

CD39⁺ regulatory T cells attenuate allergic airway inflammation

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Summary

Background The suppressive mechanism of regulatory T cells (Tregs) has remained incompletely clarified. Recent studies found that CD39 expressed by Tregs may participate in the immunoregulatory role of Tregs. CD39-induced ATP hydrolysis and/or adenosine generation contribute to the suppressive mechanism of Tregs. Previous studies suggested that ATP is involved in allergic airway inflammation by acting on type 2 purinergic (P2) receptors, but the role of CD39 and CD39⁺Tregs in allergic airway inflammation has not been elaborated.

Objective To investigate the role and underlying mechanism of CD39 expression by Tregs in allergic airway inflammation.

Methods A model of allergic asthma was developed with ovalbumin–alum in female *Cd39* wild type (*Cd39*^{+/+}) and deficient (*Cd39*^{-/-}) C57BL/6 mice. Foxp3-GFP knock-in *Cd39*^{+/+} and *Cd39*^{-/-} mice were used to sort CD4⁺GFP⁺ cells (Tregs) for exploring the role of CD39 expression by Tregs in allergic asthma. The effects of modulating CD39 activity with ARL67156 (inhibitor) or apyrase were also observed.

Results ARL67156 greatly worsened airway inflammation including increased lung inflammatory cells infiltration, goblet cell hyperplasia, and higher levels of Th2 and Th17 cytokines in bronchoalveolar lavage fluid (BALF), accompanied by an increment in transcription factor (GATA-3 and RORγt) and P2R (P2Y2, P2Y4 and P2Y6) mRNA expression in lungs. This potentiating effect was rescued by intratracheal injection of apyrase. Airway inflammation was markedly increased in *Cd39*^{-/-} mice compared to *Cd39*^{+/+} mice. In contrast to CD39⁻Tregs, CD39⁺Tregs showed stronger suppressive effects on airway inflammation. *In vitro* suppression assay suggested that CD39⁺Tregs have more potent suppressive effect on cytokines secretion from CD4⁺CD25⁻ responder T cells and the inhibitory effects were reduced by addition of adenosine A2A receptor antagonist.

Conclusion CD39 expressed on Tregs participates in the regulation of limiting allergic airway inflammation by regulating extracellular ATP and/or adenosine. CD39 may represent a new therapeutic target for asthma.

Keywords adenosine, allergic asthma, ATP, CD39, regulatory T cells

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Introduction

Asthma is a common chronic airway inflammatory disease and characterized by airway inflammation, airway hyperresponsiveness and airway remodelling [1]. Th2 cell-mediated eosinophilic inflammation is still the predominant mechanism in the pathogenesis of asthma. Apart from Th2 cells, Th17 cells and

regulatory T cells (Tregs) have been proved to contribute to airway inflammation of asthma [2, 3]. Tregs play a critical role in maintaining immune tolerance to harmless environmental allergen. Previous studies demonstrated that the frequencies of peripheral natural occurring Tregs (nTregs) and IL-10-producing Tregs from asthma patients were decreased [4], and the suppressive capacities of nTregs from asthma patients were also reduced [2]. Exogenous supplement of CD4⁺CD25⁺ nTregs not only prevented the

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development of airway inflammation and remodelling but also reversed the established airway inflammation in a murine model of asthma [5]. These data demonstrate the protective role of Tregs in allergic asthma.

However, the suppressive mechanism of Tregs is still not fully understood. Studies suggest that suppressive cytokines such as IL-10, TGF- β and IL-35, and cell surface molecules such as cytotoxic T lymphocyte antigen 4 (CTLA4), programmed cell death-1 (PD-1), and B and T lymphocyte attenuator (BTLA) are involved in the inhibitory machinery of Tregs [2, 6]. Recently, ATP hydrolysis and/or adenosine production were found to represent another suppressive mechanism of nTregs through the co-expression of CD39 and CD73 [7–9]. CD39 is mainly expressed on vascular endothelial cells and a variety of immune cells such as dendritic cells, B lymphocytes and a subset population of CD4⁺ T lymphocytes [10]. CD39 is expressed by almost all nTregs in murine [11] and by 60% nTregs in human [12]. nTregs from *Cd39*-deficient mice showed damaged suppressive function to cytokines production from responder T cells and failed to prevent allograft rejection sufficiently [7]. The frequency of circulating CD39⁺Foxp3⁺ Tregs is reduced in multiple sclerosis; CD39⁺CD4⁺CD25^{high} T cells from healthy controls suppress IL-17, whereas CD39[−]CD4⁺CD25^{high} cells generate IL-17 [13]. CD39 is also known as ecto-nucleoside triphosphate diphosphohydrolase-1 (ENTPDase1) and can degrade ATP and ADP to AMP. Extracellular ATP (eATP) concentration is very low under physiological condition. Once released, eATP exerts multiple pro-inflammatory effects by acting on type 2 purinergic receptors (P2R). ATP is sequentially degraded to AMP by CD39, and AMP is then degraded to adenosine by CD73. Adenosine suppresses the proliferation and cytokine production of responder T cells by binding to A2A receptors which are mainly expressed on responder T cells [13]. Therefore, CD39 expressed on nTregs contributes to the inhibitory effects by regulating ATP and/or adenosine.

Recent studies on murine model indicated that eATP is implicated in the pathogenesis of allergic asthma by triggering the migration of eosinophils and dendritic cells (DCs) and promoting the maturation and activation of DCs [14–17]. Building on these evidences, we hypothesized that CD39⁺Tregs limit the development of allergic asthma by the removal of eATP and/or adenosine generation. To verify this hypothesis, we determined the role of CD39 in allergic airway inflammation using a CD39 inhibitor and *Cd39*-deficient mice. In addition, we evaluated the effect of CD39⁺Tregs and CD39[−]Tregs on airway inflammation and elucidated the possible mechanism of CD39⁺Tregs limiting allergic airway inflammation in a mouse model of asthma.

Materials and methods

Animals

C57BL/6, Foxp3-GFP knock-in *Cd39*^{+/+} and *Cd39*^{−/−} mice [18, 19] were bred at the Experimental Animal Center of Wuhan University. All animal experiments were approved by the institutional animal ethics committee from Wuhan University.

