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Augmented Th17-stimulating activity of BMDCs as a result of reciprocal interaction between γδ and dendritic cells

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Abstract

Our previous studies demonstrated that γδ T cells have a strong regulatory effect on Th17 autoimmune responses in experimental autoimmune uveitis (EAU). In the current study, we show that reciprocal interactions between mouse γδ T cells and dendritic cells (DCs) played a major role in γδ regulation of Th17 responses. Mouse bone marrow-derived dendritic cells (BMDCs) acquired an increased ability to enhance Th17 autoimmune responses after exposure to γδ T cells; meanwhile, after exposure, a significant portion of the BMDCs expressed CD73 – a molecule that is fundamental in the conversion of immunostimulatory ATP into immunosuppressive adenosine. Functional studies showed that $CD73^+$ BMDCs were uniquely effective in stimulating the Th17 responses, as compared to $CD73^-$ BMDCs; and activated γδ T cells are much more effective than non-activated γδ T cells at inducing $CD73^+$ BMDCs. As a result, activated γδ T cells acquired greater Th17-enhancing activity. Treatment of BMDCs with the CD73-specific antagonist APCP abolished the enhancing effect of the BMDCs. γδ T cells more effectively induced $CD73^+$ BMDCs from the BMDCs that were pre-exposed to TLR ligands, and the response was further augmented by adenosine. Moreover, BMDCs acquired increased ability to stimulate γδ activation after pre-exposure to TLR ligands and adenosine. Our results demonstrated that both extra-cellular adenosine and TLR ligands are critical factors in augmented Th17 responses in this autoimmune disease, and the reciprocal interactions between γδ T cells and DCs play a major role in promoting Th17 responses.

Keywords

Adenosine; Autoimmunity; Adenosine receptors; APCP; BMDCs; CD73; EAU γδ T cells

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Declaration of Competing Interest

None of the authors have any conflict of interest pertaining to this work.

1. Introduction

γδ T cells can either enhance (Nian et al., 2011; Rajan et al., 2000; Spahn et al., 1999) or inhibit (Born et al., 1999; D'Souza et al., 1997; Liang et al., 2016a) an immune response. Clarifying the mechanism by which they function should allow us to manipulate immune responses more effectively. Our previous studies on the pathogenesis of experimental autoimmune uveitis (EAU) revealed that the regulatory effects of γδ T cells were closely associated with the activation status of the cells; and that activated γδ T cells have a greater enhancing effect on Th17 responses (Liang et al., 2013b, b; Liang et al., 2016a; Nian et al., 2011). In determining the factors attributed to γδ T cell activation that lead to augmented Th17 responses, our early studies showed that the regulatory effect of γδ T cells is adenosine-dependent and that interrelated γδ- and adenosine-mediated regulations are co-contributors (Liang et al., 2014a, b, 2015; Liang et al., 2016a). Adenosine showed a significant augmenting effect in γδ T cell activation, by which it enhances Th17 autoimmune responses (Liang et al., 2018, 2014b, a; Liang et al., 2016b).

Various pathological conditions are accompanied by ATP release from the intracellular to the extracellular compartment. Indeed, all mammalian cells are capable of releasing ATP (Eltzschig et al., 2004; Junger, 2011; Piccini et al., 2008; Schenk et al., 2008; Yip et al., 2009). Adenosine accumulates at inflamed sites as the result of ATP release into the extracellular environment, following dephosphorylation to ADP and AMP, and a terminal reaction converting AMP to adenosine (Sitkovsky et al., 2004; Sitkovsky and Ohta, 2005). The increase of extracellular ATP and its metabolites exerts a strong modulatory effect on immune responses and inflammation.Adenosine is an important regulatory molecule of immune responses and it affects functions of many types of immune cells, including T cells (Jin et al., 2010; Lappas et al., 2005), macrophages/DCs (Lappas et al., 2005; Naganuma et al., 2006; Panther et al., 2001), NK cells (Hoskin et al., 2008), neutrophils (Fredholm et al., 2001), platelets (Varani et al., 1996), and regulatory T cells (Ehrentraut et al., 2012; Naganuma et al., 2006; Zarek et al., 2008). During inflammation or under ischemic conditions, generation of increased amounts of adenosine in the extracellular environment modulates various biological responses, including immune responses (Fredholm et al., 2011; Haskó et al., 2008; Jacobson and Gao, 2006; Sauer et al., 2012).

Activation of autoreactive T cells is a key event by which T cells cause tissue damage and inflammation; and dendritic cells (DCs) are key cell elements causing T cell activation (Steinman et al., 1983) (Guermonprez et al., 2002). To determine whether γδ T cell regulation involves DC functional modulation, we examined the antigen-presenting (AP) effect of bone marrow-derived dendritic cells (BMDCs) on Th17 autoreactive T cells, before and after exposure to γδ T cells. We were able to show that the acquisition of augmented Th17-stimulating activity was closely associated with an increased appearance of a $CD73^+$ BMDCs among the γδ T cell-exposed BMDCS and that this activity was barely detectable before γδ T cell exposure. Comparative studies on the AP functions of BMDCs to Th1 and Th17 autoreactive T cells, before and after exposure to γδ T cells, showed that the BMDCs acquired a greater stimulating effect of Th17 responses after a pre-exposure to γδ T cells; whereas after a pre-exposure to αβ T cells, the BMDCs acquired a greater stimulating effect of Th1 responses. Subsequent studies showed that

separated $CD73^+$ and $CD73^-$ BMDCs are functionally distinct in promoting Th1- and Th17-responder T cells, and in cytokine production, including IL-12, IL-23, IL-6, and IL-1β. We also show that significantly increased numbers of $CD73^+$ BMDCs could be obtained if BMDCs were pre-treated with a TLR ligand before interacting with γδ T cells; addition to cultures with adenosine further augmented the response. Our results demonstrated that extracellular adenosine and TLR ligands are critical factors in augmented Th17 responses in this autoimmune disease. Reciprocal interaction between γδ T cells and DCs is an important pathway by which γδ T cells regulate immune responses; and generation of an increasing number of $CD73^+$ DCs is a pathogenic event leading to augmented Th17 autoimmune responses.

2. Results

2.1. Th17-promoting activity of BMDCs was enhanced after incubation with γδ T cells

Our previous studies demonstrated that γδ T cells have a stronger regulatory effect on Th17 responses than on Th1 responses (Cui et al., 2009; Liang et al., 2013b, a; Nian et al., 2011). To determine the underlying mechanisms, we questioned whether the antigen-presenting function of DCs would be altered through the effect of γδ T cells. Using mouse BMDCs cultured from GM-CSF and IL-4-containing medium (Chen et al., 2015; Liang et al., 2015), we examined the phenotypic and functional alterations of BMDCs before and after exposure to γδ T cells at a pre-determined optimal ratio of T: DC = 1:20; control sets included BMDCs exposed to the same numbers of αβ T cells.

