

Allergen-induced airway hyperresponsiveness is absent in ecto-5'-nucleotidase (CD73)-deficient mice

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Abstract Adenosine is formed from extracellular purines by ecto-5'-nucleotidase (CD73) and is an essential player in allergic airway inflammation. The contribution of adenosine and other purines to electrolyte transport and mucociliary clearance was studied in airways of allergen challenged mice. No signs for allergen-induced inflammation were found in CD73^{-/-} mice, and adenosine monophosphate (AMP) was unable to elicit airway Cl⁻ secretion in these animals. Tracheas of ovalbumin (OVA)-treated BALB/c and CD73^{+/+} mice were hyperresponsive towards methacholine when assessed by Penh and direct optical measurement of contraction. In addition Cl⁻ secretion activated by ATP and ADP was enhanced. These changes were not observed in CD73^{-/-} mice. Expression of CFTR or CLCA was unchanged upon OVA treatment of CD73 mice, suggesting enhanced Cl⁻ secretion due to upregulated purinergic pathways. Mucociliary clearance was determined by measuring particle transport in excised mouse tracheas and was strongly enhanced in OVA-challenged CD73^{+/+} mice, but remained unchanged in CD73^{-/-} mice. While mucociliary clearance is activated by allergen exposure independent of functional ecto-5'-nucleotidase, airway inflammation is largely dependent on CD73. Thus, ecto-5'-nucleotidase may provide a novel target for therapeutic intervention, probably by local application of ecto-5'-nucleotidase inhibitors through inhalation.

Keywords Ecto-5'-nucleotidase · CD73 · Asthma · Electrolyte transport · Mucociliary clearance · Chloride secretion · Chloride transport · Respiratory epithelia · Airways · P2-purinergic receptor · Adenosine · Adenosine diphosphate (ADP)

Introduction

Inflammatory airway disease such as allergic asthma is characterized by eosinophilic infiltration, increased mucous production, and bronchial hyperresponsiveness. High levels of adenosine are found in lungs of patients with asthma, which is known to cause bronchoconstriction [1, 2]. Adenosine is a potent activator of mast cells and induces release of mast cell mediators like histamine and leukotrienes leading to bronchoconstriction and inflammation [3]. In adenosine deaminase-deficient mice, lung adenosine levels reach high concentrations of around 100 μ M. The same is found in asthma patients [1]. Adenosine accumulation is associated with severe pulmonary inflammation and airway remodeling [4]. Important sources for adenosine are degradation pathways of extracellular ATP, catalyzed by nucleoside triphosphate diphosphohydrolase (NTPDase), alkaline phosphatase, and ecto-5'-nucleotidase (CD73) [5]. ATP is released from airway cells and acts similar to adenosine as an endogenous signaling molecule in immunity and inflammation [5]. In lungs of asthmatic patients and in a mouse model for asthma, increased ATP levels were shown recently. Treatment of asthmatic mice with apyrase or with P2-receptor blocker abolished eosinophilic airway inflammation, Th2 cytokine production, and bronchial hyperreactivity. Additionally, ATP itself could activate dendritic cells [6].

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While on one hand ATP serves as a pro-inflammatory factor, it is on the other hand also known that extracellular ATP enhances mucociliary clearance, which is the primary defense mechanism of the lung. ATP facilitates mucociliary clearance by inducing Cl^- secretion [7], regulating ciliary beat frequency [8], and modulating the airway surface liquid volume [9]. Given these antagonistic effects of ATP, it remains unclear whether extracellular purines support the inflammatory process in the airways or whether the beneficial effects on mucociliary clearance surpass its pro-inflammatory action. To that end, we determined inflammatory response, purine-induced Cl^- secretion, and mucociliary clearance in tracheas of ovalbumin (OVA)-challenged Balb/c and ecto-5'-nucleotidase-deficient mice (CD73^{-/-}). These results suggest that ATP directly improves mucociliary clearance, while the downstream ecto-5'-nucleotidase product adenosine causes inflammation.

Materials and methods

Allergen challenge of mice Animal studies were conducted according to the national guidelines for care and use of animals in research and the German law on protection of animals. Female BALB/c mice (6–8 weeks) were purchased from Charles River Laboratories (Sulzfeld, Germany). Generation of ecto-5'-nucleotidase (CD73)-deficient and wild-type mice on a C57BL/6J-129SvEv background were described in Castrop et al. [10]. Female/male (half/half) littermates were used for the analysis of CD73^{+/+} and CD73^{-/-} mice. Mice were sensitized to ovalbumin (OVA, Sigma) by i.p. injection of 100 µg OVA with 1 mg aluminum hydroxide gel (Sigma) on days 0 and 14. At days 21 and 24, mice were anaesthetized (ketamine 90–120 mg/kg, xylazine 6–8 mg/kg) and challenged to OVA by intratracheal instillation of 50 µg OVA in 20 µl saline. Control mice were sham sensitized with saline and aluminum hydroxide gel (Sigma) and challenged to 20 µl saline by intratracheal instillation.

Determination of enhanced pause and direct assessment of contraction Enhanced pause (Penh) was measured in unrestrained animals by barometric plethysmography using a whole body plethysmograph and IOX software from emka Technologies (Paris, France). Penh was calculated by the formula: $\text{pause} = (T_e - T_r) / T_r$ [T_e : expiratory time (ms), T_r : relaxation time, time it takes to 36% of tidal volume (ms)] and $\text{Penh} = (\text{PEF} / \text{PIF}) \times \text{Pause}$ [PEF: peak expiratory flow (ml/s), PIF: peak inspiratory flow (ml/s)] [11, 12]. Mice were placed 24 and 72 h after OVA challenge in the plethysmography, and after 15 min adaptation period, baseline readings were taken and averaged for 3 min.

Phosphate-buffered saline or methacholine in increased concentrations (3.1, 6.2, 12.5, 25, 50 mg/ml) were aerosolized into the plethysmograph for 90 s using a nebulizer head. In a time period of 6 min after each challenge, Penh reached a maximum, and a mean value of Penh around the maximum was determined. In addition, contraction and velocity of contraction of isolated trachea were assessed by direct optical control using a high resolution binocular microscope equipped with a high-speed digital camera and software (AxioVision, Zeiss, Germany).