Cell preparation

nTregs were sorted as CD4⁺GFP⁺ cells from male Foxp3-GFP knock-in *Cd39*^{+/+} and *Cd39*^{−/−} mice (6–14 weeks) using an EasyStep[™] mouse CD4⁺ T cell isolation kit (Stem Cell Technologies Inc, Canada) combination with fluorescence activating cell sorter (FACS). Responder T cells were sorted as CD4⁺CD25[−] cells from male C57B/L6 mice by FACS. The purity of cells was typically greater than 90% by FACS.

Animal experimental protocols

Female mice (6–8 weeks) were sensitized by intraperitoneal (i.p.) injection of 20 μ g OVA (Sigma-Aldrich, St Louis, MO, USA) emulsified in 2 mg alum (Pierce, Rockford, IL, USA) in a total volume of 200 μ L on days 0 and 14. Mice were then challenged by intranasal administration of 100 μ g OVA in a total volume of 50 μ L on days 25–27. Littermates only received allergen challenge as controls.

In some experiments, mice received an i.p. injection of 5 mg/kg ARL67156 (CD39 inhibitor), or PBS, or an intratracheal (i.t.) injection of 4 unit apyrase plus ARL67156 (Sigma-Aldrich) thirty minutes before each OVA challenge. In some experiments, mice received an intravenous (i.v.) injection of 1×10^6 CD4⁺GFP⁺ cells sorted from Foxp3-GFP knock-in *Cd39*^{+/+} and *Cd39*^{−/−} mice, or equal volume of PBS one hour before the first allergen challenge.

Measurement of airway hyperresponsiveness

On day 29, mice were anesthetized and inserted with a tracheostomy tube, followed by mechanical ventilation. Airway resistance (R_L) and dynamic compliance (C_{dyn}) to increasing concentrations of aerosolized methacholine (range from 3.12 to 50 mg/mL) were measured using the FinePointe RC system (Wilmington, NC, USA).

Bronchoalveolar lavage and lung histology

After performing the measurement of airway hyperresponsiveness (AHR), lungs were lavaged with a total volume of 1.5 mL PBS containing 1 mM sodium EDTA, followed by lung resection, as previously described

[20]. Cells in bronchoalveolar lavage fluid (BALF) were collected and were stained with Wright–Giemsa for differential cell counts.

Left lungs were fixed in 4% paraformaldehyde buffer, dehydrated and embedded in paraffin. Lung sections were stained with haematoxylin and eosin (HE) for evaluating inflammation and with periodic acid–Schiff (PAS) for goblet cell hyperplasia. Tissue inflammation was quantified according to a subjective scale of 0–3 [21]. PAS⁺ areas (A_{PAS}^{+}) and basement membrane perimeter (P_{bm}) were measured using Image Pro Plus 6.0 software. The degree of goblet cell hyperplasia was represented as A_{PAS}^{+} per Pbm.

Real-time quantitative PCR

For mRNA expression, total RNA from right lungs was extracted with TRIzol reagent (Invitrogen, CA, USA). cDNA was synthesized with a ReverTra Ace quantitative PCR (qPCR) RT Master Mix kit (Toyobo, Tokyo, Japan), and qPCR was performed in triplicates using a SYBR Premix Ex Taq™ kit (Takara, Tokyo, Japan) according to the manufacturer's protocols. GAPDH was used as the internal reference. The primer sequences are presented in Table 1. The data were normalized to GAPDH, and the interest gene expression was analysed using $2^{-\Delta\Delta C_t}$.

T cell culture and suppression assay

CD4⁺CD25[−] responder T cells were seeded at a density of 1×10^5 cells/well in 96-well plate precoated with 5 µg/mL anti-CD3 and cultured in an RPMI-1640

Table 1. Primer sequences and product sizes

Gene name	Primer sequences	Product size(bp)
Rorc	Sense 5'-AGCGGCTTTCAGGCTTCATG-3'	150
	Antisense 5'-CCACACCACCGTATTGCCT-3'	
Gata3	Sense 5'-AGGGACATCCTGCGGAACTGT-3'	166
	Antisense 5'-CATCTTCCGGTTTCGGGTCTGG-3'	
P2ry2	Sense 5'-AGGGTACTTCAGCACAAACCA-3'	114
	Antisense 5'-TCCTGCCTAAACTCCAGAGC-3'	
P2ry6	Sense 5'-GACAGGCAGTTATGGAGCAG-3'	101
	Antisense 5'-GGTTAGCAGCAGTCGCTTG-3'	
P2ry1	Sense 5'-TTGGAATCTGCTCCTCTGCT-3'	137
	Antisense 5'-CACGATGGGTAGTGTCTCTG-3'	
P2ry4	Sense 5'-CAGGCGATAGGATTCAAGG-3'	116
	Antisense 5'-GCTCCATACACCCACCAGAT-3'	
GAPDH	Sense 5'-TGTGTCCGTCGTGGATCTGA-3'	150
	Antisense 5'-TTGCTGTTGAAGTCGACGAG-3'	

Rorc, RAR-related orphan receptor gamma; Gata3, GATA binding protein 3; P2ry2, purinergic receptor P2Y, G-protein-coupled 2; P2ry6, purinergic receptor P2Y, G-protein-coupled 6; P2ry1, purinergic receptor P2Y, G-protein-coupled 1; P2ry4, purinergic receptor P2Y, G-protein-coupled 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

medium supplemented with 10% FBS, 1% streptomycin/penicillin, 1 mM sodium pyruvate, 2 mM L-glutamine, 5 mM Hepes and 2 µg/mL soluble anti-CD28.

Responder T cells were cocultured with different ratios of nTregs sorted from $Cd39^{+/+}$ and $Cd39^{-/-}$ mice. Responder T cells were stimulated with 10 µM adenosine, or 5 µM adenosine A2A receptor antagonist ZM241385 (Sigma-Aldrich). In addition, ZM241385 was added to coculture system. Cell culture supernatants were harvested three days later.

