

ORIGINAL ARTICLE

AIRWAY DISEASES

MicroRNA-155 modulates P2R signaling and Th2 priming of dendritic cells during allergic airway inflammation in mice

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Keywords

allergic airway inflammation; dendritic cells; microRNA-155; P2R signaling; Th2 response.

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Abstract

Background: Dendritic cells (DCs) are the professional antigen-presenting cells (APCs) in the lung. They are known to be key players in the induction and maintenance of allergic asthma by cross-linking innate and adaptive immune responses. MicroRNAs (miRNAs) are known to influence cell fate and function by translational suppression or induction of messenger RNA (mRNA) degradation. miR-155 has been shown to be a crucial regulator of the immune system. However, its function in the pathogenesis of allergic airway inflammation (AAI) is not completely elucidated yet.

Methods: Wild type (WT) and miR-155-deficient (miR-155^{-/-}) mice were used in ovalbumin (OVA) and house dust mite (HDM) models of AAI. Adoptive transfer of sensitized DCs to the lungs, migration, and T-cell priming assays were used to investigate the functional relevance of miR-155 in DCs.

Results: miR-155^{-/-} mice showed reduced eosinophilic airway inflammation compared to WT mice in both models of AAI. Furthermore, miR-155^{-/-} DCs showed limited Th2 priming capacity and failed to induce airway inflammation in allergen-exposed WT mice. miR-155 deficiency on DCs was also associated with impaired purinergic receptor signaling, as miR-155^{-/-} DCs showed reduced chemotaxis and IL-1β secretion upon stimulation with ATP, probably due to direct targeting of ectonucleoside triphosphate diphosphohydrolases (ENTPD) by miR-155.

Conclusions: miR-155 deficiency alleviates AAI by diminishing Th2 priming capacity and ATP/P2R-induced activation of DCs in mice, suggesting this miRNA as a potential therapeutic target of AAI.

Asthma is a chronic respiratory disease characterized by bronchial hyper-reactivity (BHR) to unspecific stimuli, mucous oversecretion, and bronchospasm resulting in reversible airflow

obstruction (1). The pathophysiological processes of this disease are triggered by allergen-induced eosinophilic Th2-mediated airway inflammation leading to tissue remodeling

Abbreviations

AAI, allergic airway inflammation; AID, activation-induced cytidine deaminase; APCs, antigen-presenting cells; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; BALF, broncho-alveolar lavage fluid; BHR, bronchial hyper-reactivity; BMDCs, bone marrow-derived dendritic cells; DCs, dendritic cells; ENTPD, ectonucleoside triphosphate diphosphohydrolases; HDM, house dust mite; i.p., intraperitoneal; i.t., intratracheal; IL, interleukin; LPS, lipopolysaccharide; mDC, myeloid dendritic cell; miRNA, microRNA; MLN, mediastinal lymph node; MNC, mononuclear cell; mRNA, messenger RNA; OVA, ovalbumin; P2R, purinergic type 2 receptor; SOCS1, silencing suppressor of cytokine signaling 1; Th2, T-helper cell type 2 cells; UTR, untranslated region; WT, wild type.

related to airway dysfunction (2). Recent findings suggest an important role of dendritic cells (DCs) in the initiation and maintenance phase of this Th2 response (3–6).