Histology Twenty-four and 72 h after OVA challenge, mice were sacrificed under CO_2 narcosis by cervical dislocation; the tracheas were removed and fixed according to Bouin (21 vol.% formaldehyde, 71 vol.% picric acid, 5 vol.% acetic acid). Tracheas were dehydrated and embedded in paraffin. Paraffin-embedded tissues were cut at 4 µm on a rotary microtome (Leica Mikrotom RM 2165, Wetzlar, Germany). Sections were dewaxed, rehydrated, and stained according to Pappenheim. Eosinophils were counted in 10 to 15 successive sections, and the mean number per section was calculated. Mast cells were identified by Luna staining.

Real time RT-PCR Total RNA was isolated from mouse trachea using the RNeasy Micro Kit from Qiagen (Hilden, Germany). RNA was reverse transcribed for 1 h at 37°C using oligo dT primer and M-MLV Reverse Transcriptase (Promega, Germany). Real time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in a Light Cycler (Roche, Germany), using the Quanti Tect SYBR Green PCR Kit (Qiagen, Germany). Each reaction contained 2 µl Master Mix, 1 pM of each primer (P2Y₁: 5'-CGTGGCTATCTG GATGTTTC-3', 5'- ATGAGGGCTGGTAGGGTGA-3', 114 bp; P2Y₂: 5'- CTCACCACCTCAAGAGCAG-3', 5'- CTTGTATCCCAGTTCGTC-3', 112 bp; P2Y₆: 5'- TCTT CCATCTTGCATGAGACA -3', 5'- GCCATTGTCCT GCTCCAT -3', 74 bp; A_{2B}: 5'- GAGCTCCATCTT TAGCCTCTTG -3', 5'-TGTCCCAGTGACCAAACCTT-3', 91 bp; CFTR: 5'-CAGCAGCTCAAACAAGTGA-3', 5'-TAAGCTTGTCACAAGGTGGG-3', 66 bp; β-actin: 5'-CAACGGCTCCGGCATGTG-3', 5'- CTTGCTCTGGGC CTCGTC-3', 151 bp), 2.5 mM MgCl_2 and 1 µl cDNA. After 10 min at 94°C for activation of Taq polymerase, cDNA was amplified by 15 s at 94°C, 10 s at 60°C, and 20 s at 72°C, for 50 cycles. The amplification was followed by a melting curve analysis to control the PCR products. As negative controls, water instead of cDNA was run with every PCR experiment. To verify accuracy of the amplification, PCR products were further analyzed on ethidium bromide-stained 2% agarose gels. Analysis of the data was performed using Light Cycler software 3.5.3. The ratio of the amount of target to β-actin mRNA was calculated for each sample.

Electrolyte transport measurements in trachea Mice were sacrificed under CO₂ narcosis by cervical dislocation. Tracheas were dissected, opened longitudinally on the opposite side of the cartilage free zone and were transferred immediately into an ice-cold buffer solution of the following composition (mmol/l): NaCl 145, KH₂PO₄ 0.4,

K₂HPO₄ 1.6, D-glucose 5, MgCl₂ 1, Ca-gluconate 1.3, pH 7.4. After mounting into a perfused micro Ussing chamber, apical and basolateral surfaces of the epithelium were perfused continuously with buffer solution at a rate of 5–10 ml/min (chamber volume 2 ml). All experiments were carried out at 37°C under open circuit conditions. Trans-