Detection of cytokines

The supernatants in BALF and cell culture were collected to detect cytokine levels by ELISA according to the manufacturer's protocol. The lower limit of detection was 4 pg/mL for IL-4, IL-5 and IL-17; 15 pg/mL for IFN-γ (eBioscience, San Diego, CA, USA).

Analysis of nTregs by FACS

Foxp3-GFP knock-in $Cd39^{+/+}$ and $Cd39^{-/-}$ mice were analysed for the frequency of nTregs. Mononuclear cells from the lungs and spleens were isolated as previously described [20]. Cells were stained with CD4 PE-cy5 (eBioscience, San Diego, CA, USA), and CD4⁺GFP⁺ cells were analysed by FACS.

Statistical analysis

Data analysis was performed with one-way ANOVA or Student's *t*-test using SPSS 17.0 software (IBM, New York, USA). Data were expressed as mean ± SEM. *P* < 0.05 was regarded as statistical difference.

Results

Inhibition of CD39 activity increased allergic airway inflammation

The OVA-sensitized and OVA-challenged mice showed typical asthma features including airway inflammation, goblet cell hyperplasia, higher levels of Th2 cytokines and AHR (Fig. 1). Peribronchial and perivascular inflammation was detected in HE-stained lung sections as well as PAS-positive goblet cells in OVA-sensitized and OVA-challenged mice (Fig. 1a). ARL67156, which inhibits the hydrolysis activity of CD39 and results in ATP accumulation, markedly increased inflammatory cells infiltration in the lungs and numbers of PAS-positive cells (Fig. 1b). In accordance with these results, the numbers of total cells, eosinophils and lymphocytes in BALF were significantly higher in sensitized and challenged mice treated with ARL67156 than asthma mice treated with

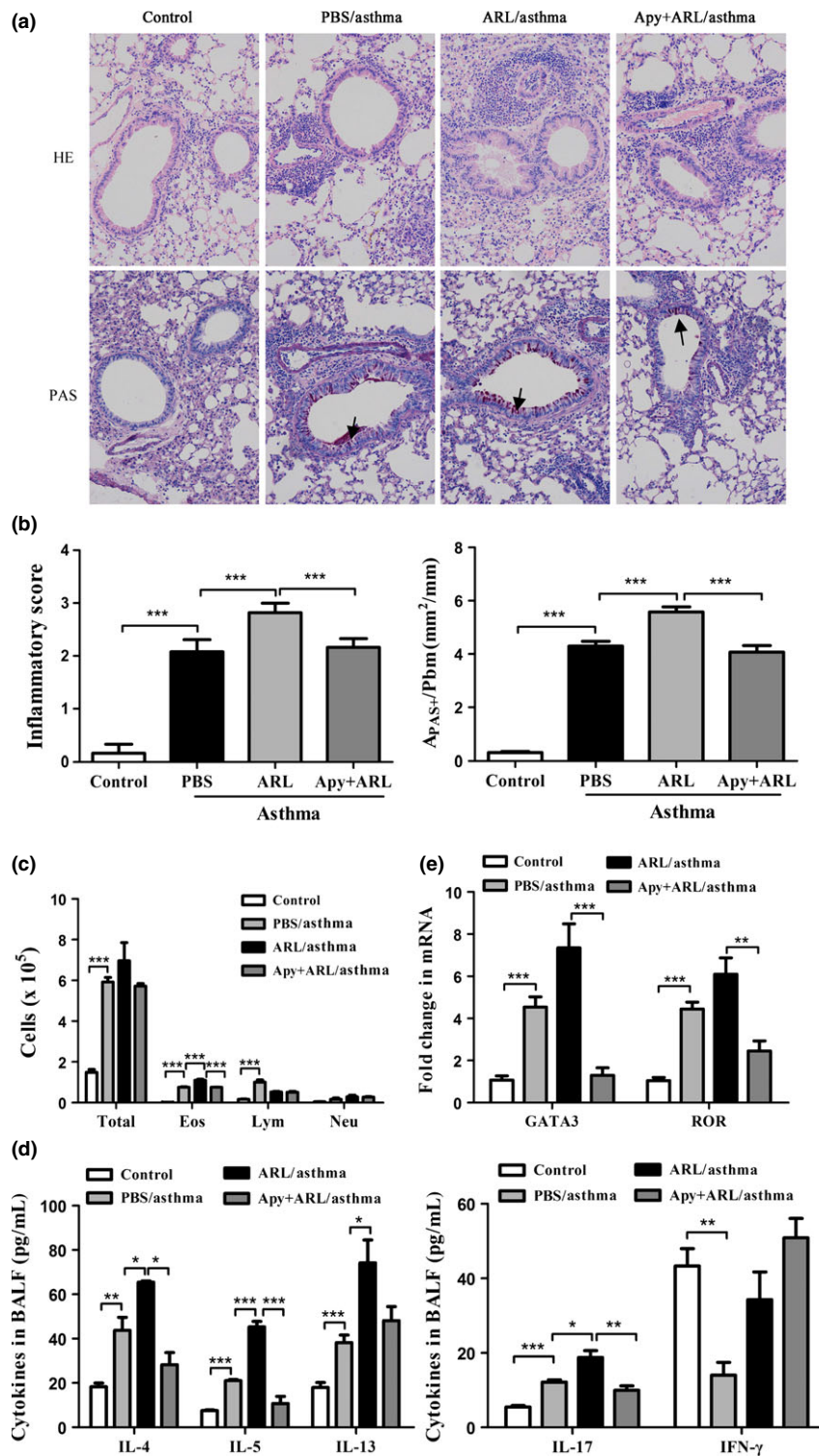


Fig. 1. Increased allergic airway inflammation in ARL67156-treated mice. Female C57BL/6 mice were sensitized at days 0 and 14 and challenged at days 25–27. Mice were received an intraperitoneal (i.p.) injection of PBS, or an i.p. injection of 5 mg/kg ARL67156 (ARL), or an i.t. injection of 4 unit apyrase (Apy) plus ARL67156 30 minutes before each challenge. Littermates only received allergen challenge as control. (a) Lung sections stained with HE and PAS (arrow) ($\times 200$). (b) Inflammatory score and PAS-positive areas per unit length were determined. BALF was analysed for (c) the number of total and different cells and (d) cytokines (IL-4, 5, 13, IFN- γ and 17) (Eos, eosinophils; Lym, lymphocytes; Neu, neutrophils). Lungs were measured for (e) GATA3 and ROR γ t and (f) P2YR mRNA. (g) Airway responsiveness to increased concentration of Mch was measured. *Control *vs* asthma, #PBS/Asthma *vs* ARL/Asthma. All data are expressed as mean $\pm$ SEM of three independent experiments ($n = 4-6$). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; # $P < 0.01$; NS, not significant.

