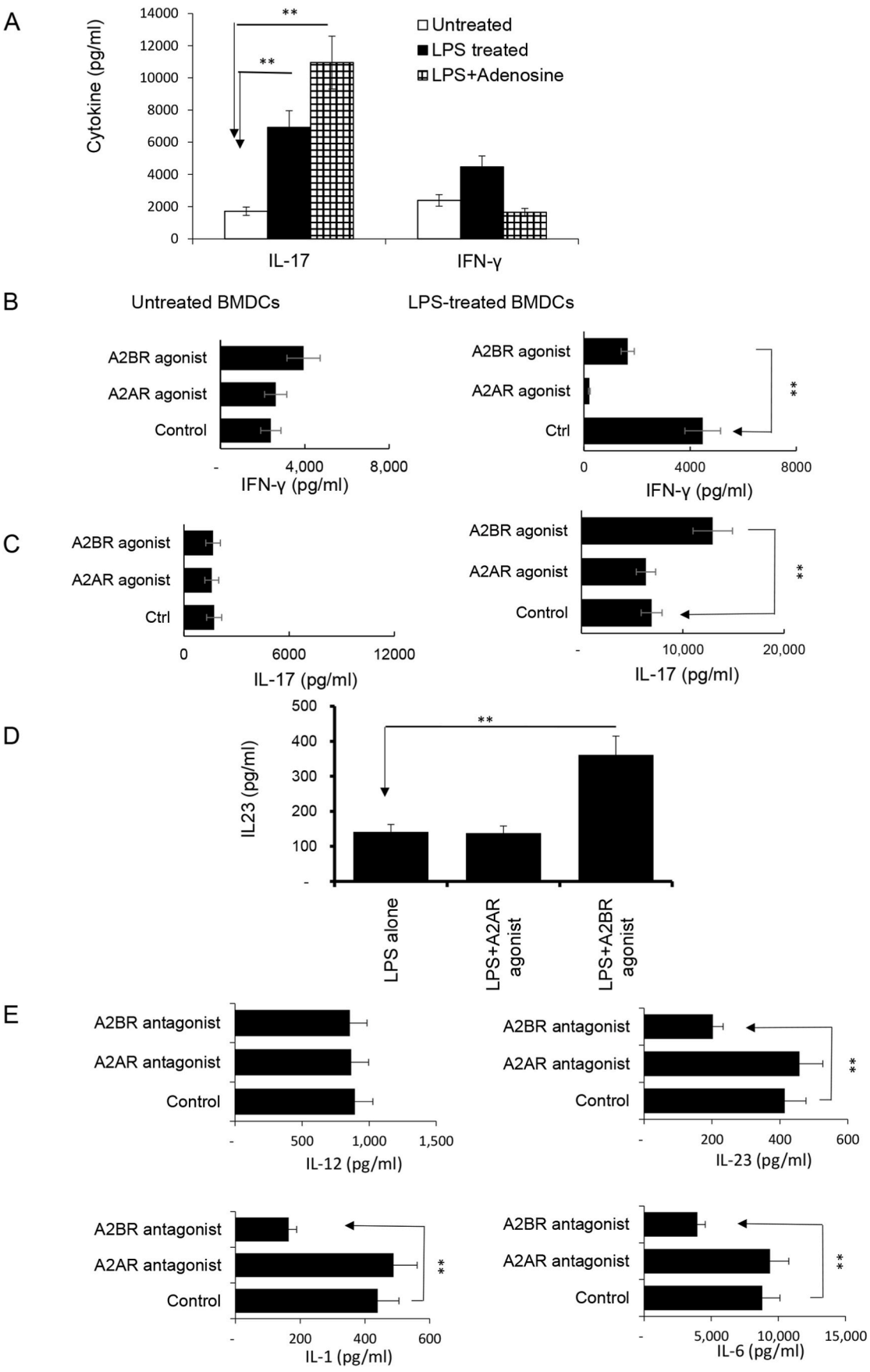

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Fig. 3. Activation of A2ARs on BMDCs inhibits Th1 responses, whereas activation of A2BRs enhances BMDCs' enhancing activity. A) LPS-treated BMDCs acquired greater stimulating activity for Th1 and Th17 responses. AP function of BMDCs were examined, before and after BMDCs were treated with LPS (100 ng/ml). B) The effect of A2AR or A2BR agonists on pro-Th1 responses of BMDCs. BMDCs were examined for AP function of pro-Th1 responses, after being treated with A2AR or A2BR agonist as indicated. ELISA assay determining the IFN-γ production of responder T cells after being stimulated by LPS-treated (right panels) and untreated (left panels) BMDCs. C) The effect of A2AR or A2BR agonists on pro-Th17 responses of BMDCs. BMDCs were examined for AP function of pro-Th17 responses, after being treated with A2AR or A2BR agonist as indicated. ELISA assay determining the IL-17 production of responder T cells after being stimulated by LPS-treated (right panels) and untreated (left panels) BMDCs. D) A2BR agonist but not A2AR agonist augmented the IL23 production by LPS-treated BMDCs.ELISA assay determining the IL-23 production of BMDCs under the effect of A2AR/A2BR agonists. LPS-treated BMDCs were treated by either A2AR agonist or A2BR agonist before LPS was added (100 ng/ml). E). Test of the A2AR or A2BR antagonist effect on a panel of cytokine production by BMDCs. ELISA assay determining the production of the pro-Th17 cytokines of BMDCs, including IL-12, IL-23, IL-1β, and IL-6. Results show that the A2BR antagonists-treated BMDCs produced decreased amounts of pro-Th17 cytokines (IL-23, IL-1β, and IL-6). The results are from a single experiment and are representative of three independent studies. **, $P < 0.01$.