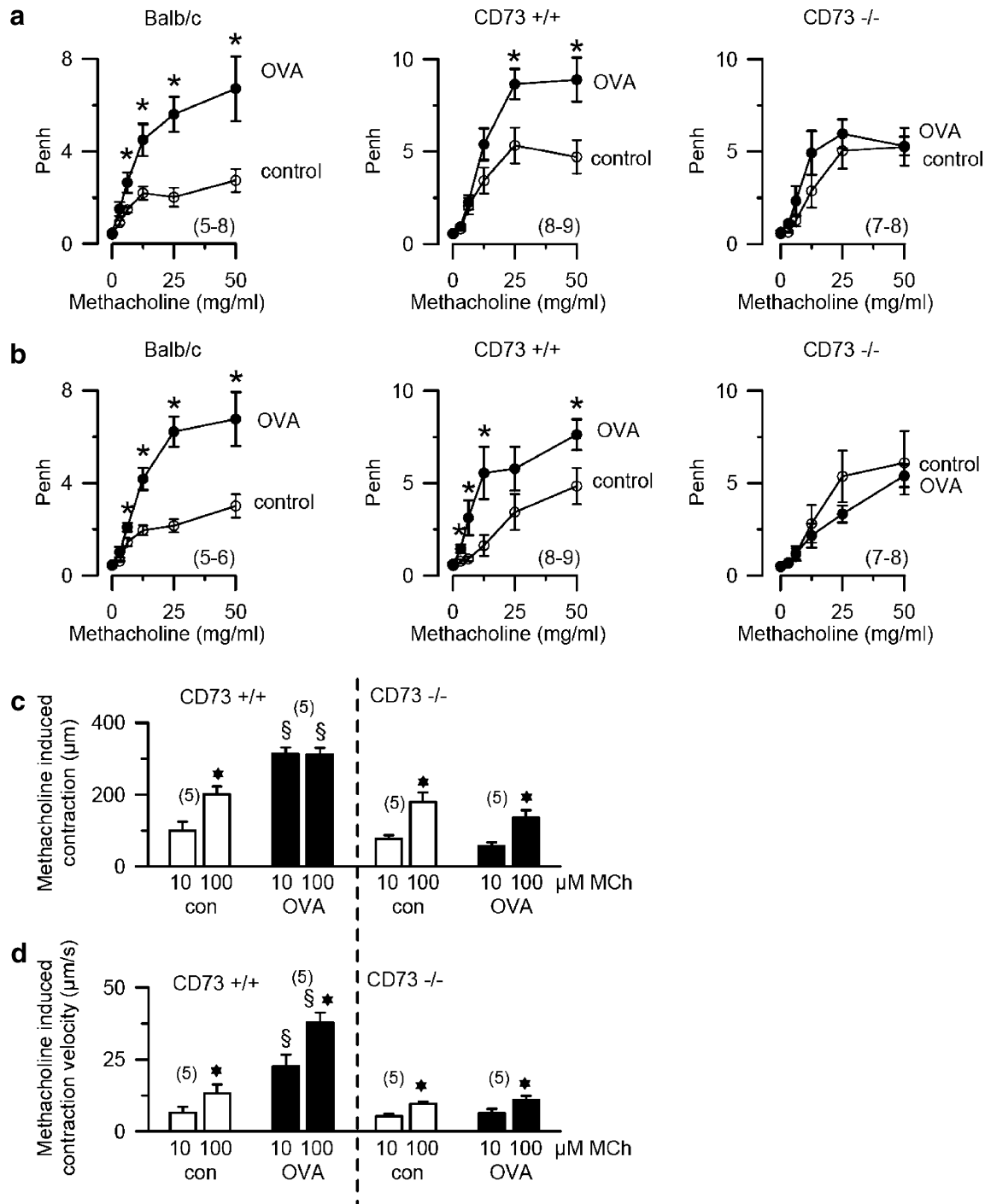


Fig. 1 Enhanced bronchoconstriction after OVA challenge is absent in CD73^{-/-} mice. **a** Increased airway resistance after methacholine challenge was determined 24 h and **b** 72 h after OVA challenge by measuring enhanced pause (Penh) using whole body plethysmogra-

phy. **c** Contraction and **d** velocity of contraction of isolated mouse tracheas of CD73^{+/+} and CD73^{-/-} mice after stimulation with 10 or 100 μM methacholine. Data are means±SEM, * (paired) and § (unpaired) indicate significant difference (*n*=number of animals)

epithelial resistance (R_{te}) was determined by applying short (1 s) current pulses ($I=0.5 \mu\text{A}$), and the corresponding changes in the transepithelial voltage (V_{te}) and basal V_{te} were recorded continuously. Values for V_{te} were referred to the serosal side of the epithelium. The equivalent short-circuit current (I_{SC}) was calculated according to Ohm's law from V_{te} and R_{te} ($I_{SC}=V_{te}/R_{te}$).

Measurement of mucociliary clearance Tracheas were mounted with insect needles onto an extra thick blot paper (Bio-Rad, Germany) and transferred into a chamber with water saturated atmosphere at 37°C . The filter paper was perfused with buffer solution at a rate of 1 ml/min and at 37°C . Polystyrene black dyed microspheres (diameter $6.51 \pm 0.58 \mu\text{m}$, Polybead®, Polyscience, Warrington, PA, USA) were washed with buffer solution ($\pm\text{ATP } 10 \mu\text{M}$). Particle solution (10 μl with around 0.5% Latex) was added onto the surface of the trachea twice, without and subsequent with ATP (10 μM). The particle transport on both conditions was visualized by taking pictures every 30 s for 10–15 min using the Zeiss stereo microscope Discovery.V12 with PlanS objective (1.0x, FWD 81 mm), Zeiss digital camera AxioCam ICc1, and the software AxioVision (Release 4.6.3, Zeiss, Göttingen, Germany). Five to 15 moving particles of different regions of the trachea were selected, and the transport was calculated by measuring the distance per time ($\mu\text{m}/\text{min}$) using the AxioVision Software (Release 4.6.3, Zeiss, Göttingen, Germany).

Materials and statistical analysis All compounds used were of highest available grade of purity from Sigma (Taufkirchen, Germany) or Merck (Darmstadt, Germany). Student's *t* test (for paired or unpaired samples as appropriate), and analysis of variance was used for statistical analysis. $P<0.05$ was accepted as significant.

Results

Reduced airway responsiveness in CD73-deficient mice Mice were sensitized to ovalbumin (OVA) with aluminum hydroxide gel and were challenged 7 and 10 days later with intratracheal instillation of OVA. To measure airway hyperresponsiveness to methacholine, enhanced pause (Penh) was determined, as a parameter for bronchoconstriction [11, 12]. Exposure of BALB/c and CD73 $+/+$ mice to methacholine induced a dose-dependent increase of Penh. The effects of methacholine on Penh were enhanced in OVA-sensitized BALB/c and CD73 $+/+$ mice 24 (Fig. 1a) and 72 h after OVA challenge (Fig. 1b). In contrast, hypersensitivity to methacholine was not found in CD73 $-/-$ mice (Fig. 1a,b). The results from Penh were

recapitulated by an independent method in which contraction and contraction velocity of isolated trachea were directly assessed by a high-resolution binocular microscope (Zeiss, Germany). Methacholine-enhanced contraction and contraction velocity dose dependently in both CD73 $+/+$ and CD73 $-/-$ mice (Fig. 1c,d). However, in contrast to CD73 $+/+$ animals, OVA-sensitized CD73 $-/-$ mice did not show enhanced contraction or contraction velocity 48 h after OVA challenge. Compared to methacholine, the effects of adenosine (100 μM) on contraction ($30 \pm 11 \mu\text{m}$) and