After a 24 h co-culture, the interacting T cells were removed and the BMDCs were collected, irradiated and seeded on to 24-well plates (5×10^5/well). Responder T cells isolated from immunized B6 mice were then added. Assessment of antigen-specific Th1 and Th17 responses were set by stimulation of responder T cells by immunizing antigen ($IRBP_{1–20}$) at a T: DC ratio of 1:20, under Th1- or Th17-polarizing conditions as we previously reported (Liang et al., 2013b; Peng et al., 2007). The AP function of BMDCs was assessed by measuring proportional IFN-γ^+ and IL-17^+ T cells among responder T cells (Cui et al., 2009; Liang et al., 2013b, a; Nian et al., 2011) and cytokine production in supernatants of responder T cells. Fig. 1 shows that BMDCs stimulated a significantly greater of IL-17^+ cells among responder T cells when they were tested after co-culture with γδ T cells, compared to control BMDCs cultured in the absence of γδ T cells (Fig. 1A lower middle panel); however, their capacity to stimulate Th1 responses (or IFN-γ^+ T cells) was not significantly affected (Fig. 1A upper middle panels). Control BMDCs also included a group of BMDCs pre-exposed to the same number of αβ T cells. The BMDCs in this group stimulated a greater number of IFN-γ^+ cells among the responder T cells (Fig. 1A, upper right panel), but not IL-17^+ T cells (Fig. 1A, upper lower panel). Cytokine tests agreed evaluating IFN-γ^+ and IL-17^+ T cell numbers by showing that the γδ T cell-exposed BMDCs stimulated significantly greater amounts of IL-17 but not IFN-γ from responder T cells from four separate assays (Fig. 1B); whereas the αβ T cells-exposed BMDCs stimulated greater amounts of IFN-γ but not IL-17 from the responder T cells (Fig. 1C).

2.2. Increased appearance of CD73^{+} DCs among the γδ-exposed BMDCs

To determine the cellular and molecular mechanisms by which γδ shaped the AP function of BMDCs, we examined the expression of T cell activation surface molecules on BMDCs to both before and after exposure to γδ T cells. Our results showed that the γδ-exposed BMDCs expressed increased levels of CD86 (Fig. 2A) and CD73 (Fig. 2B), but not MHC II molecules (Fig. 2A); by contrast, αβ-exposed BMDCs expressed increased levels of MHC II molecules but not CD86 and CD73 (Fig. 2A). Examination of CD73 was included because our previous observations showed that extracellular adenosine levels and CD73 molecules expressed on immune cells were crucially involved in Th17 responses (Liang et al., 2014a, b, 2015; Liang et al., 2016a). We observed, unexpectedly, that a significant portion of the BMDCs co-expressed CD73 after exposure to γδ T cells, whereas the untreated BMDCs as well as those exposed to αβ T cells remained CD73 negative (Fig. 2B). Cytokine-production tests showed that γδ-exposed BMDCs produced significantly increased amounts of IL-23 and IL-1β, whereas αβ-exposed BMDCs produced significantly increased amounts of IL-12 (Fig. 2C).

To determine whether CD73 expression on BMDCs was related to their Th1- and Th17-stimulating effect, we separated CD73^{+} and CD73^{-} BMDCs using a MACS column (Fig. 3A). The AP function (Fig 3B) and cytokine-producing activity (Fig. 3C) of separated CD73− and CD73^{+} BMDCs was determined both before and after exposure to γδ T cells. The results showed that CD73^{+} BMDCs stimulated a significantly greater number of IL-17^{+} autoreactive T cells after exposure to γδ T cells, whereas CD73− BMDCs did not. Measurement of cytokine-producing capability of CD73^{+} and CD73− BMDCs, after LPS stimulation and in the absence or presence of AMP, showed that under LPS stimulation CD73− BMDCs produced even more IL-12 and IL-23 as compared to CD73^{+} BMDCs. However, after addition of AMP only CD73^{+}, but not CD73−, BMDCs produced significantly higher amounts of IL-23 (Fig 3C, right panels). IL-12 production was the opposite. AMP inhibited IL-12 production by CD73^{+}, but not CD73−, BMDCs (Fig 3C, left panels) suggesting that CD73 expression affected BMDC responses to AMP. Since CD73 degrades AMP to adenosine, enhanced IL-23 cytokine production by CD73^{+} BMDCs leads to augmented Th17 responses. To further determine whether CD73 is the functional molecule responsible for BMDCs' selective Th1- and Th17-stimulating activity, we also examined the AP function of γδ-exposed CD73$^{+/+}$ BMDCs, with or without treatment with APCP – an antagonist that specifically blocks CD73 function (Jin et al., 2010; Liang et al., 2014b). As demonstrated in Fig. 3D, APCP treatment completely abolished augmented Th17 responses by γδ-treated BMDCs.

We questioned whether BMDCs isolated from CD73$^{-/-}$ mice (CD73$^{-/-}$ BMDCs) were functionally different from those isolated from wt-B6 mice (CD73$^{+/+}$ BMDCs). Fig. 4A demonstrated that the Th17-stimulating function of CD73$^{+/+}$ and CD73$^{-/-}$ BMDCs (lower panels compared to the upper panels) were indistinguishable before exposure to γδ T cells. After interacting with γδ T cells, the Th17-promoting effect of CD73$^{+/+}$ BMDCs was much greater increased, even though minimal increased Th17 promoting effect were also seen in CD73$^{-/-}$ BMDCs. The effect on Th1 responses showed no difference with either CD73$^{+/+}$ or CD73$^{-/-}$ BMDCs (Fig. 4B). Indeed, measurement of cytokine production by responder

T cells under the same experimental setting supported this conclusion by showing that the Th17 responses stimulated by $CD73^{+/+}$ BMDCs was augmented by addition of AMP and partially blocked by APCP (upper panels), whereas the Th17 responses stimulated by $CD73^{-/-}$ BMDCs was not augmented by addition of AMP (lower panels) (Fig. 4C).

