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Attenuated allergic airway inflammation in *Cd39* null mice

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Abstract

Background—Extracellular ATP is known to accumulate in the lung, following allergen challenge, and contributes via activation of purinergic receptors on dendritic cells (DC), to the development of allergic airway inflammation (AAI). Extracellular ATP-levels in the airways are normally tightly regulated by CD39. This ectonucleotidase is highly expressed by DC purified from skin (Langerhans cells) and bone marrow, and has been shown to modulate DC adaptive/haptenic immune responses. In this study, we have evaluated the impact of Cd39 deletion and associated perturbation of purinergic signaling in AAI.

Methods—Standard OVA-alum and house dust mite (HDM) bone marrow derived DC (BMDC) dependent models of AAI were used to study effects of Cd39. Migration assays, time lapse microscopy and T-cell priming assays were further used to determine functional relevance of Cd39 expression on BMDC in the setting of immune and Th2-mediated responses in these models.

Results—*Cd39*^{−/−} mice exhibited marked increases in BALF ATP-levels but paradoxically exhibited limited AAI in both OVA-alum and HDM models. These pathophysiological abnormalities were associated with decreased myeloid DC activation and chemotaxis towards ATP, and were linked to purinergic receptor desensitization responses. Further, *Cd39*^{−/−} DCs exhibited limited capacity to both prime Th2-responses and form stable immune synaptic interactions with OVA-transgenic naïve T-cells.

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Author contributions

Each named author contributed to the design of the study, its implementation and/or the writing of the manuscript.

Conflict of interest

None of the authors has a conflict of interest with respect to the study.

Conclusions—*Cd39*-deficient DCs exhibit limited capacity to induce Th2 immunity in a DC-driven model of AAI in vivo. Our data demonstrate a role of CD39 and perturbed purinergic signaling in models of AAI,

Keywords

Asthma; CD39 ectonucleotidase; Chemotaxis; Dendritic cells; T-cell priming

Introduction

Asthma is an important clinical condition, characterized by chronic inflammatory disease of the airways. This disease is associated with variable and recurring symptoms such as reversible airflow obstruction, bronchial hyperreactivity (BHR) to unspecific stimulus, excessive mucus production and bronchospasm. Increased Th2-responsiveness and subsequent involvement of mast cells, eosinophils, NK-T-cells and dendritic cells (DC) contribute to the persistent inflammatory state in the airways, leading to extensive tissue remodeling [1]. Compelling findings point to important roles for the myeloid DC (mDC) in the initiation phase and maintenance of allergic airway inflammation (AAI) [2–5].

Recently, extracellular nucleotides (such as ATP) - also via activation of the inflammasome pathway [6], - have gained attention as important mediators in AAI. Following allergen challenge, extracellular ATP accumulates in the lungs of asthmatic patients as well as animals in experimental models of AAI. In these latter settings, interference with ATP/type 2 purinergic receptor (P2R)-pathways has been shown to attenuate AAI [4, 6, 7].

Extracellular nucleotide-levels are tightly regulated by CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1/ENTPDase1) [8], the dominant vascular and immune cell ectonucleotidase in the lung and at other sites. CD39 catalyzes the phosphohydrolysis of ATP and ADP to AMP [9], which is then, in turn, metabolized by ecto-5'-nucleotidase to adenosine [10].

Compelling data indicate important roles of CD39 in the regulation of immune function [11–16]. Interestingly, *Cd39*-deficiency can be linked to both attenuation, e.g. experimental hepatitis or pancreatitis [15, 16] or worsening of inflammatory diseases in vivo e.g. transplant rejection [17], experimental diabetes [18] and colitis [19], depending on the innate or adaptive immune response, cellular signaling and kinetics of responsiveness. Furthermore, inhibition and/or deficiency of *Cd39* results in decreased migration capacity of neutrophils and monocytes/macrophages, perturbation of regulatory T-cell and/or Langerhans cellular function [12, 13, 20, 21].