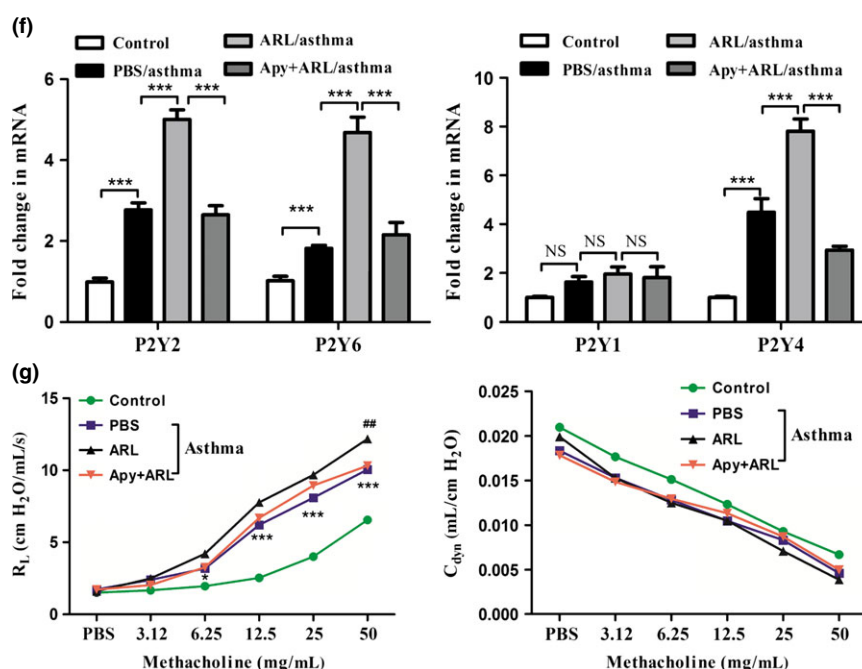


Fig. 1. Continued.

PBS (Fig. 1c). The levels of IL-4, IL-5, IL-13 and IL-17 in BALF were higher in sensitized and challenged mice treated with ARL67156 than that in PBS-treated mice (Fig. 1d), accompanied by an increase in GATA3 and ROR γ t mRNA expression in the lungs (Fig. 1e). Then, we determined whether ARL67156-worsened allergic airway inflammation was linked with up-regulated expression of P2R mRNA in the lungs. mRNA expression of P2Y2, P2Y4 and P2Y6 was markedly elevated in OVA-sensitized and challenged mice received ARL67156 treatment in contrast to mice received PBS treatment (Fig. 1f). Increased airway resistance to methacholine and decreased compliance were found in OVA-sensitized and challenged mice in comparison to control mice (Fig. 1g). However, no significance in R_L and C_{dyn} was observed among different treatment groups.

Apyrase from potato has the same function of scavenging ATP as CD39. We evaluated whether pretreatment with apyrase could rescue ARL67156-enhanced airway inflammation. As expected, we found apyrase reduced eosinophilia in the lungs and BALF. In addition, goblet cell hyperplasia and the levels of Th2 and Th17 cytokines as well as transcriptional factors were also reduced by apyrase (Fig. 1). Overall, these data indicate a protective role of CD39 in allergic airway inflammation.

Cd39-deficient mice had increased allergic airway inflammation

Next, we assessed whether airway inflammation and AHR are increased in $Cd39^{-/-}$ mice. As shown in

Fig. 2, OVA-sensitized and OVA-challenged $Cd39^{-/-}$ mice showed a significant increase in inflammatory cells infiltration in the lungs and BALF as well as goblet cell hyperplasia when compared to OVA-sensitized and OVA-challenged $Cd39^{+/+}$ mice (Fig. 2a–c). Interestingly, not only the levels of IL-4, IL-5 and IL-17 but also the level of IFN- γ in BALF were significantly higher in $Cd39^{-/-}$ mice than that in $Cd39^{+/+}$ mice (Fig. 2d). No difference was detected in R_L and C_{dyn} between two groups (Fig. 2e). These results suggest that $Cd39$ deficiency results in increased airway inflammation.

A reduction in the frequency of nTregs is observed in $Cd39^{-/-}$ mice

We sought to determine whether the frequency of nTregs was abnormal, which led to increased airway inflammation in $Cd39^{-/-}$ mice. As shown in Fig. 3a and b, $Cd39^{-/-}$ mice demonstrated a significant reduction in the numbers of nTregs from spleen and lung mononuclear cells (MNCs) compared to $Cd39^{+/+}$ mice under physiological condition. The frequencies of nTregs from spleen MNCs (Fig. 3c) and lung MNCs (Fig. 3d) were decreased during allergic airway inflammation. No difference of nTregs from lung and spleen MNCs was detected between $Cd39^{-/-}$ and $Cd39^{+/+}$ mice during allergic asthma. These data demonstrate that the frequencies of systemic and local nTregs are decreased in $Cd39^{-/-}$ mice, and the numbers of systemic and local nTregs are reduced during allergic airway inflammation.