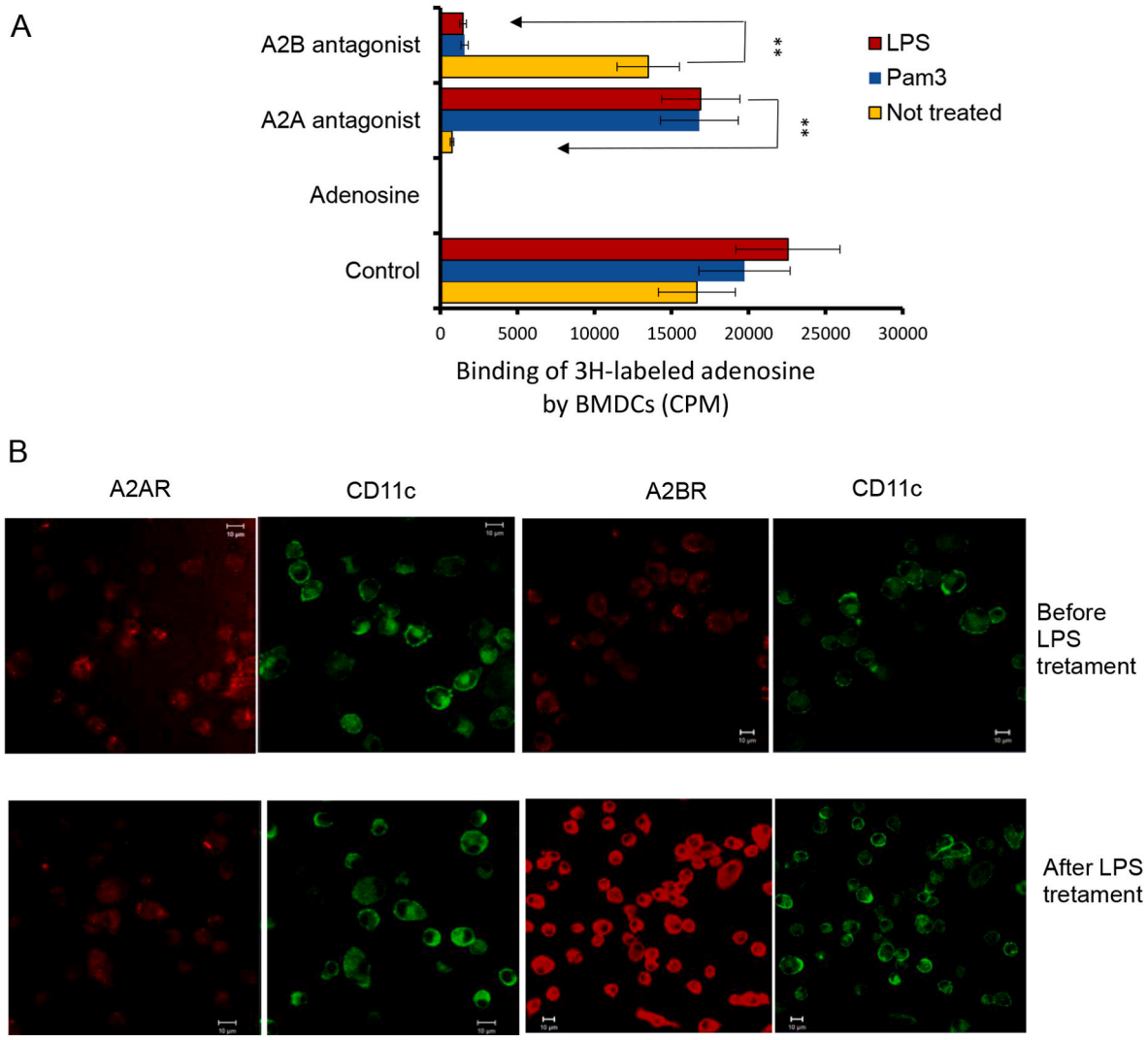

Fig. 4. TLR ligand exposure changes the adenosine receptor usage of BMDCs leading to enhanced Th17 response. A) 3H-labeled adenosine binding test. BMDCs were tested for binding of a 3H-label adenosine, before (control) or after incubation with unlabeled adenosine (10 μM), A2AR antagonist (SCH 58261, 100 nM) or A2B antagonist (MRS 1754, 100 nM). After 30 min preincubation with antagonist of the A2AR (SCH 58261, 100 nM), A2BR (MRS 1754, 100 nM), BMDCs were incubated for 1 h with radiolabeled adenosine (3H-adenosine, 100 nM). The amount of labeled adenosine bound was measured. The results are from a single experiment and are representative of three independent studies. **, $P < 0.01$. B) Immunocytochemical analyses revealed that BMDCs express increased A2BRs after TLR ligand exposure. (A) Light phase of untreated BMDCs. (B &C) A2A/A2B immunocytochemical staining of BMDCs. (C) Light phase of LPS-treated BMDCs (E–F) Immunocytochemical staining of LPS-treated BMDCs. Fluorescence label test. BMDCs, with or without a prior treated with LPS (100 ng/ml) and/or Pam3 (100 ng/ml) were fixed with 4% paraformaldehyde and then blocked in 5% BSA and 0.3% Triton X-100. Rabbit anti-A2BR antibody (1:100 dilution) as a primary antibody at 4 °C overnight and FITC anti-rabbit secondary antibody (1:200) and PE-conjugated CD11c antibody (1:200) as a secondary antibody was used.