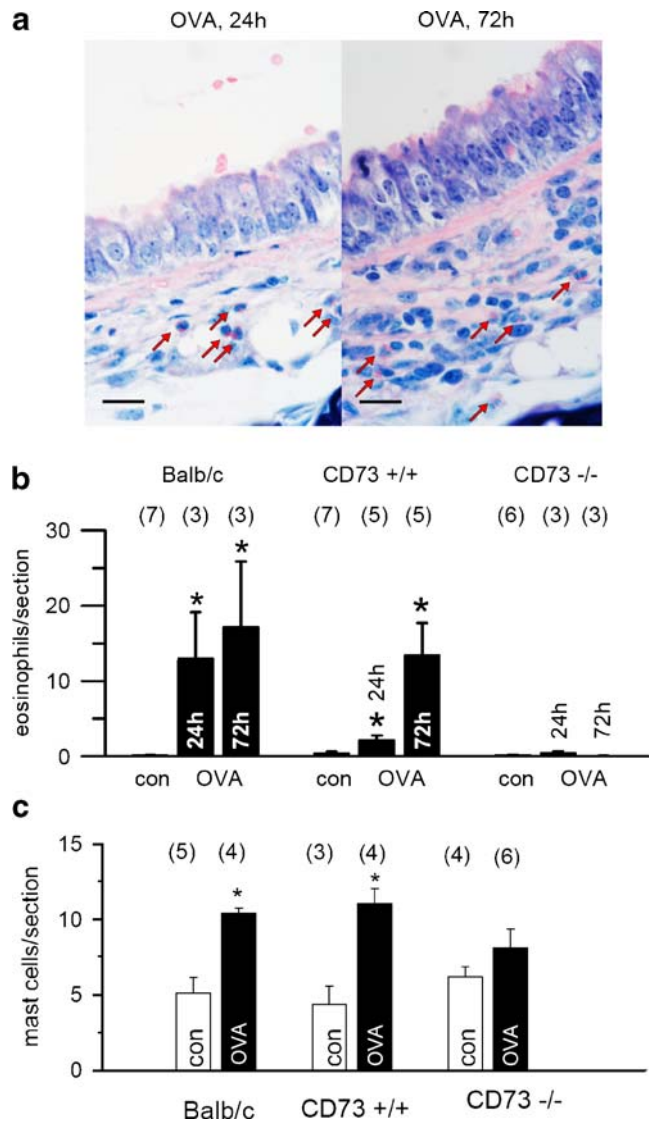


Fig. 2 OVA-induced airway inflammation is absent in CD73 $-/-$ mice. **a** Papanheim staining of trachea from OVA-sensitized BALB/c mice 24 and 72 h after OVA challenge. Increased infiltration of eosinophils (arrows) was detected. Bar=20 μm . **b** The number of eosinophils per section was determined from trachea of control and OVA challenged BALB/c, CD73 $+/+$ and CD73 $-/-$ mice. **c** The number of mast cells per section was determined (Luna) from trachea of control and OVA-challenged BALB/c, CD73 $+/+$, and CD73 $-/-$ mice. Data are means $\pm$ SEM, * $P<0.05$ (n =number of animals)

contraction velocity ($1.6 \pm 0.7 \mu\text{m}$; $n=5$) of isolated trachea were relatively small and were therefore not further examined. OVA-sensitized BALB/c and CD73^{+/+} mice showed an increased infiltration of eosinophils 24 and 72 h after OVA challenge, suggesting an asthma-like airway inflammation (Fig. 2). In contrast, infiltration of the airways with eosinophils was absent in CD73^{-/-} mice (Fig. 2b). Moreover, mast cell infiltration was quantified using Luna staining and was found to be enhanced in OVA-sensitized Balb/c and CD73^{+/+} animals but not in CD73^{-/-} mice (Fig. 2c). These results suggest a lack of allergic inflammatory airway response in the absence of ecto-5'-nucleotidase.

Purine-dependent electrolyte transport is increased in OVA-challenged BALB/c mice It was shown that allergen challenge causes accumulation of extracellular ATP in the airways [6]. We therefore investigated the effects of purines on electrolyte transport in tracheas of BALB/c mice, using a micro Ussing chamber. Apical application of ATP (100 μM), ADP (100 μM), AMP (100 μM), or adenosine (100 μM) induced a rapid and transient voltage deflection, followed by a steady-state change of the transepithelial voltage (V_{te} ; Fig. 3a). Thus, extracellular purines triggered transient and steady-state equivalent short-circuit currents (I_{SC}), due to activation of airway Cl^- secretion [13]. In