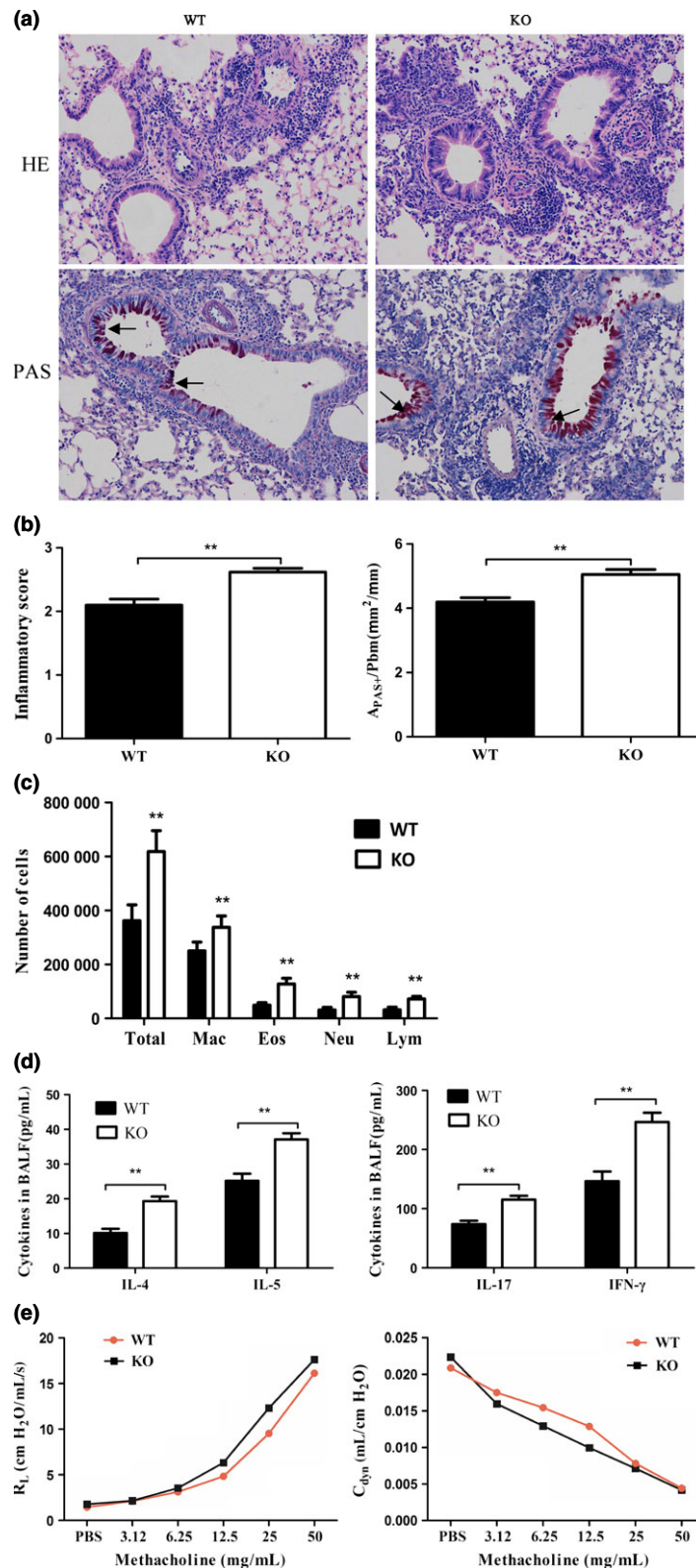


Fig. 2. Enhanced allergic airway inflammation in $Cd39^{-/-}$ mice. Female C57BL/6 $Cd39^{+/+}$ and $Cd39^{-/-}$ mice were sensitized at days 0 and 14 and challenged at days 25–27. (a) Lung sections stained with HE and PAS (arrow) ($\times 200$). (b) Inflammatory score and PAS-positive areas per unit length were determined. BALF was analysed for (c) the number of total and different cells and (d) cytokines (IL-4, 5, 13 and 17) (Eos, eosinophils; Lym, lymphocytes; Neu, neutrophils). (e) Airway responsiveness to increased concentration of Mch was measured. All data are expressed as mean $\pm$ SEM from three independent experiments ($n = 4$). ** $P < 0.01$.