3.6. BMDCs acquired greater γδ T cell-stimulating activity when A2BRs are activated

As our previous studies demonstrated that augmented Th17 responses are closely associated with increased γδ activation (Liang et al., 2013, 2016a; Nian et al., 2012), we questioned whether increased Th17-promoting activity of BMDCs is associated with increased γδ stimulation by BMDCs as a result of adenosine exposure. BMDCs were cultured in the absence or presence of A2AR or A2BR agonists before they were co-cultured with MACS-sorted $CD3^+$ T cells, at a ratio of T:DC

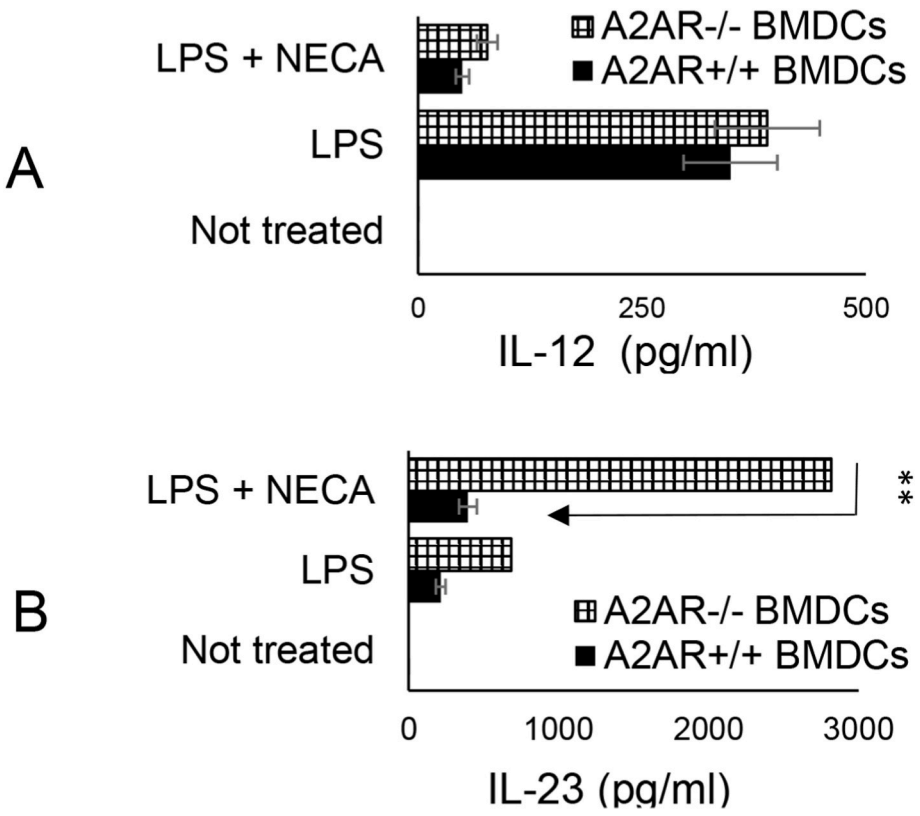

Fig. 5. A2AR$^{-/-}$ BMDCs are more sensitive to NECA induced IL-23 secretion. A2AR−/− BMDCs were cultured from A2AR−/− mouse and A2AR+/+ BMDCs from B6 mouse. IL-12 (A) and IL-23 (B) production of BMDCs were assessed by ELISA, after BMDCs were stimulated LPS or LPS & NECA. The results are from a single experiment and are representative of three independent studies. **, $P < 0.01$.