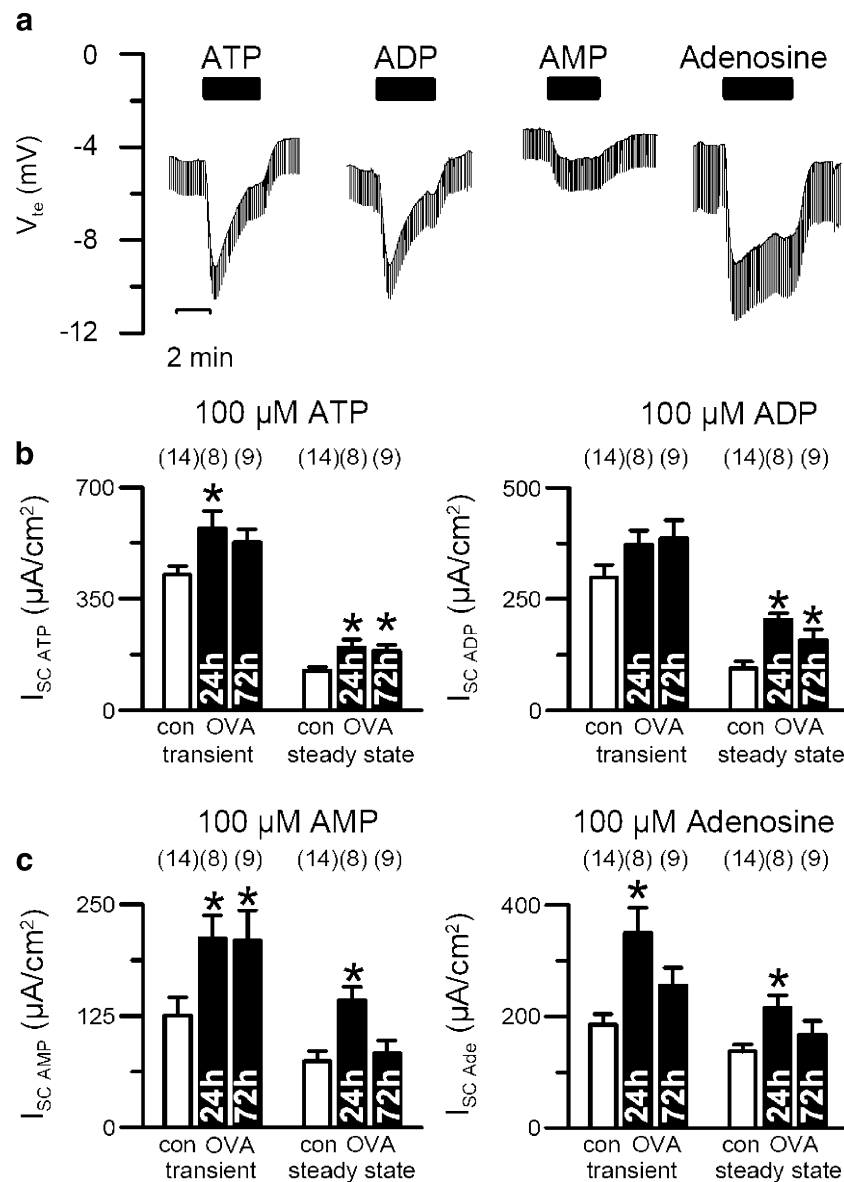


Fig. 3 Purine-stimulated Cl^- secretion is enhanced in trachea of OVA-challenged BALB/c mice. **a** Original recordings of purine-stimulated Cl^- secretion in trachea of BALB/c mice using a micro-Ussing chamber. Apical application of ATP (100 μM), ADP (100 μM), AMP (100 μM), or adenosine (100 μM) caused a transient

and steady-state increase of the transepithelial voltage V_{te} . **b** Summary of the ATP-, ADP-, AMP-, or adenosine-induced transient and steady-state equivalent short-circuit current (I_{SC}) in control and OVA-sensitized BALB/c mice 24 and 72 h after OVA challenge. Data are means $\pm$ SEM, $*P < 0.05$ (n = number of animals)

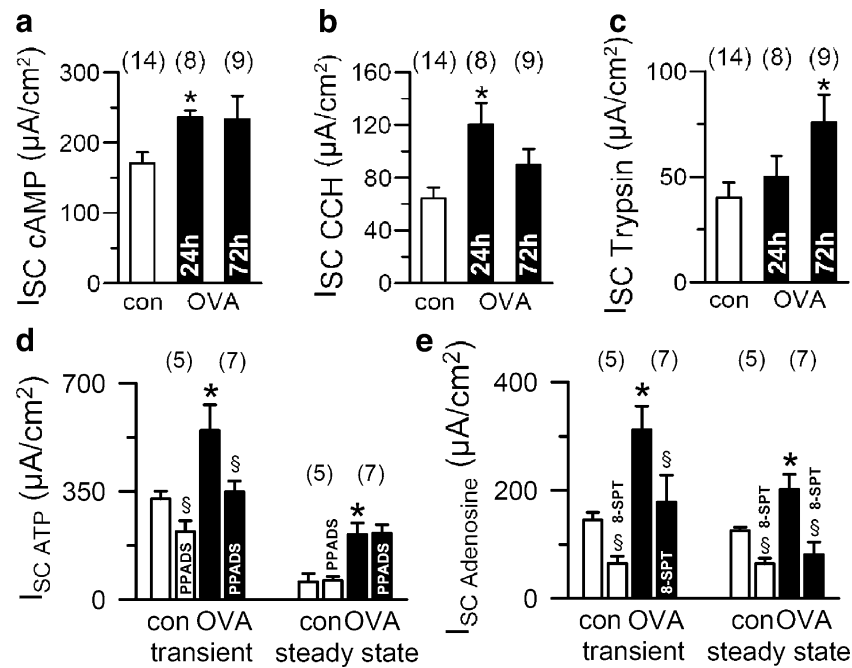


Fig. 4 CFTR-, carbachol-, and trypsin-dependent Cl^- secretion is enhanced in tracheas of OVA-challenged BALB/c mice. **a** Summary of the IBMX (100 μ M), forskolin (2 μ M)-induced equivalent short-circuit current (I_{SC} cAMP) in control and OVA-sensitized BALB/c mice 24 and 72 h after OVA challenge. **b** Summary of the carbachol (CCH, 100 μ M)-induced steady-state equivalent short-circuit current (I_{SC} CCH) in control and OVA-sensitized BALB/c mice 24 and 72 h after OVA challenge. **c** Summary of the trypsin (1 μ M)-induced equivalent short-

circuit current (I_{SC} CCH) in control and OVA-sensitized BALB/c mice 24 and 72 h after OVA challenge. **d** ATP-induced transient Cl^- secretion in OVA-sensitized animals was inhibited by the P2Y₆-specific inhibitor PPADS (100 μ M). **e** Adenosine-activated transient and steady-state transport in OVA-sensitized animals was blocked by the specific inhibitor of A_{2B} receptors, 8-SPT (100 μ M). Data are means $\pm$ SEM, * P < 0.05 (n = number of animals)

OVA-sensitized and OVA-challenged BALB/c mice, increased response to stimulation with ATP, ADP, and AMP were observed, and both transient and steady-state activation of Cl^- secretion was augmented. The response to adenosine was significantly enhanced only 24 h after OVA challenge (Fig. 3b). In addition to enhanced purinergic

responses, Cl^- secretion activated by IBMX (100 μ M) and forskolin (2 μ M) and carbachol (100 μ M)-induced Cl^- secretion were enhanced in BALB/c mice after OVA challenge along with an enhanced response of protease-activated receptors (PAR) type 2 upon stimulation with trypsin (1 μ M; Fig. 4a–c). Enhanced Cl^- secretory response