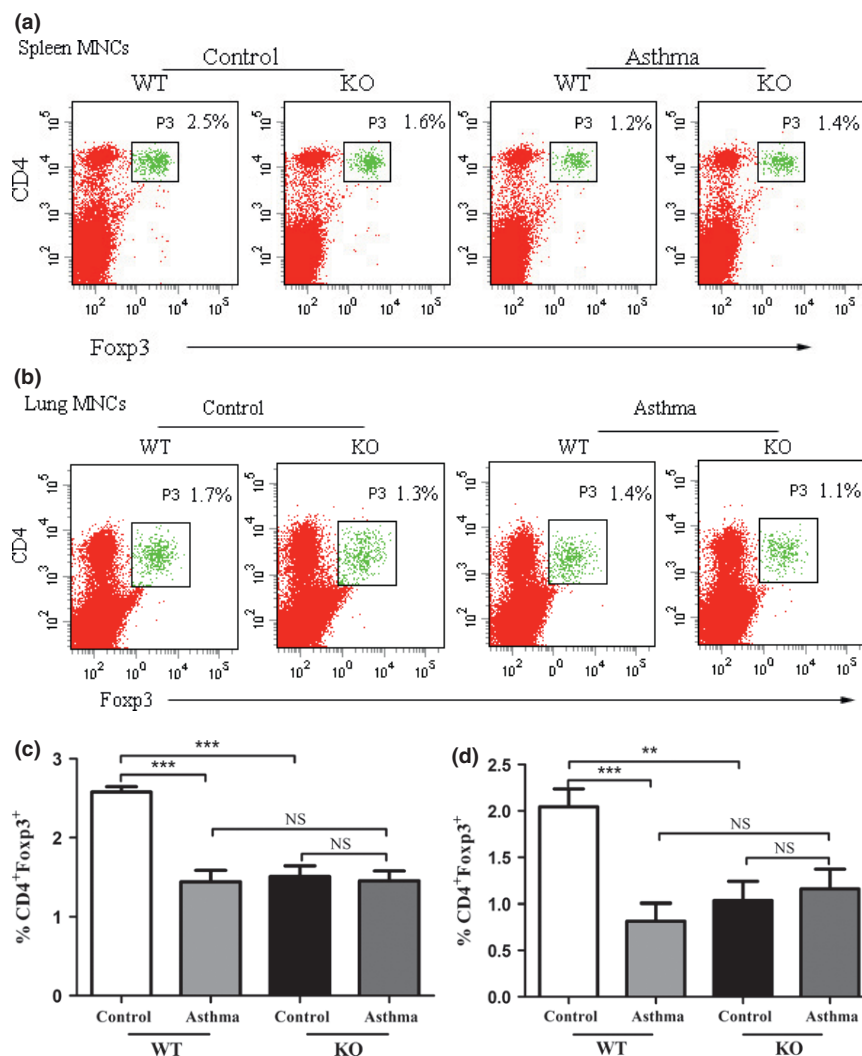


Fig. 3. A reduction in regulatory T cells in *Cd39*^{-/-} mice. Female C57BL/6 *Cd39*^{+/+} and *Cd39*^{-/-} mice were sensitized at days 0 and 14 and challenged at days 25–27. (a) Representative dot plots of CD4⁺GFP⁺ cells (Tregs) from spleen mononuclear cells (MNCs) in *Cd39*^{+/+} and *Cd39*^{-/-} mice. (b) Representative dot plots of Tregs from lung MNCs in *Cd39*^{+/+} and *Cd39*^{-/-} mice. (c) The frequency of spleen Tregs was reduced during allergic airway inflammation and was reduced in *Cd39*^{-/-} mice. (d) The frequency of lung Tregs was reduced during allergic airway inflammation and was reduced in *Cd39*^{-/-} mice. Data are mean $\pm$ SEM ($n = 6$) of 2 independent experiments ($n = 6$). Representative dot plot was shown from a single mouse. *** $P < 0.001$.

Impaired capacity of CD39⁻ Tregs to limit allergic airway inflammation

We evaluated whether *Cd39* deficiency in nTregs influences their suppressive effect on allergic airway inflammation. We adoptively transferred CD4⁺GFP⁺ cells (nTregs) sorted from *Cd39*^{+/+} and *Cd39*^{-/-} mice into OVA-sensitized *Cd39*^{+/+} mice prior to the first OVA challenge by i.v. injection. Histological analysis demonstrated that nTregs alleviated airway inflammation and goblet cell hyperplasia (Fig. 4a,b). In BALF, a reduced number of inflammatory cells (Fig. 4c) and lower levels of Th2 cytokines (Fig. 4d) were observed in mice received nTregs. Apart from down-regulation of Th2 cytokines, CD39⁺Tregs decreased IL-17 in BALF,

whereas CD39⁻Tregs had no significant effect on IL-17 level and neutrophils number (Fig. 4c,d). Compared to mice without cell transfer, mice received nTregs showed a decreased airway resistance (Fig. 4e). No significance in R_L and C_{dyn} was found in mice received CD39⁻Tregs and CD39⁺Tregs (Fig. 4e). These data demonstrated that nTregs from *Cd39*^{-/-} mice displayed impaired suppressive capacities to limit allergic airway inflammation.

Adenosine mediated the suppressive effect of CD39⁺ Tregs

To further explore the underlying inhibitory mechanism of CD39⁺ Tregs, we then evaluated the effects of adenosine and ZM241385, an adenosine A2A receptor antagonist, on cytokine production from responder