= 10:1, followed by assessing the proportional numbers of γδ and αβ T cells among the CD3$^+$ responder T cells. The activation of γδ T cells is assessed by IL-17 production by responder γδ T cells (Cui et al., 2009) (Liang et al., 2013) (Liang et al., 2016a). The responder γδ T cells were isolated from immunized B6 mice by auto-MACS column. The results show that untreated BMDCs have little, if any, stimulating effect on γδ T cells, as measure by IL-17 production. LPS-treated BMDCS stimulated significantly increased amounts of IL-17 from responder γδ T cells the produced IL-17 was further increased if, BMDCs were additional exposed to A2BR, but not A2AR agonist (Fig. 6A). As an alternative test, we co-cultured CD3$^+$ responder T cells with BMDCs that were pretreated with LPS and/or A2AR/A2BR agonists. Following a 2-day coculture the proportional number of γδ T cells among the responder T cells were counted. The results in Fig. 6B show that the proportional numbers of γδ T cells was significantly higher after stimulation by LPS-treated BMDCs (Fig. 6B, lower panels), as compared to LPS-untreated (Fig. 6B, upper panels). Moreover, the A2BR, but not A2AR, agonist further augmented γδ-stimulating effect of LPS-treated, but not untreated, BMDCs, suggesting that LPS and A2BR agonist have a synergistic effect in rendering BMDCs to acquire increased γδ-stimulating activity. The results also showed that A2BR agonist alone does not appreciably enable BMDCs to acquire increased γδ-stimulating activity and A2AR agonist was ineffective in generate a synergistic stimulating effect with LPS.

To determine whether A2BR activation is also a promoting effect of γδ T cell activation in vivo, groups (n = 6) immunized mice were administered with A2AR and A2BR agonists, respectively. At the peak of induced responses (D 13 post immunization), the proportional numbers of γδ T cells were estimated among CD3$^+$ T cells by FACS analysis. The results in Fig. 6C show that CD3$^+$ T cells of mice received a single dose of A2BR agonist contained significantly increased numbers of γδ T cells, whereas those mice received a single dose of A2AR agonist contained significantly decreased numbers of γδ T cells, when compared to the mice in control group received neither A2AR nor A2BR agonists.

4. Discussion

Adenosine is an extracellular purine nucleoside signaling molecule, which governs cell and tissue function both in health and disease. Inflammatory responses are accompanied by increased adenosine release of many types of immune cells (Eltzschig et al., 2003; Lennon et al., 1998). Adenosine is formed after the degradation of its precursor, adenosine 5′ triphosphate (ATP), released in to extracellular compartment from the cell after stressful and injurious events, and is degraded to adenosine via a cascade of ectonucleotidases, including CD39 (nucleoside triphosphate diphosphorylase [NTPDase]) and CD73 (5′-ectonucleotidase [Ecto5′NTase]) (Haskó et al., 2009; Yegutkin, 2008). The levels of extracellular adenosine increase greatly during inflammation (Eltzschig et al., 2012; Ohta and Sitkovsky, 2001; Sitkovsky et al., 2004). Adenosine accumulates in the extracellular space in response to metabolic stress and cell damage (Haskó et al., 2008) and elevations in extracellular adenosine are found in conditions of ischemia, hypoxia, inflammation and trauma (Linden, 2001) (Fredholm et al., 2001a). Cells of the immune system including neutrophils, mast cells, endothelial cells, regulatory T cells and platelets have been appreciated as the most prodigious sources of extracellular adenosine (Eltzschig et al., 2008). Oxygen radicals such as H_2O_2 also promote increased adenosine release from cells (Morabito et al., 1998).

Studies have shown that adenosine could generate either anti- or pro-inflammatory effects and that “timing” is important factor for successful therapeutic interference (Ko et al., 2021; Liang et al., 2014a, 2016b; Zhou et al., 2009, 2011). Adenosine is anti-inflammatory at early or quiescent stages of immune responses but turned to be pro-inflammatory at alternative disease stages (Ko et al., 2021; Liang et al., 2014a, 2016b; Zhou et al., 2009, 2011). Our previous study