2.3. Role of adenosine in γδ-DC interaction

Given previous findings (Antonioli et al., 2013; Ben Addi et al., 2008; Novitskiy et al., 2008; Yang et al., 2009), including those of our own studies (Chen et al., 2015; Liang et al., 2015), that adenosine metabolism has a great impact on DC differentiation and function, we examined the role of adenosine in γδ-DC interaction. Adenosine deaminase (ADA) is an enzyme that converts adenosine into functionally inactive molecules (North and Cohen, 1978; Ullman et al., 1976). We previously reported that administration of a single dose of ADA to EAU-prone B6 mice inhibited the Th17 pathogenic T cell responses, as well as the development of EAU (Liang et al., 2016b). To determine whether depletion of an excessive amount of endogenously generated adenosine by ADA would modulate γδ-DC interaction, and thus, prevent augmented Th17 responses we examined the AP function of γδ-exposed BMDCs, with or without prior treatment with ADA. As demonstrated in Fig. 5A&B, approximately 50 % of the Th17-enhancing effect of the BMDCs induced by pre-exposure to γδ T cells was reduced by pretreatment with ADA, as measured by $IL\text{-}17^{+}$ T cell responses among responder T cells. Administration of 1 U/mL of ADA was effective in *in vitro* tests, while a further increase in the ADA dose did not further increase the blocking effect (not shown).

2.4. Activated γδ T cells were more active in inducing $CD73^{+}$ BMDCs

Given our previous finding that activated γδ T cells strongly enhance Th17 responses (Liang et al., 2017, 2013b; Nian et al., 2011), we wished to determine whether activated γδ T cells more effectively induce $CD73^{+}$ BMDCs, leading to augmented Th17 responses. Activated and non-activated γδ T cells were isolated from immunized and naïve B6 mice, as our previous studies showed that a majority (>60 %) of the γδ T cells in immunized mice were activated, whereas those in naïve mice remain non-activated (Liang et al., 2013b, a). Our results showed that the BMDCs pre-exposed to activated γδ T cells had a much greater effect in inducing $CD73^{+}$ BMDCs. The stronger promoting effect was most easily demonstrated with a low γδ/BMDCs ratios. As demonstrated in Fig. 5C, $CD73^{+}$ BMDCs increased >20-fold after exposure to γδ T cells. When the ratio of γδ/BMDCs declined to 1:20, however, activated γδ T cells remained functionally potent, whereas the effect of resting γδ T cells declined dramatically.

2.5. Reciprocal interactions between BMDCs and γδ T cells

γδ T cells can be activated in the absence of TCR ligation (Petermann et al., 2010; Rincon-Orozco et al., 2005; Sutton et al., 2009; Wesch et al., 2006). They can be activated by cytokines including IL-1β, IL-7, and IL-23 (Nian et al., 2011; Petermann et al., 2010; Sutton et al., 2009), TLR ligands (Nian et al., 2011), and DCs (Liang et al., 2012, 2013a; Liang et al., 2013b). To examine whether the γδ-stimulating effect of BMDCs was affected by inflammatory molecules like TLR ligands and adenosine, BMDCs were tested for their stimulating effect on γδ T cells, with or without prior treatment with TLR ligand and/or

adenosine. $CD3^+$ T cells isolated from immunized B6 mice were taken as responder T cells. After a two-day incubation, the proportional number of αβ and γδ T cells in the responder T cells were assessed. As demonstrated in Fig. 6, BMDCs have a weak stimulating effect on γδ T cell expansion before exposure to TLR ligands. After exposure to TLR ligands, the stimulating effect increased significantly (13.1 % versus 4.8 % of γδ T cells) and was further increased (from 13.1 to 27.2%) when BMDCs received prior exposure of both TLR ligand and adenosine.

3. Discussion

Multiple lines of evidence demonstrated that γδ T cells have a regulatory effect on immune responses (Chien et al., 2014; Girardi, 2006; Huber et al., 2000; Kabelitz et al., 2007; Paul et al., 2014; Poccia et al., 2005). We have previously shown that γδ T cells have a strong regulatory effect on Th17 responses in autoimmune disease, and that this effect is determined by the activation status of γδ T cells (Cui et al., 2009; Liang et al., 2013b, a; Nian et al., 2011, 2010). In this study, we examined the regulatory mechanisms of γδ T cells; determined the underlying mechanisms by which activation shapes the γδ T cells' regulatory activity; and determined the mechanism by which γδ T cells distinctively affect Th17 and Th1 responses (Cui et al., 2009; Liang et al., 2013b, a; Nian et al., 2011, 2010). We show that interaction between γδ T cells and DCs is an important pathway to promote Th17 responses. BMDCs cultured in the prototypic GM-CSF + IL-4-containing medium were superior APCs for Th1 responses, but not as effective at Th17 responses. However, after exposure to γδ T cells, they became strongly effective in stimulating Th17 responses. Such an observation increases our understanding of the role of DCs in the balance between Th1 and Th17 responses in pathogenic disease processes.

The results of examining the phenotypic and functional changes in BMDCs, before and after exposure to γδ T cells, showed that the increased Th17-promoting activity after exposure to γδ T cells was closely associated with the appearance of $CD73^+$ BMDCs, and that this enhancing activity was greatly diminished when BMDCs received prior treatment with the CD73 antagonist APCP (26). These results suggest that the enhanced Th17-promoting activity of BMDC on γδ T cells in autoimmune responses is attributed to generation of inducible $CD73^+$ DCs. The fact that activated γδ T cells are much better able to induce $CD73^+$ BMDCs, thereby leading to augmented autoimmune responses, supported our previous findings (Liang et al., 2013b, b; Liang et al., 2016a; Nian et al., 2011).

The purinergic system is an evolutionally selected regulatory system that modulates immune responses (Hasko et al., 2008; Junger, 2011). Release of ATP into the extracellular space is elicited by tissue injury or damage, such as that related to inflammation. Under physiological conditions ATP is present primarily within cells. Almost all mammalian cell types care capable of releasing ATP in response to tissue injury or damage (Junger, 2011). Once released into the extracellular space ATP is hydrolyzed in a stepwise manner into ADP, AMP, and finally adenosine, by ectonucleotidases, including CD73 and CD39, which are expressed on surfaces of various immune and non-immune cells (Doherty et al., 2012; Raczkowski et al., 2018; Sidibé and Imhof, 2015; Yegutkin, 2008). The regulatory effect of

adenosine on immune responses has been well established (Augusto et al., 2013; Csoka et al., 2008; Ibrahim et al., 2011; Jacobson and Gao, 2006).