Table 1 Expression of purinergic receptors and CFTR in OVA-challenged mice

Strain	Treatment	<i>n</i>	P2Y ₁	P2Y ₂	P2Y ₆	A _{2B}	CFTR
BALB/c	Control	6	100.0 $\pm$ 7.6	100.0 $\pm$ 6.6	100.0 $\pm$ 3.9	100.0 $\pm$ 7.1	100.0 $\pm$ 18.3
	OVA 24 h	6	113.7 $\pm$ 11.6	110.8 $\pm$ 12.7	152.5 $\pm$ 12.2*	189.5 $\pm$ 20.9*	200.1 $\pm$ 100.7
	OVA 72 h	7	112.1 $\pm$ 8.9	100.6 $\pm$ 9.4	136.2 $\pm$ 11.5*	169.6 $\pm$ 20.8*	238.2 $\pm$ 37.8*
CD73 ^{+/+}	Control	7	100.0 $\pm$ 12.9	100.0 $\pm$ 13.5	100.0 $\pm$ 9.0	100.0 $\pm$ 19.8	100.0 $\pm$ 16.5
	OVA 24 h	7	109.2 $\pm$ 6.5	137.7 $\pm$ 11.0*	86.8 $\pm$ 2.8	102.6 $\pm$ 18.7	98.1 $\pm$ 13.1
CD73 ^{-/-}	Control	8	100.0 $\pm$ 12.2	100.0 $\pm$ 7.3	100.0 $\pm$ 9.4	100.0 $\pm$ 15.0	100.0 $\pm$ 16.3
	OVA 24 h	7	123.9 $\pm$ 23.9	125.0 $\pm$ 17.6	139.6 $\pm$ 24.3	129.7 $\pm$ 17.2	87.4 $\pm$ 16.6
	OVA 72 h	8	97.3 $\pm$ 12.0	104.3 $\pm$ 5.0	115.3 $\pm$ 14.4	149.5 $\pm$ 22.1	86.6 $\pm$ 14.3

Expression of mRNA of P2Y₁, P2Y₂, P2Y₆, and A_{2B} receptor and CFTR were quantitatively measured in single trachea by real-time RT-PCR. Data are means $\pm$ SEM

* P < 0.05.

in the airways of OVA-challenged BALB/c mice was paralleled by an increase in mRNA expression for the purinergic receptors P2Y₆ and A_{2B}, and CFTR Cl⁻ channel, as demonstrated by real-time RT-PCR (Table 1). RT-PCR analysis of CLCA proteins (CLCA 1-4) and CFTR in Balb/c, CD73^{+/+}, and CD73^{-/-} mice indicated no changes in expression levels after OVA treatment, apart from an increase in CFTR mRNA in Balb/c mice (data not shown). The significance of the increase in P2Y₆ and A_{2B} receptor transcripts is further supported by the fact that ATP-induced transient Cl⁻ secretion in OVA-sensitized animals was inhibited by the P2Y₆-specific inhibitor PPADS (100 μ M), while adenosine activated transient and steady-state transport was blocked by the specific inhibitor of A_{2B} receptors, 8-SPT (100 μ M; Fig. 4d,e). Taken together, it is unlikely that changes in Cl⁻ channel expression cause enhanced purinergic ion secretion after ovalbumin treatment.

AMP-activated Cl⁻ secretion depends on ecto-5'-nucleotidase (CD73) activity Extracellular ATP is rapidly degraded to AMP by NTPDase (e.g., apyrase, CD39) and alkaline phos-

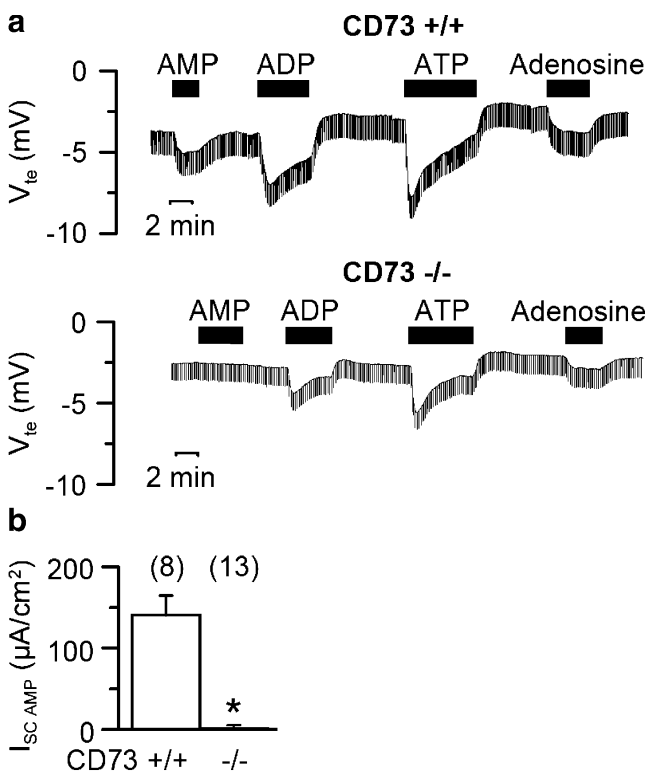


Fig. 5 AMP-induced Cl⁻ secretion is dependent on ecto-5'-nucleotidase (CD73) activity. **a** Original using chamber recordings of trachea from CD73^{+/+} and CD73^{-/-} mice stimulated with apical ATP (100 μ M), ADP (100 μ M), AMP (100 μ M), or adenosine (100 μ M). **b** Summary of the AMP (100 μ M)-induced steady-state equivalent short-circuit current ($I_{SC AMP}$) in CD73^{+/+} and CD73^{-/-} mice. Data are means $\pm$ SEM, * P <0.05 (n =number of animals)

phatase. The conversion of AMP to adenosine is mediated by alkaline phosphatase or ecto-5'-nucleotidase (CD73) [14]. In tracheas of CD73-deficient mice (CD73^{-/-}) apical application of ATP (100 μ M), ADP (100 μ M), and adenosine (100 μ M) activated Cl⁻ secretion that was significantly reduced when compared to CD73^{+/+} mice (Figs. 5 and 6). In contrast to the other purines, even a high concentration of AMP (100 μ M) was not able to induce Cl⁻ secretion in CD73^{-/-} mouse trachea (Fig. 5). It remains currently unclear why purinergic responses are reduced in CD73^{-/-} airways; however, the results clearly demonstrate the essential role of ecto-5'-nucleotidase for generation of the secretagogue and pro-inflammatory factor adenosine.