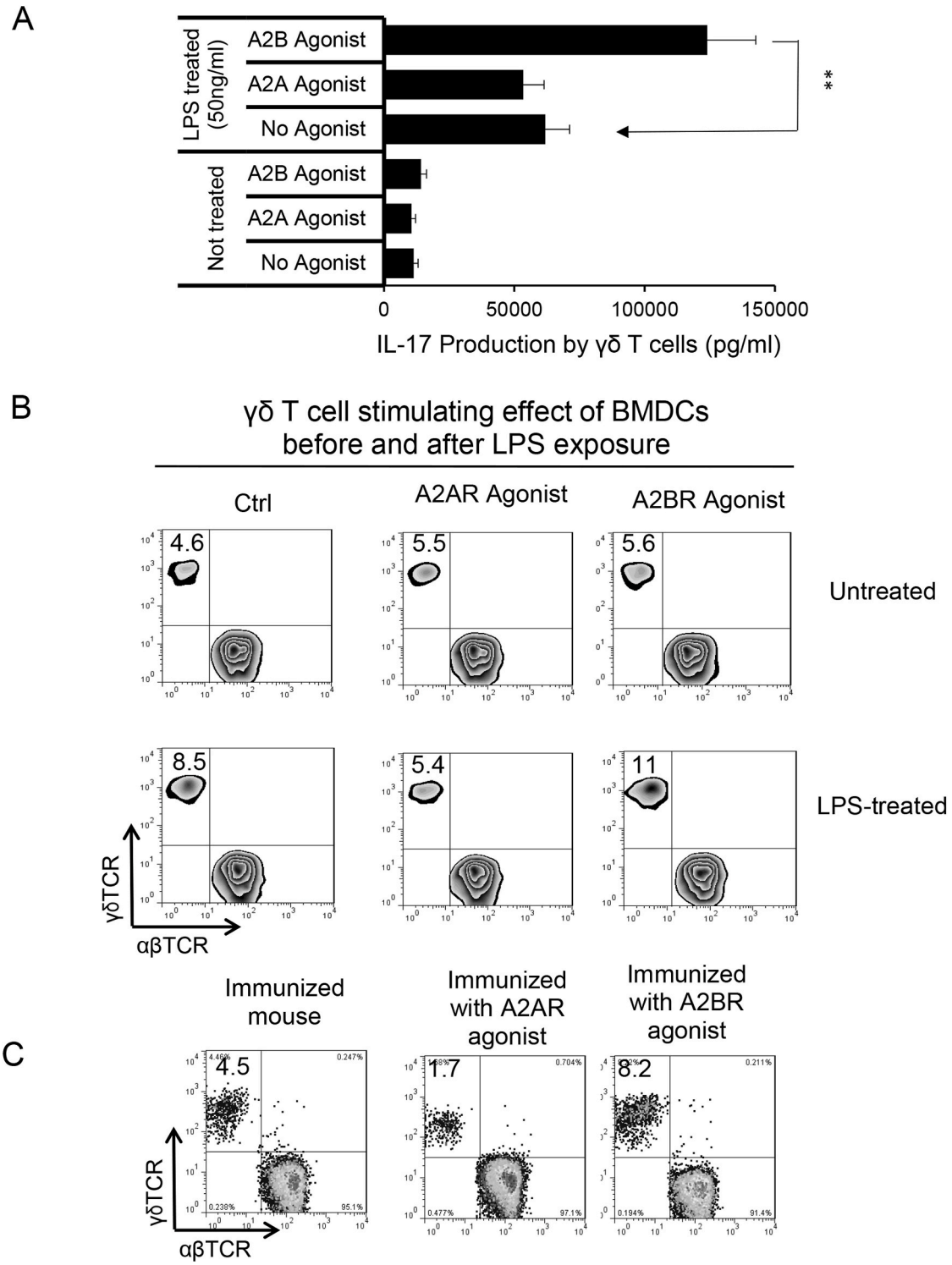

Fig. 6. BMDCs acquired greater γδ T cell-stimulating activity when A2BRs are activated. A) After incubation with LPS-treated BMDCs γδ T cells produced increased amounts of IL-17 which was further augmented by treatment of BMDCs with A2BR, but not A2AR agonist. γδ T cells were separated from immunized B6 mice (M&M). They (2 × 105/well) were incubated with BMDCs, at ratio of DC:T = 1:10, with or without pretreated as indicated. IL-17 in the co-cultured of γδ T cells and BMDCs was assessed by ELISA after 48h. The results are from a single experiment and are representative of three independent studies. **, $P < 0.01$. B) BMDCs treated with A2BR agonist acquired augmented γδ T cell-stimulating activity. LPS-treated (lower panel) or untreated (upper panels) BMDCs were additionally treated with A2AR (CGS 21680, 100 nM)- or A2BR agonist (BAY 60–6538, 100 nM) before they were co-culture with responder T cells at ratio of DC:T = 1:10. Two days later, responder T cells were collected and the proportional numbers of CD3+γδ TCR + cells were estimated after labeling with FITC-anti-αβTCR and PE-anti-γδTCR and analyzed by FACS. C) EAU-prone mice In vivo administered with A2BR agonist have significantly increased number of γδ T cells. Groups (n-3) of B6 mice were left untreated or administered with a single dose of A2AR (CGS 21680, 1 mg/kg)- or A2BR agonist (BAY 60–6538, 1 mg/kg) at the time they were immunized. 13 days post immunization, the $CD3^+$ T cells were enriched by anti-MACS column. The proportional numbers of CD3+γδ TCR + cells were estimated after labeling with FITC-anti-αβTCR and PE-anti-γδTCR and analyzed by FACS.

investigated the treatment effect on EAU pathogenesis of adenosine deaminase (ADA) — an enzyme that degrades adenosine, in EAU (Liang et al., 2016b). The results showed that the protective effect is timing restricted — ADA was protective when administered at ongoing phase of EAU but was ineffective if administered during quiescent disease stages (Liang et al., 2016b). We also observed administration to EAU-inducing mice with a non-selective AR agonist, NECA, had an inhibitory effect on both Th1 and Th17 responses, but only when NECA was injected at early stages of disease (Ko et al., 2021). To determine the factors that are contributed to this "timing effect" and to the pro- and anti-inflammatory effect of