Our attention to the study of adenosine's effect on γδ regulation was prompted by reports that this molecule affects the immune function of a wide range of cells, such as lymphocytes (Jin et al., 2010; Lappas et al., 2005; Wilson et al., 2011), polymorphonuclear leukocytes (Fredholm et al., 2001; Hasko and Cronstein, 2004), and macrophages/DCs (Hasko et al., 2009; Lappas et al., 2005; Naganuma et al., 2006; Panther et al., 2001). The outcome of those studies suggested that adenosine analogues suppress Th1 responses. Indeed, such a suppressive effect of adenosine has been exploited in initiating immunotherapies for cancer (Antonioli et al., 2013; Vijayan et al., 2017) and autoimmune diseases (Cronstein et al., 1993; Eckle et al., 2008; Flogel et al., 2012; Hasko et al., 2008; Ibrahim et al., 2011; Jacobson and Gao, 2006; Luijk et al., 2008). Since previous studies examining adenosine's effect on immune responses have not adequately examined Th17 responses, we wished to determine whether existing findings of the adenosine effect on immune responses would apply to Th17 responses as well. Using a reproducible EAU model, our recent studies showed that Th17 pathogenic responses differed greatly in their response to (Liang et al., 2018, 2014b) γδ- and adenosine-mediated regulation when compared to Th1 pathogenic responses. Unlike previous observations that adenosine was mainly inhibitory in Th1 responses, its effect on Th17 responses is often enhancing (Liang et al., 2014b).

We have previously demonstrated that adenosine played an important role in skewing DC differentiation (Chen et al., 2015; Liang et al., 2015; Zhao et al., 2016). Since CD73 is pivotal in the conversion of ATP into adenosine (Beavis et al., 2012; Rabinovich et al., 2007), and since immune cells that express different levels of CD73 are functionally distinct (Naganuma et al., 2006; Zarek et al., 2008), we wished to determine whether adenosine affected γδ-DC interaction. Our results showed that prior treatment of BMDCs with ADA - an enzyme that degrades adenosine into functionally inactive molecules (North and Cohen, 1978; Ullman et al., 1976), diminished the Th17-stimulating activity of BMDCs, indicating that adenosine is crucially involved in the regulatory function of γδ T cells (Liang et al., 2014b, 2015; Liang et al., 2016a).

In summary, our studies demonstrated that the γδ T cell-mediated immunoregulation is strongly affected by reciprocal interactions between adenosine- and γδ T cells. γδ T cell stimulated DCs have a greater ability to promote Th17 pathogenic T cell responses. After exposure to TLR ligand and adenosine BMDCs acquired increased γδ-stimulating effect, and thus, promoted γδ activation. Furthermore, both TLR ligands and extracellular adenosine appear to play an important role in the balance of Th1 and Th17 pathogenic responses with their reciprocal interactions leading to augmented autoimmune responses.

4. Conclusion

In this study we present experimental evidence that mouse bone marrow-derived dendritic cells (BMDCs) acquired an increased ability to enhance Th17 autoimmune responses after exposure to γδ T cells; meanwhile, after exposure, a significant portion of the BMDCs expressed CD73 – a molecule that is fundamental in the conversion of immunostimulatory

ATP into immunosuppressive adenosine. $CD73^+$ BMDCs were uniquely effective in stimulating the Th17 responses, as compared to $CD73^-$ BMDCs and treatment of BMDCs with the CD73-specific antagonist APCP abolished the enhancing effect of the BMDCs. Moreover, BMDCs acquired increased ability to stimulate γδ activation after pre-exposure to TLR ligands and adenosine and the reciprocal interactions between γδ T cells and DCs play a major role in promoting Th17 responses.

5. Materials and methods

5.1. Animals and reagents

Female C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME); 12- to 16-week-old mice were used in all studies. $CD73^{-/-}$ knockout mice obtained from Jackson Laboratory are available in our laboratory (Liang et al., 2016a). All mice were housed and maintained in the animal facilities of the University of California Los Angeles. Institutional approval (Protocol number: ARC#2014–029-03A) was obtained from the Institutional Animal Care and Use Committee of the Doheny Eye Institute, University of California Los Angeles, and institutional guidelines regarding animal experimentation were followed. Veterinary care was provided by IACUC faculty. Any immunized animal that displayed joint swelling was either humanely euthanized or administered an analgesic (buprenorphine, 0.1 mg/kg sc. twice daily or ketoprofen, 5 mg/kg sc. daily) until the swelling resolved. By the end of the study, mice were euthanized by cervical dislocation after a lethal injection of ketamine and xylazine before tissue collection.

Recombinant murine IL-12 and IL-23 were purchased from R & D Systems (Minneapolis, MN). FITC-, PE- or allophycocyanin-conjugated antibodies against the mouse αβ TCR, IFN-γ, IL-17, Foxp3 and isotype control antibodies were purchased from e-Bioscience (San Diego, CA). ATP was purchased from Sigma-Aldrich (St. Louis, MO). The non-selective AR agonist 50-N-ethylcarboxamidoadenosine (NECA) (Csóka et al., 2012; Ryzhov et al., 2011) and the CD73 inhibitor α, β-methylene ADP (APCP) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2. Prepare γδ T cells

Non-activated and activated γδ T cells were separated from either naïve B6 mice or $IRBP_{1–20}$-immunized B6 mice, respectively (Liang et al., 2013b, a), by positive selection using a combination of FITC-conjugated anti-TCR-δ antibody and anti-FITC antibody-coated Microbeads, followed by separation using an auto-MACS.

5.3. Prepare autoreactive T cells from mice immunized with autoantigen ($IRBP_{1–20}$)

B6 mice were subcutaneously injected with an emulsion containing 200 μg of the human interphotoreceptor retinoid-binding protein (IRBP) peptide ($IRBP_{1–20}$, Sigma-Aldrich) in PBS and CFA (Difco, Detroit, MI) at 6 spots at the tail base and on the flank, followed by i.p. injection of 300 ng of pertussis toxin. αβ T cells used were purified from the spleen or draining lymph nodes of immunized mice at day 13 post-immunization using an auto-MACS separator system, as described previously [29]. The purity of the purified cells was >95 %, as determined by flow cytometric analysis using PE-conjugated antibodies

against αβ T cells. The cells were co-cultured for 48 h with irradiated (5000 Rad) spleen cells (1.5×10^6/well) as APCs and $IRBP_{1–20}$ (10 μg/mL) in a 24-well plate under either Th1 (culture medium supplemented with 10 ng/mL of IL-12) or Th17 polarized conditions (culture medium supplemented with 10 ng/mL of IL-23) (Liang et al., 2013b, a). Cytokine (IFN-γ and IL-17) levels in the serum and 48 h of culture supernatants were measured by ELISA (R & D Systems).