The importance of CD39 functionality in AAI remains unexplored. Our aim in the present study was to elucidate the role of mDC-expressed CD39 in these systems. We demonstrate here for the first time that *Cd39*^{-/-} mice exhibit attenuated AAI and that this end-result is linked to perturbation of ATP induced migration of mDC in vivo with aberrant OVA-specific T-cell mediated priming in vitro.

Material and Methods

Mice

Female C57BL/6, *Cd39*^{-/-} and *Cd39*^{+/+} littermates [17] and OVA-Tcr transgenic OT-II mice, all on a C57BL/6 background, were bred at the University Freiburg. All experiments were performed according to institutional guidelines of the animal ethics committee from the City of Freiburg.

OVA-alum model of allergic airway inflammation (AAI)

Mice were sensitized to OVA (Worthington Biochemical) via intraperitoneal (i.p.) injection on days 0 and 7, and challenged with OVA aerosols (1% OVA in PBS) on days 19–21, for 30 minutes [3] [6, 7]. On day 22, BALF and mediastinal lymph node (MLN) were collected, followed by lung resection and storage in OCT freezing medium.

Determination of bronchial hyper-responsiveness

Dynamic resistance and compliance to increasing concentrations of aerosolized methacholine (0, 3mg/ml, 10mg/ml, 30mg/ml, and 100mg/ml) were measured in anesthetized, intubated and mechanical ventilated (Minivent 485, Hugo-Sachs, March, Germany) mice as previously described [6, 7].

House dust mite (HDM) induced allergic airway inflammation

WT and *Cd39*^{-/-} mice were anesthetized and injected intratracheally (i.t.) with either 100µg HDM extract (Greer Laboratories) or vehicle (PBS) as a negative control on day 0 and day 7. On day 14 animals were challenged with an i.t. injection of HDM (100µg). Animals were assessed for the classical features of AAI such as airway hyper responsiveness, inflammation and remodeling and cytokines levels of HDM- re-stimulated MLN-cells on day 17, as previously described [7, 22].

Generation of bone marrow derived dendritic cells (BMDCs)

DCs were prepared as previously described [7]. Briefly, bone marrow cells from WT and *Cd39*^{-/-} mice were grown in RPMI 1640 with 10% FCS (Biocell Laboratories), 1% gentamicin, 2-mercaptoethanol and recombinant murine GM-CSF (200 IU/ml). On days 3, 6, and 8, the medium was refreshed. FACS analysis showed that the purity of BMDCs was greater than 90%.

AAI and adoptive transfer of bone marrow derived DCs from WT and *Cd39*^{-/-}

WT and *Cd39*^{-/-} mice were anesthetized and received an i.t. instillation of 1×10^6 vehicle-WT-DCs, OVA-WT-DCs, vehicle-*Cd39*^{-/-} DCs or OVA-*Cd39*^{-/-} DCs respectively, as described [6] (see supplementary methods). On days 10–12, mice were exposed to OVA aerosols (30 min). Mice were sacrificed 24 hours after the last aerosol.

Migration assay in vivo

80µl of Alexa-488 labeled-OVA (10mg/ml), with or without ATP (100µM) was administered i.t. to *Cd39*^{-/-} and WT mice. Control mice received 80µl of vehicle. At 24–36 hours after

injection, migrating DCs were enumerated in the lung and mediastinal LN as CD11c⁺ MHCII⁺ cells carrying Alexa-488 labeled material.

Quantitative PCR

Quantitative PCR was performed on LightCycler 480 (Roche) using the fast-blue+ UNG kit (Eurogentec). *Cd39* and *β2-microglobulin* primers/probes were designed using Beacon Designer v7.50 (Premier Biosoft). Percent *reference gene* (*RG*) values for the *gene of interest* (*GOI*) were calculated using the formula: %*RG* = $100 \times 2^{(-Ct)}$. Cumulative standard deviations calculated using the formula: $SD = 100 \times 2^{(-Ct)} \times ((\ln 2 \times SD_{RG})^2 + (\ln 2 \times SD_{GOI})^2)^{1/2}$ [23].