adenosine, we examined the role of DCs – the principal antigen presenting cells critically involved in T cell responses. Engagement of distinct adenosine receptors was interpreted as a possible cause for adenosine's pro- or anti-inflammatory effects. Studies have shown that activation of the high affinity A2ARs is inhibitory (Linden, 2001; Zarek et al., 2008), whereas activation of the low affinity A2BRs is pro-inflammatory (Haskó et al., 2008) (Mustafa et al., 2007; Zhou et al., 2010) (Wei et al., 2013) (Kolachala et al., 2008a; Zaynagetdinov et al., 2010). It is likely that in early stages of inflammation only the high affinity A2ARs are activated whereas the low affinity A2BRs remain silent when the adenosine levels are low (Fredholm et al., 2001b). In the current study we propose an additional hypothesis that the pro- or anti-inflammatory effect of adenosine could be correlated to switched use of adenosine receptors on DCs induced by TLR ligand.

Using a binding assay, in which the binding of ^{3}H-labeled adenosine to BMDCs, in the absence or presence of antagonists specific for A2AR or A2BR, and before and after an exposure to TLR ligand were examined. We were able to show that the adenosine binding to BMDCs is mainly blocked by A2AR antagonist; however, after exposure to TLR ligand the adenosine binding to BMDCs is mainly blocked by A2BR antagonist. Concurrently, adenosine effect on AP function of BMDCs turned to be pro-inflammatory. The finding of the binding study was supported by the fluorescence labeled staining which show that untreated BMDCs dominantly express A2ARs whereas after TLR ligand treatment A2BR expression become dominant. Functional assays also showed that A2BR agonist become much more effective in enhancing antigen-presenting function of BMDCs after TLR ligand exposure. We also observed that the Th1 and Th17 responses activated by adenosine receptor activated BMDCs are dissociated. The enhancing effect is mainly seen in Th17 responses, whereas the suppressive effect seen in Th1 responses. At lease, a part of such biased effect, is attribute to that A2BR receptor engagement has a unique effect altering cytokine producing capability of BMDCs. Cytokines such as IL-1β and IL-23 was enhanced whereas IL-12 was suppressed. As a result, dual treatment with LPS and adenosine enabled BMDCs to acquire greatly increased Th17 promoting activity.