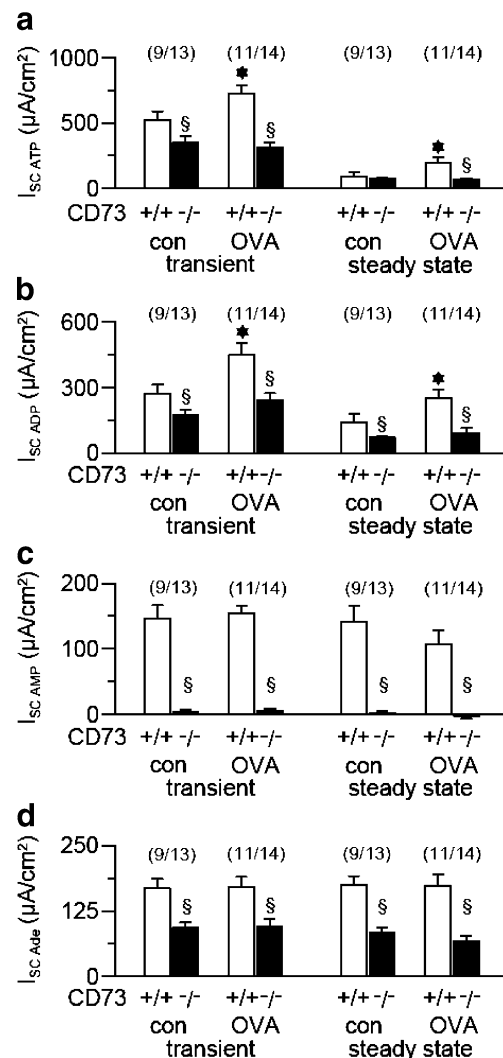


Fig. 6 ATP- and ADP-induced Cl⁻ secretion is attenuated in OVA-challenged CD73^{-/-} mice. **a** Summary of ATP (100 μ M), **b** ADP (100 μ M), **c** AMP (100 μ M), or **d** adenosine (100 μ M)-induced transient and steady-state equivalent short-circuit current (I_{SC}) in control and OVA-challenged CD73^{+/+} and CD73^{-/-} mice. Data are means $\pm$ SEM, * P <0.05 to control mice, § P <0.05 to CD73^{+/+} mice (n =number of animals)

ATP- and ADP-induced Cl^- secretion is attenuated in airways of OVA-challenged $\text{CD73}^{-/-}$ mice Ion transport induced by the nucleotides ATP, ADP, AMP, and adenosine was further examined, after we found no differences in baseline transport in tracheas of $\text{CD73}^{+/+}$ and $\text{CD73}^{-/-}$ (Supplement 1). In $\text{CD73}^{+/+}$ mice, ATP and ADP stimulate transient and steady-state Cl^- secretion that is increased 24 h after OVA challenge, whereas the effects of AMP and adenosine remained unchanged. In contrast, transient and steady-state Cl^- transport activated by ATP or ADP was unchanged in OVA-treated $\text{CD73}^{-/-}$ mice (Fig. 6). Purinergic receptors were found to be upregulated in OVA-sensitized BALB/c (P2Y_6 , $\text{A}_{2\text{B}}$) and $\text{CD73}^{+/+}$ (P2Y_2) mice. This was not found in $\text{CD73}^{-/-}$ mice (Table 1).

The mucociliary clearance in mouse trachea is enhanced in OVA-challenged mice The mucociliary clearance is an essential defense mechanism of the lung [15, 16]. Purines are important regulators of the electrolyte transport and are enhanced during airway inflammation. It is suggested that purines influence mucociliary clearance by controlling electrolyte transport and water movement [7–9]. To investigate the role of purines for mucociliary clearance in normal and in inflamed airways, we measured the ciliary transport of small (6 μm) polystyrene microspheres in iso-

lated tracheas ex vivo in a humidified chamber. In non-inflamed (non-challenged) control tracheas, basal particle transport was similar in BALB/c, $\text{CD73}^{+/+}$, and $\text{CD73}^{-/-}$ mice. Mucociliary particle transport was remarkably increased in all three mouse strains after apical application of ATP (10 μM ; Fig. 7a–c). OVA challenge (24 h) caused a strong increase of the particle transport in BALB/c, $\text{CD73}^{+/+}$, and $\text{CD73}^{-/-}$ mice, which was not further elevated by ATP (Fig. 7). In paired experiments with tracheas from sensitized Balb/c mice and control mice, we found that ATP release is enhanced in the sensitized epithelium, since increased particle transport was fully inhibited by the nonspecific inhibitor suramin (Fig. 7d). Thus, mucociliary clearance is not affected by a defect in the ecto-5'-nucleotidase and does not rely on the presence of adenosine.

Discussion

The present results suggest that ATP release during airway inflammation has two major functions: (1) it promotes an acute defense mechanism by increasing mucociliary clearance; and (2) it induces an immune response by activation