Histology and Immunohistochemistry

4μm thick frozen lung sections were stained with hematoxylin and eosin for histological analysis. Immunohistochemistry for CD39 was carried out with a guinea-pig anti-mouse antibody as previously described [24, 25] (<http://ectonucleotidases-ab.com>). For detection, biotinylated anti-guinea-pig secondary antibody was used with Vectastin Elite ABC Kit (Vector Laboratories).

Flow cytometry

BAL cells were stained with I-Ad/I-Ed FITC, CCR3 PE, CD3 and CD19 PE/Cy5 and CD11c APC antibodies. Differential cell counts were analyzed by flow cytometry as previously described [6, 7]. For analysis of DC maturation, bone marrow cells were stained with FITC-labeled anti-I-Ad/I-Ed, PE-labeled anti-CD40, anti-CD80, anti-CD83, anti-CD86, and with APC-labeled anti-CD11c antibodies [6, 7]. Dead cells were excluded using propidium iodide. Acquisition was performed on a FACS Calibur flow cytometer and the analysis was made using FlowJo software.

ATP and cytokine measurements in BALF

The BALF ATP-level and cytokine concentrations (IFN-γ, IL-4, IL-5, and IL-13) were measured using by ATP Lite (Perkin Elmer) or ELISA kits (R&D Systems, Minneapolis, USA) respectively as previously described [4].

Immune synapse formation and time lapse microscopy

OVA- or PBS-pulsed WT and *Cd39*^{-/-} BMDC were stained with FITC-labeled anti-MHCII antibody and plated. Four hours later, purified CD4⁺-OT-II-T-cells that were stained with anti-TCR antibody followed by streptavidin-Cy5 were added. The imaging started after 2 hours and images have been taken every 5 minutes. The ZEISS Cell Observer inverted microscope with incubation chamber and Axiovision software (Zeiss) was used for imaging and Imaris Software (Bitplane AG, Zurich, Switzerland) for synapse analysis. (see supplementary methods).

Results

Lung CD39 expression is down regulated in AAI

OVA-alum and HDM-driven AAI was induced in wild type mice. One day (OVA) or three days (HDM) after the last allergen challenge, mice were euthanized and the expression of CD39 in the lungs was determined by qPCR and IHC. In both models, the induction of AAI was associated with substantial down-regulation of CD39 expression on mRNA (Fig. 1A–B) and protein level (Fig. 1C–D).

Cd39^{−/−} deficiency attenuates AAI

Cd39^{−/−} and WT mice were OVA or sham sensitized, followed by OVA challenge, as previously described [7]. OVA-sensitized, but not of sham-sensitized, WT-animals resulted in an increase in BALF ATP-levels (Fig. 2A), BALF-eosinophilia (Fig. 2B), enhanced Th2-cytokine production by MLN cells (Fig. 2C), peribronchial inflammation (Fig. 2D) as well as BHR to methacholine (Fig. 2E–F).

Somewhat surprisingly, despite increased intrapulmonary ATP levels and the observed down-regulation of CD39 during AAI, *Cd39*^{−/−} mice presented a milder asthma phenotype as seen by significant decrease in all cardinal features of asthma.

Also in the HDM-driven model of AAI, despite the increased pulmonary ATP-levels, *Cd39*^{−/−} mice showed a milder phenotype of AAI, as determined by BAL eosinophilia, Th2-cytokine levels lung tissue infiltration, collagen deposition, mucus hyper-production and BHR to methacholine (Fig. 3A–E).

Cd39^{−/−} DC and ATP induced migration in vitro and vivo

Defective ATP-induced migration in *Cd39*^{−/−} neutrophils and monocytes/ macrophages [20] has been observed. The migratory capacity of endogenous WT and *Cd39*^{−/−} mDCs towards ATP (100μM) in vivo was addressed in these studies. ATP induced recruitment of blood DCs to the lungs was significantly decreased in *Cd39*^{−/−} compared to WT animals (Fig. 4A). In addition, in in vitro experiments, immature BMDCs derived from *Cd39*^{−/−} show significantly decreased migration capacity, when compared to immature BMDC generated from WT animals (Fig. 4B)

Cd39^{−/−} DC show decreased capacity for priming Th2 immunity in vivo

To test the involvement of CD39 in Th2-cell priming capacity of DC, OVA-specific OT-II-T-cells were injected intravenously into WT mice followed by an i.t. administration of OVA- or PBS-pulsed WT or *Cd39*^{−/−} BMDCs. Mice receiving OVA-pulsed *Cd39*^{−/−} DCs showed significant lower levels of the Th2-cytokines (IL-4, IL-5, IL-13) in the MLN, as compared to mice receiving OVA-pulsed WT DCs (Fig. 5A).