5.4. Intracellular staining and FACS analysis

For intracellular staining, T cells (2×10^5 in 100 μl of PBS) harvested 5 days post in vitro stimulation with the immunizing peptide and APCs were incubated for 4 h with 50 ng/mL of PMA, 1 μg/mL of ionomycin, and 1 μg/mL of brefeldin A (Sigma-Aldrich), then were washed, fixed, permeabilized overnight with Cytofix/Cytoperm buffer (eBioscience), intracellularly stained with antibodies against IFN-γ and IL-17, and analyzed on a $FACS_{calibur}$ flow cytometer.

5.5. Generation of BMDCs

BMDCs were generated by incubation of bone marrow cells for 5 days in the presence of 10 ng/mL of recombinant murine GM-CSF and IL-4 (R&D Systems), as described previously (Inaba et al., 1992). Cytokine (IL-1, IL-6, L-12 and IL-23) levels in the culture medium were measured by ELISA. To determine antigen-presenting function, BMDCs were incubated in a 24-well plate with responder T cells isolated from immunized B6 mice under Th1- or Th17-polarizing conditions. Forty-eight hours after stimulation, IFN-γ and IL-17 in the culture medium were measured by ELISA. 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 as described above.

5.6. Experimental setting for T cell-dendritic cell interaction and measurement of Th1 and Th17 responses

In 6-well plates, 2×10^6/well BMDCs were co-cultured with 2×10^5/well γδ or αβ T cells at a ratio of T: DC = 1:10. The T cells were removed after 24 h and the collected BMDCs were irradiated (5000 Rad), recounted, and seeded to a 24-well plate (3×10^4 /well). Responder $CD3^+$ cells (1×10^6/well) isolated from immunized TCR-$\delta^{-/-}$ mice were then added. Five days later, the percentage of IFN-γ^+ and IL-17^+ T cells among the responder T cells was determined after an intracellular staining followed by FACS analysis, as described previously (Liang et al., 2014a).

5.7. ELISA measurement of cytokine

ELISA kits (E-Bioscience) were used to measure serum IFN-γ and IL-17 levels on day 13 post-immunization and in the 48 h culture supernatants of responder T cells isolated from $IRBP_{1–20}$-immunized B6 or TCR-$\delta^{-/-}$ mice on day 13 post-immunization. Purified $CD3^+$ T cells from $IRBP_{1–20}$-immunized TCR-$\delta^{-/-}$ mice were stained with CFSE (Sigma-Aldrich) as described previously. Briefly, the cells were washed and suspended at 50×10^6 cells/mL in serum-free RPMI 1640 medium; incubated at 37 °C for 10 min with gentle shaking with a final concentration of 5 μM CFSE; then washed twice with, and suspended in, complete

medium, stimulated with immunizing peptide in the presence of APCs, and analyzed by flow cytometry.

5.8. Statistical analysis

The results in the figures are from a representative experiment, which was repeated 3–5 times. The statistical significance of differences between groups in a single experiment was initially analyzed by ANOVA, and, if a statistical significance was detected, the Student–Newman–Keuls post-hoc test was subsequently used. A P value less than 0.05 was considered statistically significant (indicated as **).

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Abbreviations:

AR	adenosine receptor
ATP	adenosine triphosphate
A2AR	adenosine 2A receptor
APCP	α,β-methylene adenosine 5′-diphosphate
EAU	experimental autoimmune uveitis
IRBP	interphotoreceptor retinoid-binding protein
NECA	5′-N- ethylcarboxamidoadenosine

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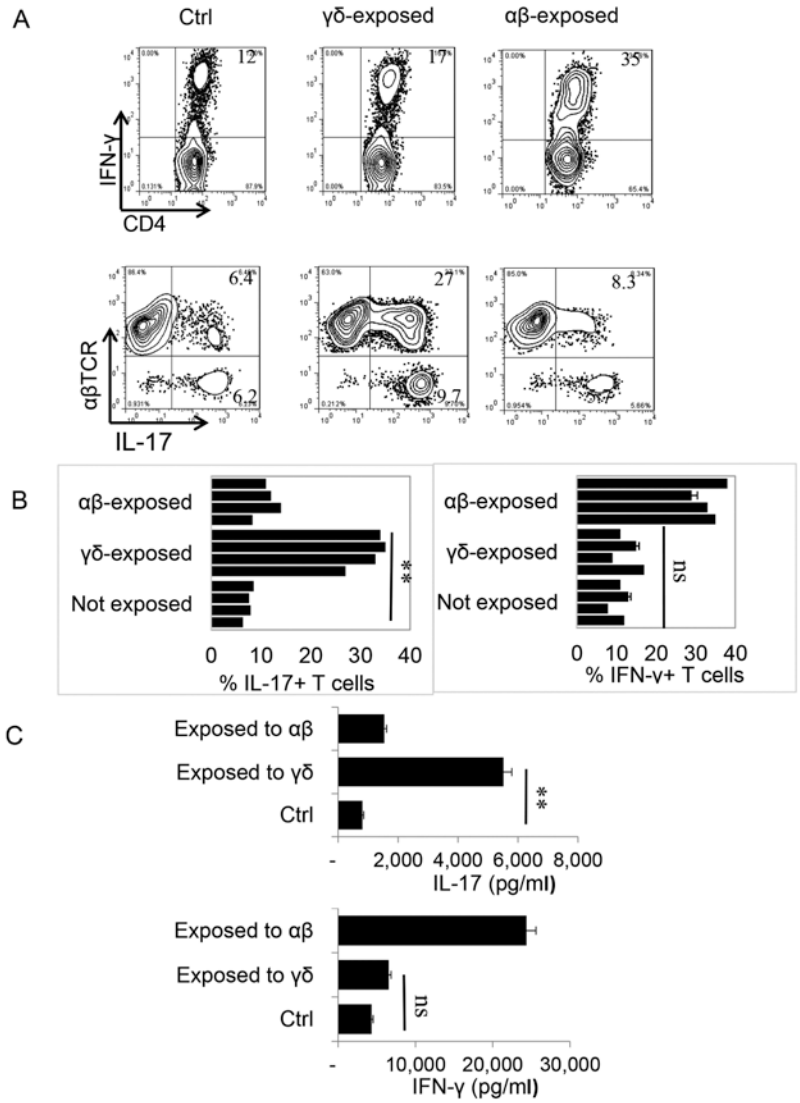

Fig. 1. Bone marrow dendritic cells (BMDCs) acquired an enhanced Th17-promoting effect after exposed to γδ T cells, whereas pre-treatment with αβ T cells resulted in an augmented Th1-promoting activity.