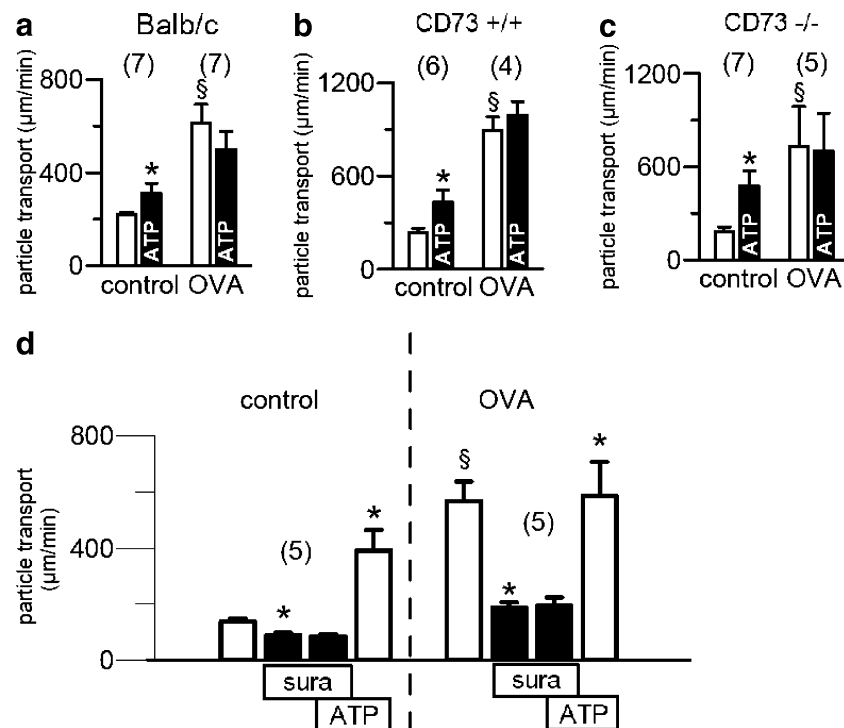


Fig. 7 Mucociliary clearance is enhanced 24 h after OVA-challenge mice. Mucociliary clearance was determined by measurement of the transport of polystyrene microspheres (diameter $6 \pm 0.58 \mu\text{m}$) in isolated trachea. **a** Summarized effects of apical application of ATP (10 μM) in control and OVA-challenged BALB/c, **b** $\text{CD73}^{+/+}$, and **c**

$\text{CD73}^{-/-}$ mice. **d** Particle transport in control Balb/c mice and OVA-sensitized Balb/c mice. Experiments with suramin (100 μM) and ATP (10 μM) suggest enhanced ATP release in OVA-sensitized Balb/c mice under baseline conditions. Data are means $\pm$ SEM, $*P < 0.05$ to absence of ATP, $\$P < 0.05$ to control mice (n =number of animals)

of mast cell degranulation [5, 17, 18]. In a recent study, Idzko et al. supplied evidences that ATP is an important mediator in the pathogenesis of asthma. In the airways of allergen-challenged asthma patients, ATP levels were found to be elevated which correlated well to an increased number of eosinophils in bronchoalveolar lavage fluid. In airways of OVA-sensitized and OVA-challenged Balb/c mice, an increased level of ATP could also be detected. Airway inflammation was absent in these mice, if P2 receptors were blocked or extracellular ATP was eliminated by instillation of apyrase [6]. We also found infiltration of eosinophils and mast cells in the tracheal mucosa of OVA-challenged Balb/c mice and an increased sensitivity to methacholine which caused bronchoconstriction (Figs. 1 and 2). Ussing chamber experiments showed elevated Cl^- secretion induced by purines and proteases, 24 and 72 h after OVA challenge (Figs. 3 and 4). It was shown that purinergic receptors like P2X_7 , P2Y_1 , and P2Y_2 were upregulated in inflammatory cells and in the vasculature during chronic inflammation [19]. In trachea of OVA-challenged Balb/c mice, we found up-regulation of P2Y_6 and $\text{A}_{2\text{B}}$ (Table 1). Mice with a C57BL/6J-129SvEv background ($\text{CD73}^{+/+}$) behaved slightly differently in that only ATP and ADP-induced Cl^- secretion was enhanced, and P2Y_2 receptors were upregulated. This may reflect a different susceptibility to allergic airway inflammation in different mouse strains [20]. Increased sensitivity to ADP, i.e., enhanced ADP-induced Cl^- secretion in inflamed tracheas may be due to ATP formation catalyzed by ecto-nucleoside diphosphokinase [5]. ATP is an important modulator of mucociliary clearance [7–9]. Thus, increased release of extracellular ATP and up-regulation of purinergic receptors is likely to support mucociliary clearance during inflammation. As shown in Fig. 7, apical application of ATP increased mucociliary particle transport in the trachea of both Balb/c and $\text{CD73}^{-/-}$ mice, while in OVA-challenged mice, particle transport was strongly enhanced and not further activated by ATP. ATP release and enhanced Cl^- secretion therefore contribute to mucociliary clearance and strengthen the importance of this lung defense mechanism.

Extracellular degradation of ATP to AMP is catalyzed by NTPDase-1, -2, -3, -8, or alkaline phosphatase [5]. AMP is further hydrolyzed to adenosine in the presence of nucleotide pyrophosphatase/phosphodiesterase (NPP2, autotoxin) or alkaline phosphatase. The key enzyme for the formation of adenosine, however, is the ecto-5'-nucleotidase (CD73) [5]. The results obtained in Ussing-chamber experiments demonstrate that AMP induces Cl^- secretion through adenosine formation by CD73 (Fig. 5). Thus, degradation of AMP to adenosine by CD73 is an essential step during development allergen-induced inflammation: Lack of CD73 prevented airway hyperresponsiveness to methacholine, infiltration of eosinophils, and

increased P2Y_2 receptor expression. Lack of the dominant Langerhans cell-associated ecto-NTPDase in $\text{CD39}^{-/-}$ mice caused accumulation of extracellular ATP and desensitization of P2 receptors, while allergic contact hypersensitivity was severely attenuated [21]. Activation of Cl^- secretion by stimulation with purines was attenuated in $\text{CD73}^{-/-}$ mice in the present study, which may be caused by receptor desensitization. This could also be part of the reason why allergen-induced inflammation is absent in $\text{CD73}^{-/-}$ mice, apart from the absence of pro-inflammatory adenosine. Along this line, Idzko et al. showed that ATP enhances Th2 immunity by causing myeloid antigen-presenting dendritic cell (mDC) recruitment and activation. ATP also enhances the capacity of mDCs to prime for Th2 response directly [22]. The present results indicate that ATP release and adenosine formation by CD73 are key steps for development of airway inflammation after allergen exposure. While mucociliary particle transport is supported by ATP release from airway epithelial cells, airway inflammation depends on the ecto-5'-nucleotidase, which may offer a novel target for therapeutic intervention, probably by local application of ecto-5'-nucleotidase inhibitors through inhalation.

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