To elucidate the relevance in context of AAI, we next used a DC-driven model of AAI. I.t. application of OVA-pulsed WT DCs, but not PBS-pulsed WT DCs, into WT animals leads to BAL-eosinophilia, peribronchial and perivascular inflammation, and Th2-cytokines

production by re-stimulated MLN cells (Fig. 5B–D). Of note, in animals receiving OVA-pulsed *Cd39*^{−/−} DCs, all classical features of AAI were significantly decreased.

In contrast OVA-pulsed WT DCs were able to readily sensitize *Cd39*^{−/−} animals (Fig. 5E–F). These suggest that specific expression of CD39 on DC alone contributes in the sensitization phase but not during the effector phase of AAI.

The observed limitation in Th2-priming appeared to be not due to a defect in OVA-induced maturation of *Cd39*^{−/−} DC, as assessed by the surface expression of CD40, CD83, CD80 and CD86 (data not shown).

CD39-expression on DCs is involved in regulating synapse formation with T-cells

Finally the involvement of CD39-expression on DC in the formation of dendrites and in establishing stable interactions with T-cells was studied. The time of interaction between purified CD4⁺ OT-II cultured with OVA-pulsed *Cd39*^{−/−} DC was significantly shorter, as compared to OVA-pulsed WT-DC and OT-II (Fig. 5G).

Discussion

Compelling studies point to an important role of extracellular nucleotides and associated ectonucleotidases, such as CD39, in regulating immune responses [13]. Deletion of *Cd39*, resulting in an imbalance between extracellular nucleotides and nucleosides, has been shown to adversely impact the outcomes of several acute and chronic inflammatory disorders, e.g. DSS-induced colitis [19] renal ischemia-reperfusion injury [26] and diabetic nephropathy [27], amongst others. These and other studies suggest that augmenting CD39-associated NTPDase activity might be a useful therapeutic strategy in such inflammatory states.

Recently we reported that allergen challenge of asthmatics and sensitized mice results in accumulation of airway ATP-levels, which then acts via activation of P2-receptors and contributes to cardinal features of AAI [4, 7]. CD39 hydrolyzes the proinflammatory molecule ATP to initiate an enzymatic cascade that ultimately produces the anti-inflammatory molecule adenosine via CD73 [11], and a down regulation of CD39-expression/activity has been observed in inflamed tissues [13] including AAI (Fig. 1). Therefore one might speculate that *Cd39*-deficiency would in fact aggravate AAI.

Here we report that despite increased BALF ATP-levels, *Cd39*-deficiency is associated with amelioration of all cardinal features of AAI in mice, including eosinophilic airway inflammation, goblet-cell hyperplasia, Th2-cytokine production, and BHR to inhaled methacholine.

This unexpected result could be partially explained by aberrant functions and impaired migration of blood DC towards ATP. Recently, we reported that ATP contributes to the recruitment of DCs to the lung during AAI via P2Y2R-signaling. The migration of immature blood DC to lung and then subsequently to the MLN is an integral part of both primary and secondary immune response to inhaled allergens [5]. Consequently, in a manner similar to previously reported compounds which interfere with the migration capacity of DC [3], AAI is substantively decreased in *P2Y2R*^{−/−} animals [4, 7]. While the migration of skin DC to

draining lymph nodes in the model of contact dermatitis is not impacted in *Cd39*^{-/-} mice [21], recent studies suggest a more general disordered ATP-induced migration of monocytes and macrophages in these animal. This phenotype has been linked to desensitization of P2YR-subtypes [20, 21]. Therefore, our observation that *Cd39*^{-/-} DCs were unable to migrate in response to ATP in vivo and in vitro, also suggests desensitization of select P2YR in *Cd39*^{-/-} DCs that impact AAI.