(A&B) BMDCs were generated by incubation of bone marrow cells for 5 days in GM-CSF and IL-4-containing medium, γδ T cells and $CD3^+$ responder αβ T cells were prepared from $IRBP_{1–20}$-immunized B6 mice (day 13) by MACS sorting. For pre-treatment of DC with γδ T cells, 10^6/well BMDCs were cultured in 6-well plate, with or without γδ or αβ T cells, at a T: BMDC ratio of 1:20. The BMDCs were collected after 24 h, irradiated, and re-seeded into 24-well plates. For AP function test, BMDCs, with or without prior exposure to αβ/γδ T cells were cocultured with responder T cells in the presence of the immunizing peptide ($IRBP_{1–20}$) and indicated BMDCs. Five days after stimulation, the responder T cells were then double-stained with anti-mouse αβTCR and PE-anti-mouse IL-17/IFN-γ and analysed by flow cytometry. Results of four separate assays were pooled and shown (B).

(C) ELISA test of IFN-γ and IL-17 production of the responder T cells activated in the presence of BMDCs with or without exposure to γδ T cells. Two days after in vitro

stimulation, under either Th1 or Th17 polarized conditions, a fraction of the culture supernatants of the responder T cells were collected for ELISA test. The numbers indicated are calculated from triplicated samples. Data are from a single experiment, representative of three independent experiments. **, $P < 0.05$; ns, not significant.

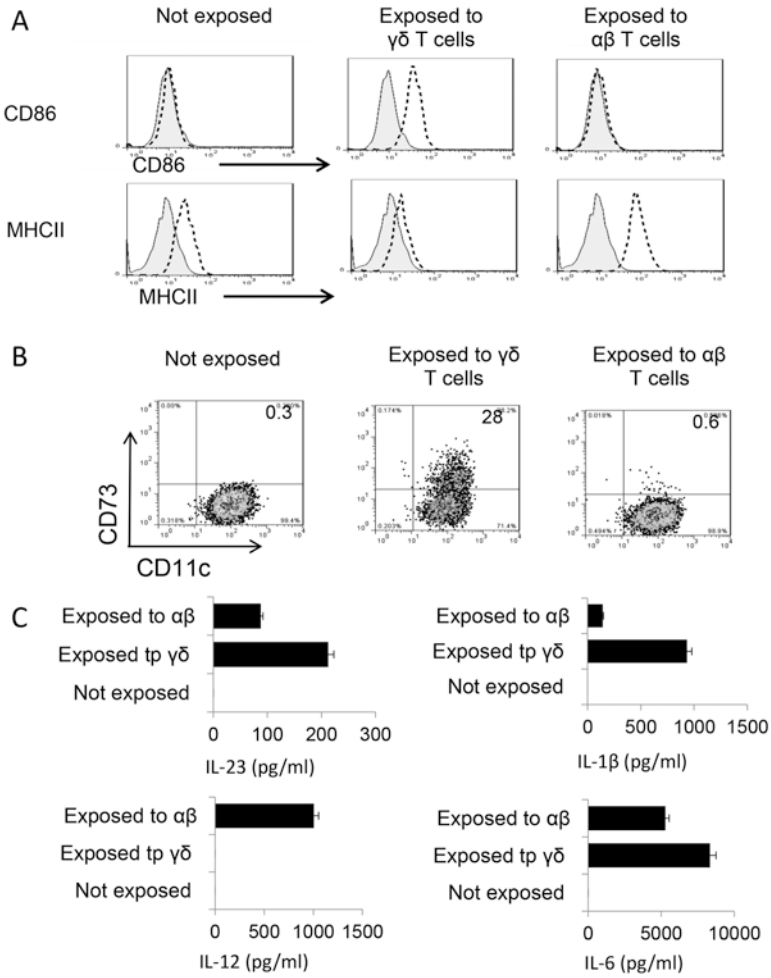

Fig. 2. A significant numbers of bone marrow derived cells (BMDCs) became CD73^{+} after exposure to γδ T cells.

A) BMDCs were stained for expression of CD86 and MHC class II molecules, with or without a prior exposure to γδ (middle panels) or αβ (right panels) T cells.

B) BMDCs were stained for expression of CD73 molecules, with or without prior exposure to γδ (middle panels) or αβ (right panels) T cells.

C) Cytokine production by BMDCs without prior exposure to γδ or αβ T cells.

The results are from a single experiment (n = 6) and are representative of three independent studies.

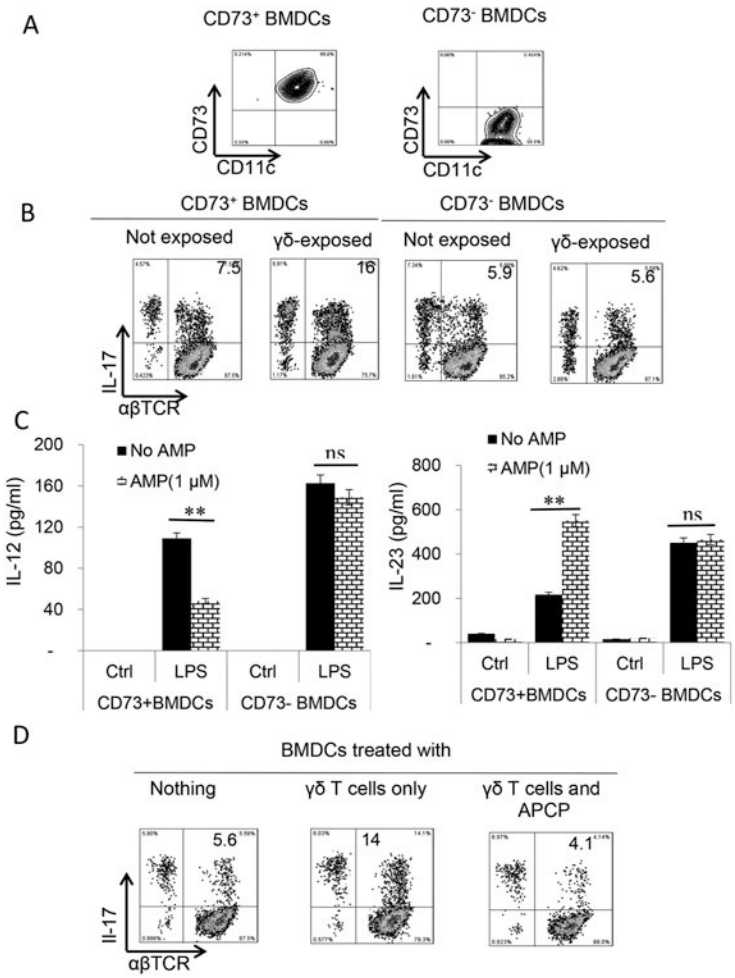

Fig. 3. Functional test of separated CD73$^{+/+}$ and CD73$^{-/-}$ BMDCs.