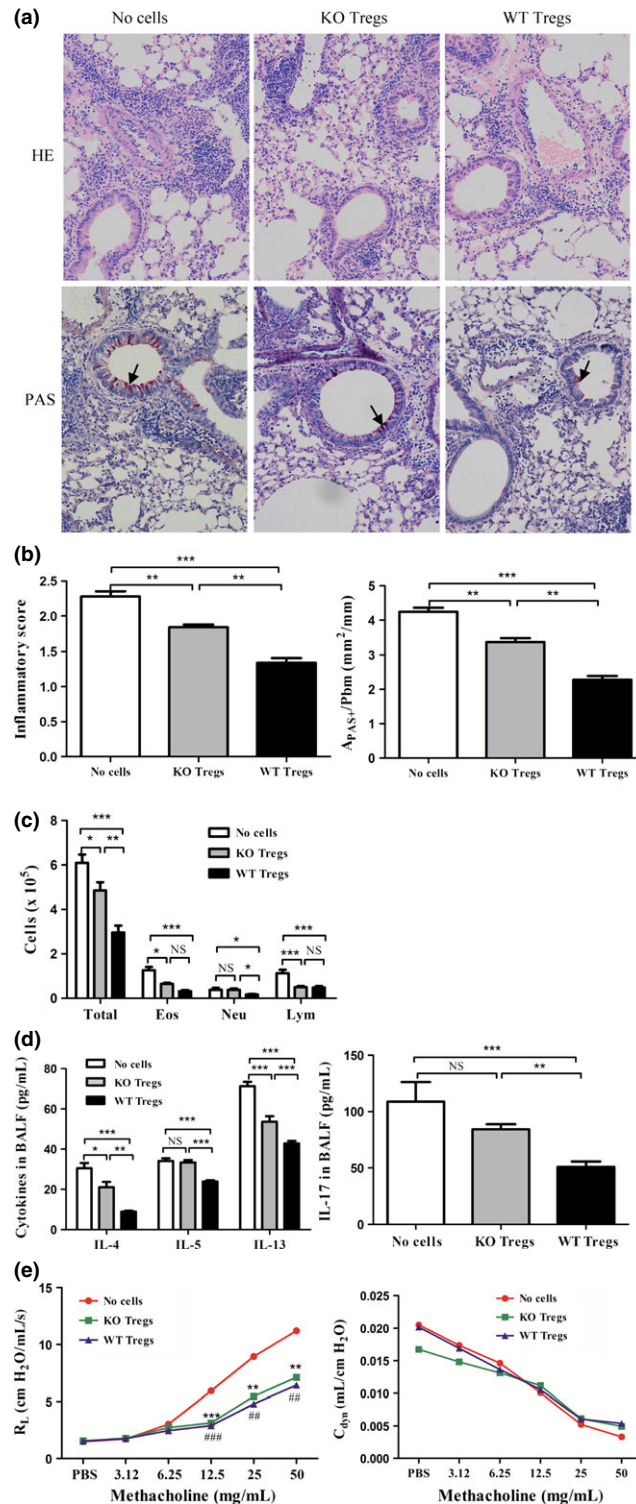


Fig. 4. Tregs from $Cd39^{-/-}$ mice insufficiently limit allergic airway inflammation. Female C57BL/6 mice were sensitized at days 0 and 14 and challenged at days 25–27. Mice were received an intravenous (i.v.) injection of 1×10^6 $CD4^+GFP^+$ cells sorted from Foxp3-GFP knock-in $Cd39^{+/+}$ and $Cd39^{-/-}$ mice, or an i.v. injection of PBS one hour before the first allergen challenge. (a) Lung sections stained with HE and PAS (arrow) ($\times 200$). (b) Inflammatory score and PAS-positive areas per unit length were determined. BALF was analysed for (c) the number of total and different cells and (d) cytokines (IL-4, 5, 13 and 17) (Eos, eosinophils; Lym, lymphocytes; Neu, neutrophils). (e) Tregs significantly decreased airway resistance. * $CD39^{-/-}$ Tregs vs No cells; # $CD39^{+/+}$ Tregs vs No cells. Data are expressed as mean $\pm$ SEM of 3 independent experiments ($n = 5$). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ### $P < 0.001$; ## $P < 0.01$.

T cells. We found that adenosine at the concentration of 10 μM efficiently inhibited IL-4 and IL-17 production from activated CD4⁺CD25⁻ cells and ZM241385 alone had no effect on cytokines production (Fig. 5a). Next, we compared the suppressive effects of CD39⁺ and CD39⁻ Tregs on cytokine production. As shown in Fig. 5b, the suppressive effects of Tregs on cytokines were enhanced with increasing numbers of Tregs. CD39⁺Tregs effectively decreased the production of IFN- γ , IL-4 and IL-17 from responder T cells, whereas CD39⁻Tregs had attenuated inhibitory effects on IFN- γ and IL-4 production, but undetectable inhibitory effect on IL-17 production. In addition, blocking adenosine A2A receptor with ZM241385 decreased the inhibitory effect of CD39⁺Tregs on IL-4 and IL-17 production (Fig. 5b).

Discussion

In our present study, we showed the protective effect of CD39 on allergic airway inflammation in a mouse model of allergic asthma. Our data showed that increased airway inflammation was observed when CD39 activity was inhibited or CD39 expression was deficient, whereas allergic airway inflammation was attenuated in mice received CD39⁺Tregs.

Our previous study demonstrated that CD39 expression in lungs was reduced at mRNA and protein level, and apyrase limited airway inflammation in a mouse model of allergic asthma [22]. In *Cd39*^{+/+} mice, inhibition of CD39 hydrolysis activity with ARL67156 increased airway inflammation, which was concerned in that ARL67156 could prolong the effect of ATP on P2R by preventing ATP hydrolysis when CD39 is present in