During the sensitization of AAI phase, mDCs play a crucial role is the priming of naïve T-cells into allergen-specific Th2 effector cells. Interestingly, Th2-cell differentiation in the MLN following intratracheal injection of OVA-pulsed *Cd39*^{-/-} DCs is impaired, suggesting involvement of CD39 in priming of naïve OT-II T-cells. These observations are in line with a previous report by us, demonstrating an impaired ability of *Cd39*^{-/-} DCs to stimulate hapten reactive T-cells [21]. This defect is associated with resistance of *Cd39*^{-/-} mice to the development of delayed contact hypersensitivity.

To further elucidate a potential involvement of CD39 on DCs in T-cell priming during AAI, OVA-pulsed WT and *Cd39*^{-/-} DCs were adoptively transferred into the lungs of naïve animals, followed by OVA challenge. In parallel with this hypothesis, WT animals receiving OVA-pulsed *Cd39*^{-/-} DCs showed a significant reduction in cardinal features of AAI as compared to animals receiving OVA-pulsed WT DCs.

The induction and maintenance of Th2-immunity in the lungs depends upon adequate migration and antigen-processing function in association with maturation of lung DCs [2]. However, in accordance with previously observations, the OVA-induced expression of CD40, CD80, CD83, CD86, and MHCII did not differ between immature or mature BMDCs from WT and *Cd39*^{-/-} animals.

The immune synapse is considered as a distinct extracellular space shared by a lymphocyte and DC, which is closely linked to antigen presentation. This special junctional structure serves as a microenvironment where various molecule clusters interact with each other leading to the activation of the T-cell, based on the pairing of TCR and presented antigen. Using time lapse imaging, we observe a significant shorter time of interaction between OTII T-cells and OVA- *Cd39*^{-/-} DCs compared to WT-DCs, while the total number of synapse formation did not differ. The formation of the immune synapse is equally important as the proper duration of the interaction, allowing full-scale activation of the T-cell upon antigen recognition. In fact, both parameters are strictly dependent on capabilities of the DCs [28]. There is increasing evidence concerning autocrine and paracrine involvement of ATP in modulating cellular functions. ATP release, leading to the activation of P2-receptors, supports the establishment of a basal stimulation status that facilitates subsequent activation and regulation of many signal transduction pathways [29].

Recent studies suggest that ATP-P2R-signaling within the immune synapse plays a role in T-cell activation upon antigen representation [30, 31]. In this context, involvement of ATP binding to P2X1R and P2X4R-subtypes, have been shown as a part of T-cell activation process [32].

Previous studies have reported that P2R-subtypes are prone to ATP-mediated desensitization [33]. Further, CD39 is palmitoylated and subsequently is localized to lipid rafts that comprise and regulate immune synapse [34, 35]. These data suggest that antigen presentation occurs under specific ATP-concentrations regulated by CD39, P2-receptors and Pannexin-1 within or adjacent to the immune synapse. Thus, our data might indicate that contributions of DC expressed CD39 in the regulation of the ATP/ADP levels within the immune synapse may be crucial in the determination of the activation status of the T-cells.

In summary, we demonstrate that genetic deletion of *Cd39* is associated with decreased AAI in mice. Absence of CD39 is associated with aberrant P2-signaling, which modulate DC migration and limits the capacity of these cells to prime Th2 responses in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AAI	Allergic airway inflammation
ATP	Adenosine-5'-Triphosphate
BALF	broncho-alveolar lavage fluid
P2R	purinergic receptors
BHR	bronchial hyper-reactivity
BMDC	bone marrow-derived dendritic cell
CD39 / ENTPD1	ectonucleoside triphosphate diphosphohydrolase-1
HDM	house dust mite (<i>Dermatophagoides pteronyssinus</i>)
MLN	mediastinal lymph node
OVA	ovalbumin

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Figure 1.