A) Purity test of separated CD73^{+} and CD73^{-} BMDCs. CD11c^{+}CD73^{+} and CD11c^{+}CD73- BMDCs were separated from γδ T cell-treated BMDCs, using a MACS-column and dually stained with CD11c and CD73 followed by FACS analysis.

B) Compare AP function of CD73^{+} and CD73^{-} BMDCs. Responder T cells were prepared from IRBP$_{1-20}$-immunized B6 mice. They were stimulated by CD73$^{+/+}$ (left panels) or CD73$^{-/-}$ BMDCs (right panels), with or without a γδ T cell exposure (detailed in Fig.1 legend), under Th17 polarizing conditions. The responder T cells were then double-stained with anti-mouse αβTCR and PE-anti-mouse IL-17/IFN-γ and analysed by flow cytometry.

C) Cytokine-producing ability of separated CD73$^{+/+}$ and CD73$^{-/-}$ BMDCs. CD73$^{+/+}$ and CD73$^{-/-}$ BMDCs were assessed for production of IL-12 and IL-23, before and after treatment with LPS (100 ng/mL) or/and AMP (1 μM). ** $P < 0.05$; ns, not significant.

D) Treatment of BMDCs with CD73 specific antagonist APCP abolished the enhancing effect on Th17 responses. AP functions of γδ-exposed BMDCs were determined with or without a prior treatment of BMDCs with APCP (3 μM) after BMDCs were exposed to γδ T cells.

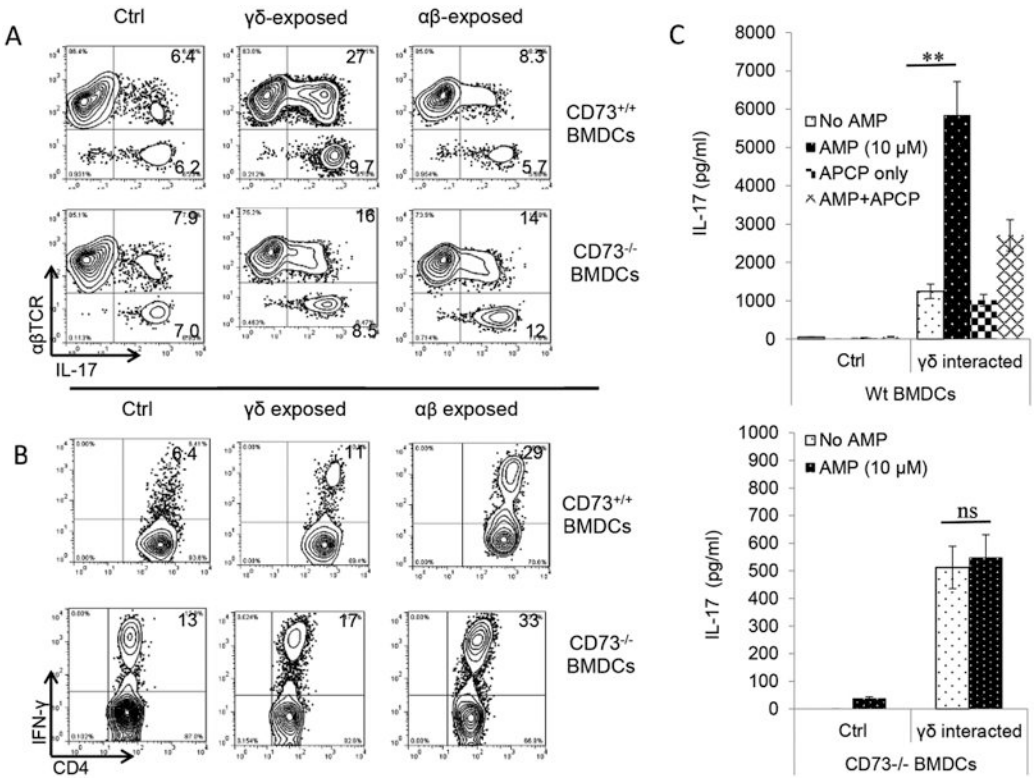

Fig. 4. CD73$^{-/-}$ BMDCs resisted the γδ-inducing enhancing effect.

A&B) Compared Th1- and Th17-promoting effects of CD73$^{+/+}$ and CD73$^{-/-}$ BMDCs. CD73$^{+/+}$ and CD73$^{-/-}$ BMDCs were isolated from B6 mice and CD73$^{-/-}$ mice, respectively. Responder αβ T cells were prepared from IRBP$_{1-20}$-immunized B6 mice. AP function of BMDCs was determined, before and after an exposure to γδ and αβ T cells, under Th1 (A) and Th17 (B) condition. The results show that the CD73$^{-/-}$ BMDCs have a limited increased Th17-promoting activity after exposure to γδ T cells, as compared to CD73$^{+/+}$ BMDCs. C) The cytokine assessment of the BMDCs' AP function. Two days in vitro stimulation by CD73$^{+/+}$ (upper panels) or CD73$^{-/-}$ BMDCs (lower panels), under Th17 polarized conditions, and in the presence or absence of AMP, APCP, or AMP plus APCP. Culture supernatants of the responder T cells were collected for ELISA test. **, $P < 0.05$; ns, not significant.