Usage of adenosine receptors change by myeloid cells has been previously found. For example, under hypoxic condition AR expressed on myeloid cells changed greatly with the A2BR express increased significantly (Panther et al., 2001) (Yang et al., 2009). Upregulation of A2B receptors in gut tissue was also found in human and murine colitis (Kolachala et al., 2005). In addition, adenosine receptor change was also observed when myeloid cells exposed to cytokines such as IFN-γ and TLR ligand (Cohen et al., 2015). Additional factors, such as LPS (Néemeth et al., 2003), TNF-α (Khoa Nguyen et al., 2003; Kolachala et al., 2005), IL-1β (Khoa Nguyen et al., 2003) IFN-γ (Khoa Nguyen et al., 2003) (Xaus et al., 1999), free radicals (St. Hilaire et al., 2008) and the endogenous agonist adenosine (Sitaraman et al., 2002) have all been shown to increase the expression of A2B receptors. It is worth mentioning that the enhancing effect of adenosine on Th17 responses is accomplished via a sum of effects elicited by adenosine effect on various cellular responses important for T cell activation, including αβ T cells, γδ T cells, DCs and regulatory T cells. Adenosine is also an important molecule modulating DC differentiation (Erdmann et al., 2005; Lappas et al., 2005; Ohta et al., 2006; Sevigny et al., 2007) into a unique subset that has a Th17-stimulating effect (Panther et al., 2001, 2003; Wilson et al., 2011). In addition, adenosine exposed DCs showed a greater stimulating effect on γδ T cell activation leading to enhanced Th17 responses. We have previously reported that activated γδ T cells acquire greatly increased adenosine binding capability (Liang et al., 2014b) and preferential binding of adenosine by γδ T cells re-distributes adenosine binding among various immune cells, with diminished binding by αβ T cells favors augmented αβ T cell responses (Liang et al., 2014b, 2018). In this study we show that adenosine exposed BMDCs acquire augmented γδ-stimulating activity of BMDCs, via which Th17 responses are further promoted.

Both Th1 and Th17 autoreactive T cells are pathogenic in autoimmune uveitis in patients (Amadi-Obi et al., 2007) and in animal models (Korn et al., 2009; Kroenke et al., 2008; Luger et al., 2008; Peng et al., 2007b). Continue efforts in identification of mechanisms of the pro- and anti-inflammatory effects of adenosine on immune responses should allow us to manage desired and undesired effect of adenosine more effectively and thus improve the therapeutic goal of adenosine-based treatment of diseases. A better knowledge and understanding of the functional conversion of adenosine should facilitate adenosine-mediated immunotherapies.

5. Conclusion

A better knowledge and understanding of the functional conversion of adenosine should facilitate adenosine-mediated immunotherapies. The cellular and molecular basis for enhancing and/or inhibiting the effects of ATP/adenosine remain to be further determined and the outcome of such studies should improve currently available therapies, including adenosine- and γδ T cell-based immunotherapies.

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Data sharing statement

The main data supporting the findings of this study are available within the paper.

CRediT authorship contribution statement

Deming Sun: Formal analysis, Writing – original draft, Data curation, Designed research, performed the experiments and analyzed data, wrote the manuscript, All authors contributed to the article and approved the submitted version. **Hui Shao:** Formal analysis, Data curation, Designed research, performed the experiments and analyzed data, All authors contributed to the article and approved the submitted version. **Henry J. Kaplan:** Writing – original draft, Designed research, wrote the manuscript,All authors contributed to the article and approved the submitted version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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