CD39 expression is down regulated in OVA-alum and HDM-driven AAI. In both AAI models expression of CD39 is decreased at both mRNA (A–B) and protein (C–D) levels, as determined by qPCR and IHC, respectively. Numbers of mice - 6–8 per group * $p < 0.01$, ** $p < 0.001$.

Figure 2.

Cd39^{-/-} animals are partially protected against OVA-alum induced allergic airway inflammation. (A) BALF-ATP level and (B) BALF cell differential counts of WT and *Cd39*^{-/-} mice. (C) Cytokine production by MLN cells restimulated with OVA and quantified by ELISA. Mean of 6–8 mice per group # $p < 0.01$ *Cd39*^{+/+} PBS/OVA and *Cd39*^{+/+} OVA/OVA versus *Cd39*^{-/-} PBS/OVA and *Cd39*^{-/-} OVA/OVA animals; * $p < 0.01$ *Cd39*^{+/+} OVA/OVA versus *Cd39*^{-/-} OVA/OVA. (D) HE staining of lung sections. (E) Bronchial hyper-responsiveness (BHR) to various doses of methacholine measured by changes in

resistance [R]. Mean of 6–8 mice per group # $p < 0.01$ $Cd39^{+/+}$ and $Cd39^{-/-}$ PBS/OVA animals vs. $Cd39^{+/+}$ and $Cd39^{-/-}$ OVA/OVA animals; * $p < 0.05$ $Cd39^{+/+}$ OVA/OVA vs. $Cd39^{-/-}$ OVA/OVA.

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Figure 3.

Cd39-deficient mice in HDM induced model of allergic airway inflammation. (A) BALF-ATP level and (B) BALF cell differential counts of WT and *Cd39*^{-/-} mice. (C) Th2 cytokine production by MLN cells restimulated with HDM and quantified by ELISA. (D) HE staining of lung sections. (E) Bronchial hyper-responsiveness (BHR) to various doses of methacholine, as measured by changes in resistance [R]. Mean of 6–8 mice per group **p* < 0.05, # *p* < 0.01 *Cd39*^{+/+} PBS/HDM and *Cd39*^{+/+} HDM/HDM versus *Cd39*^{-/-} PBS/HDM

and *Cd39*^{-/-} HDM/HDM animals; *p<0.01 *Cd39*^{+/+} HDM/HDM versus *Cd39*^{-/-} HDM/HDM

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Figure 4. Unresponsiveness of blood *Cd39*^{-/-} DC in migrating towards ATP in vivo. (A) Effect of ATP on the absolute number of DCs in the lungs of WT and *Cd39*^{-/-} animals. (B) Immature BMDC from *Cd39*^{-/-} show significantly decreased capacity to migrate towards ATP in vitro. Mean of 6 mice /group *p<0.05

Figure 5.

Effect of CD39 on T cell priming capacity and synapse formation of DCs in vivo and in vitro. (A) Mice were instilled i.t. with PBS- or OVA-pulsed WT or $Cd39^{-/-}$ BMDC, on day two, OVA-specific naïve $CD4^{+}$ T-cells were injected i.v. On day 6, MLN cells were isolated and cultured for 4 days. Levels of IL-4, IL-5 and IL-13 in the supernatants were determined by ELISA. Data are mean $\pm$ SEM, n=6 animals per group *p<0.05. (B) BALF cell differential counts of WT and $CD39^{-/-}$ mice received OVA-pulsed WT or $Cd39^{-/-}$ DCs i.t. (C) Production of Th2 cytokines (IL-4, IL-5, IL-13) in the MLN. (D) HE staining of lung

sections. (E, F) Sensitization of *Cd39*^{-/-} animals by OVA-pulsed WT DCs. Mean of 6–8 mice/group *p<0.05 PBS-DCs versus OVA-DCs; #p<0.05 WT-OVA-DCs versus *Cd39*^{-/-} OVA-DCs. (G) Immune synapse formation between WT/*Cd39*^{-/-} BMDCs and CD4⁺ T-cells from OT-II mice; *p<0.05.

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