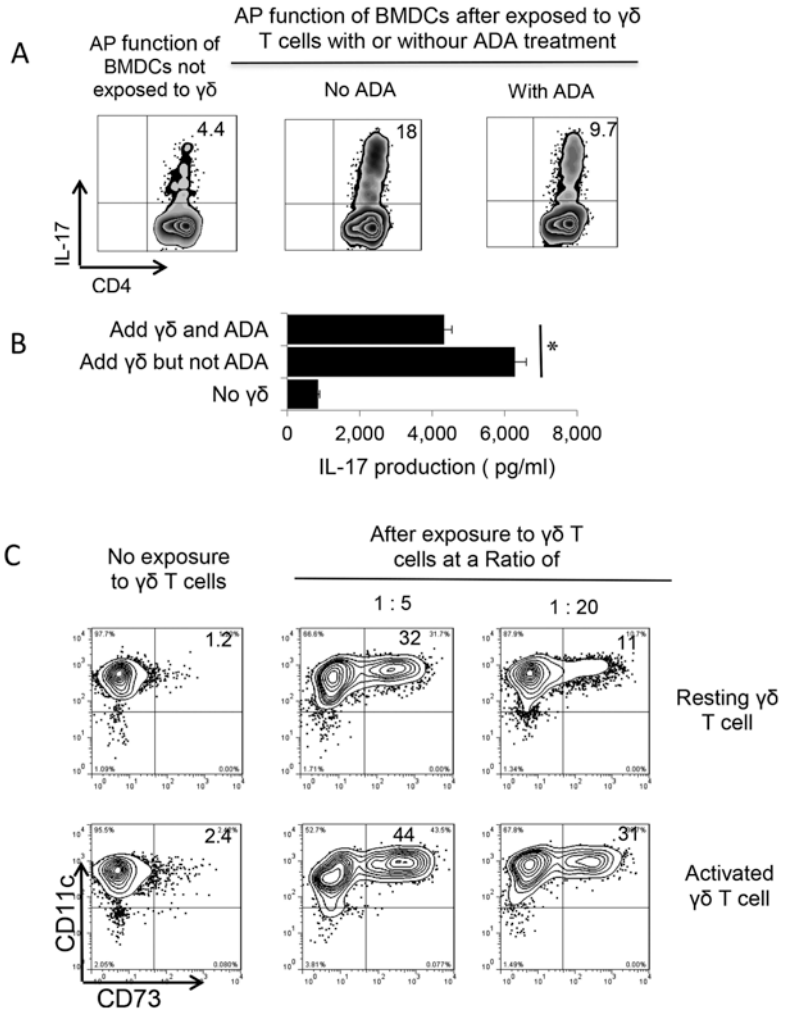

Fig. 5. γδ-DC interactions were affected by adenosine.

A&B) Treatment of γδ-DC interacting cells with ADA – an adenosine-degradation enzyme - reduced the enhancing effect of Th17 responses. γδ-DC interacting cultures were tested for Th17-promoting effects, with or without treatment of ADA (1U), as evaluated by IL-17$^+$ cells in the responder T cells (A), and the produced cytokines by the responder T cells (B). **, $P < 0.05$.

C). Activated γδ T cells were a more potent inducer of CD73$^+$ bone marrow derived cells (BMDCs). BMDCs were cultured with γδ T cells at various ratios (from 0 to 1:5). After 24 h, BMDCs were collected, irradiated (3000 Rad), and re-seeded into 24-well plates for AP function test. The BMDCs were then double-stained with anti-mouse CD73 and PE-anti-mouse CD11c and analyzed by flow cytometry.

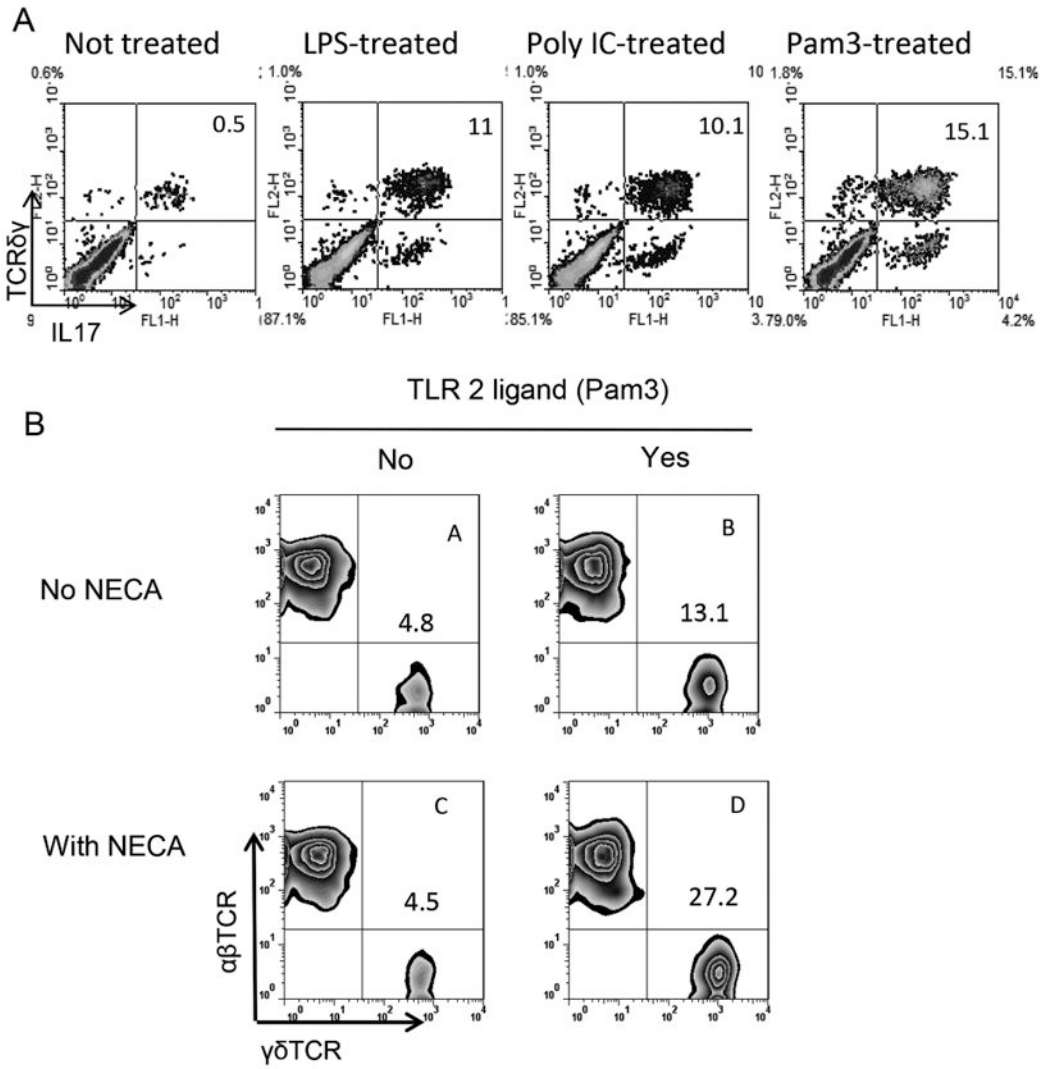

Fig. 6. BMDCs gained increased γδ-stimulating activity after treatment with TLR ligand and adenosine.

A) Responder T cells obtained from IRBP-immunized B6 mice were co-cultured with BMDCs (T:DC ratio = 10:1) which were pre-exposed to TLR4 (LPS), TLR3 ligand (Poly IC) or TLR2 ligand (Pam3), as indicated.

B) Synergistic effect between adenosine analogue NECA and TLR3 ligand (Pam3) in augmenting BMDCs' γδ-stimulating activity. BMDCs were treated with a Pam3 (right panels) or remained untreated (left panels), and with or without NECA (100 nM) treatment for 24 h., before co-cultured with responder CD3+ T cells. After 48 h, the responder T cells were stained with PE-anti-αβ and FITC-anti-γδ antibodies, followed by FACS analysis.