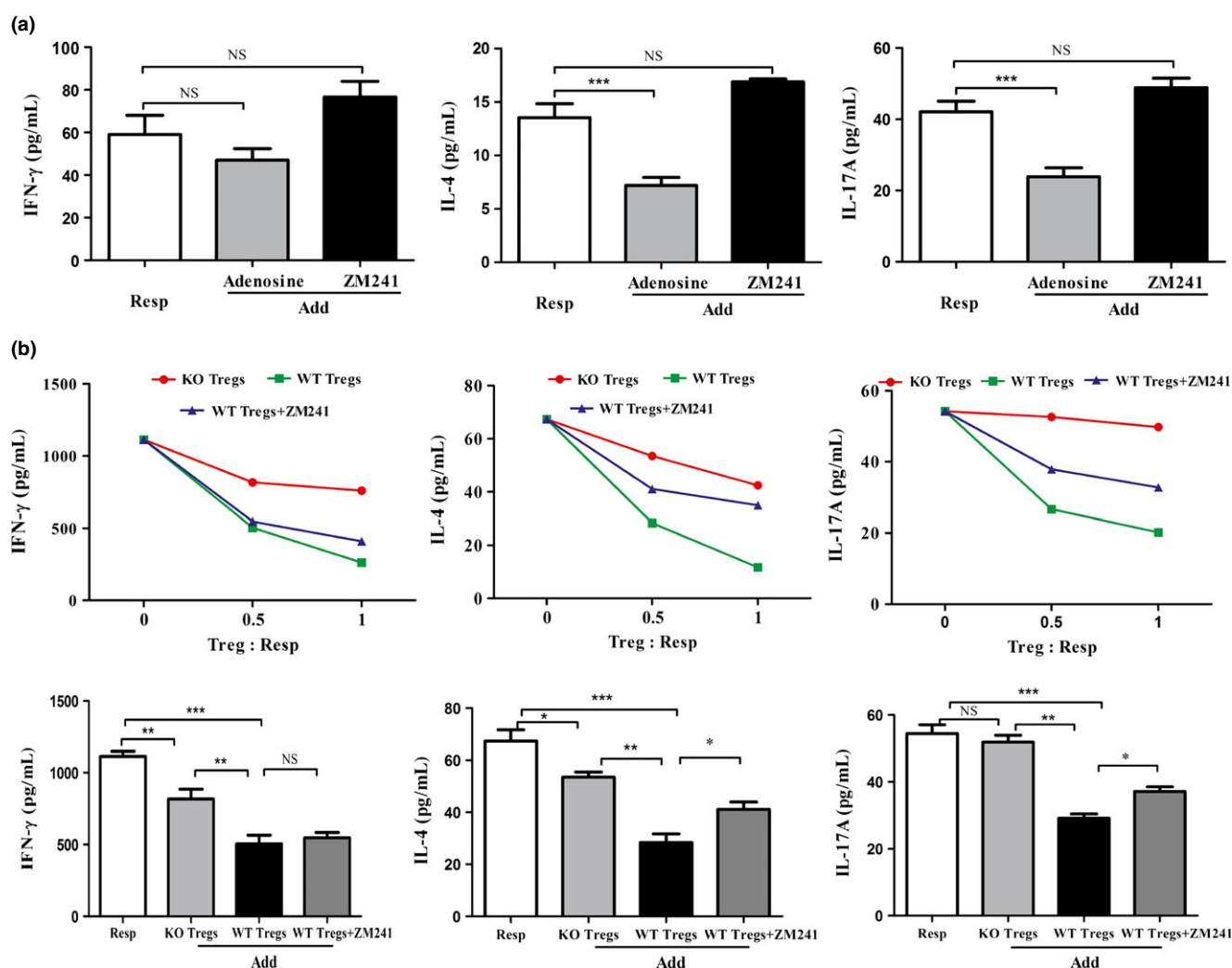


Fig. 5. CD39 expressed on Tregs exerts the inhibitory effect by adenosine. (a) Adenosine (10 μM) inhibited cytokines from activated responder T cells, whereas adenosine A2A receptor antagonist (ZM241, 5 μM) had no significant effect on cytokines. (b) The effect of CD39⁺Tregs and CD39⁻Tregs on cytokines were determined and the underlying suppressive mechanism of CD39⁺Tregs was evaluated by adding ZM241. Upper: average value of 3 independent experiments. Lower: mean $\pm$ SEM ($n = 3$). Resp: responder T cells; *** $P < 0.001$ ** $P < 0.01$ * $P < 0.05$ NS, not significant

cell microenvironment [23]. In mice, P2Y1, P2Y2, P2Y4 and P2Y6 are expressed in lungs [24, 25]. Recent studies have demonstrated P2YRs such as P2Y2, P2Y4 and P2Y6 are involved in the pathogenesis of allergic asthma by binding to natural ligand ATP. It is found that P2Y2 receptors mediate DCs and eosinophils recruitment into the lungs [14], and P2Y4 and P2Y6 receptors mediate bronchospasm by acting on airway epithelial cells [26]. In addition, P2Y6 receptors mediate airway inflammation and airway remodelling by acting on lung structural cells [27]. Based on these evidences, inhibition of CD39 hydrolysis activity worsened allergic airway inflammation, whereas previous supplement with apyrase alleviated allergic airway inflammation, which was associated with modulating the interaction of ATP and P2YR.

We found that increased airway inflammation in *Cd39*^{-/-} mice. However, Idzko and colleagues observed attenuated airway inflammation in *Cd39*^{-/-} mice, which was attributed to purinergic receptor desensitization in *CD39*^{-/-} DCs including defective migration of DCs, impaired priming Th2 response and unstable immune synaptic interactions [28]. *In vitro* migration assay showed no significance was detected between *Cd39*^{+/+} and *Cd39*^{-/-} DCs towards ATP (Fig. S1). Interestingly, *Cd39*^{-/-} DCs led to an earlier onset of experimental autoimmune encephalomyelitis (EAE) and worsened EAE [29], which seemed as if *Cd39*^{-/-} DCs has normal even stronger function under Th1 cell and Th17 cell-mediated immunity. Whether the function of *Cd39*^{-/-} DCs is discrepant under different immune response needs still to be further investigated.

The frequencies of local and systemic nTregs were decreased during allergic airway inflammation, which indicated appropriate number of nTregs was required for preventing allergic airway inflammation. A reduction in local and systemic nTregs was observed in *Cd39*^{-/-} mice. Compared to nTregs from *Cd39*^{+/+} mice, nTregs from *Cd39*^{-/-} mice had weaker suppressive capacities to cytokines secretion, especially IL-17 from responder T cells and insufficiently limited allergic airway inflammation. This is consistent with data from an autoimmune gastritis that demonstrated Th17 cells were more difficult to be suppressed than Th1 and Th2 cells by polyclonal Tregs [30]. A reduction in *CD39*⁺Tregs and impaired suppressive capacity to Th17 cells were also observed in tuberculosis pleural effusion [31] and

autoimmune hepatitis [32]. *In vitro* assay confirmed adenosine from *CD39*⁺Tregs was an underlying inhibitory machinery. Overall, defective number and/or function of nTregs resulted in increased airway inflammation in *Cd39*^{-/-} mice, and *CD39* expressed on Tregs contributes to the integral suppressive function of Tregs by modulating ATP and/or adenosine.

Down-regulation of *CD39* activity on Tregs resulted in tipping the balance to Th17 response [33], whereas up-regulation of *CD39* expression by circulating Tregs improved the sensitivity of glucocorticoids to Th17 cell-mediated severe asthma [34]. *CD39* is expressed on *CD4*⁺*Foxp3*⁺ and *CD4*⁺*Foxp3*⁻ cells. Therefore, we analysed the expression profiles of *CD39* in these two populations during allergic airway inflammation. We found *CD39* expression by *CD4*⁺*Foxp3*⁺Tregs from lung MNCs was significantly reduced in OVA-sensitized and OVA-challenged *Cd39*^{+/-} mice compared to control mice (Fig. S2a), which was consistent with other independent studies [34, 35]. Unexpectedly, an increase in *CD39* expression by *CD4*⁺*Foxp3*⁻ cells from lung MNCs was observed (Fig. S2b). Consistent with our result, previous studies showed that an increased expression by responder cells from synovial MNCs dampened inflammation induced by ATP in juvenile idiopathic arthritis [36].

However, AHR to methacholine was unchanged in OVA-sensitized and OVA-challenged mice received different treatments in the present study. It seemed that airway inflammation did not change together with AHR in parallel, which was supported by other independent studies [37, 38].

In conclusion, *CD39* mediates the protective role of *CD4*⁺*Foxp3*⁺Tregs in attenuation of allergic airway inflammation at least in part by regulating ATP and/or adenosine.

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Conflict of interest

The authors have no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The migration capacities of $Cd39^{+/+}$ and $Cd39^{-/-}$ DCs toward ATP.

Figure S2. CD39 expression on lung $CD4^{+}Foxp3^{+}$ and $CD4^{+}Foxp3^{-}$ T cells was analyzed by FACS.