MicroRNAs (miRNAs) are short (21–23 base pairs) single-stranded RNA molecules that mediate posttranscriptional gene silencing mainly by binding to the 3'-untranslated region (UTR) of target messenger RNA (mRNA) leading to degradation or inhibition of translation (7–9). Over the last years, numerous studies identified a crucial role of miRNAs in regulating innate and adaptive immune response in carcinogenesis and various inflammatory disorders such as arthritis, atherosclerosis, and dermatitis (10). miR-155, which is among the most intensively studied immune system-related miRNAs, has been implicated in allergic disorders. miR-155 is highly expressed on activated B cells, T cells, DCs, and macrophages and is required for their cellular functions for innate and/or adaptive immune response (11). The human *miR-155* gene is mapped to a region on chromosome 21q21, associated with asthma, pollen sensitivity, and atopic dermatitis susceptibility (12–14). For instance, by targeting activation-induced cytidine deaminase (AID) and transcription factor PU.1 mRNA of B cells, it regulates germinal center formation, antibody secretion, and class switch recombination (11, 15–17). Furthermore, miR-155 modulates development and activation of various T-cell subsets; for example, by targeting c-Maf, a potent transactivator of the IL-4 promoter, miR-155 attenuates Th2-cell responses (11), while it positively regulates Th1 (and Th17) proliferation via silencing suppressor of cytokine signaling 1 (SOCS1) (18). Consequently, miR-155-deficient T cells are intrinsically biased toward Th2 differentiation, and interestingly, up to 50% of aging miR-155-deficient mice spontaneously develop asthma-like lung inflammation (11). With regard to macrophages, miR-155 has been shown to target IL-13 receptor alpha, SOCS1 and SHIP1 mRNA, thus regulating M1/M2 balance toward pro-Th1/M1 macrophages (19). These data suggest that overexpression of miR-155 might decrease Th2-driven AAI. By modulating c-FOS and DC-SIGN expression, miR-155 regulates DCs functions that are crucial in the induction and maintenance of AAI (20, 21). Additionally, miR-155-deficient DCs showed an impaired T-cell priming capacity upon lipopolysaccharide (LPS)-induced maturation (11). Moreover, miR-155-deficient mice have been reported to be partially protected against OVA-induced eosinophilic airway inflammation in a Th2-dependent manner (22). Therefore, we focused on the role of miR-155 in DCs during the initiation phase and maintenance of AAI, rather than the actual adaptive immune response mediated by Th2 cells.

Material and methods

Mice

C57BL/6 wild type, *B6/miR-155^{-/-}* mice on C57BL/6J background (B6Cg-MiR-155^{tm1.1Rsky}/J) were bred at the Freiburg University. All experiments were approved by the University Committee on the Use and Care of Laboratory Animals and performed according to the institutional guidelines of the national animal ethics committee (G-12/10, X-12/02H).

Generation of bone marrow-derived dendritic cells

Bone marrow cells were isolated from tibia and femur of miR-155^{-/-} and WT mice by flushing the medullary cavity. Cells were cultivated in RPMI medium, containing 10% fetal calf serum (FCS; Biocell Laboratories), 1% penicillin/streptavidin, beta-mercaptoethanol and recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF, 200 IU/ml). On days 3 and 6, medium was refreshed. OVA (100 µg/ml), HDM (10 µg/ml), and ATP (100 µM) stimulation of the bone marrow-derived dendritic cells (BMDCs) was performed on day 8 for 24 h. Purity of BMDCs was >90% as determined by FACS.

DCs migration assay *in vitro* and *in vivo*

In vitro migration assay was performed in a 24-well plate with 5-µm transwell chamber (Corning, Amsterdam, Netherlands) in triplicates. Naïve BMDCs (10⁵ cells/well) were added into the upper compartment, whereas the lower compartment was loaded with different concentrations of ATP. The plate was incubated at 37°C, 5% CO₂ for 2 h. Chemotactic index was calculated by dividing number of cells from each lower compartment by the number of migrated cells in medium control.

For *in vivo* migration assay, C57BL/6 and miR-155^{-/-} mice received an intratracheal (i.t.) instillation of 80 µl Alexa-647-conjugated OVA (10 mg/ml; Life Technologies, Darmstadt, Germany) either with or without additional ATP (100 µM; Sigma-Aldrich, Taufkirchen, Germany). Control mice received 80 µl PBS. Thirty-six hours later, migrated DCs were detected in the lung and quantified by FACS as CD11c⁺ MHCII⁺ Alexa-647⁺ cells.

Statistical analysis

Data are shown as mean ± SEM. Analysis was performed using one-way ANOVAS with additional Bonferroni correction in GraphPad Prism 5 software (GraphPad software, La Jolla, CA, USA). *P* values lower than 0.05 were considered statistically significant.

Results

miR-155 deficiency attenuates OVA- and HDM-induced airway inflammation in mice

In both OVA- and HDM-driven AAI models, allergen-challenged WT mice showed an increased miR-155 expression compared to naïve mice (Fig. 1A) in line with recent results (22, 23). miR-155 deficiency was accompanied by an astonishing decrease in all cardinal features of AAI compared to WT. A reduced number of broncho-alveolar lavage fluid (BALF) eosinophils, lymphocytes, and neutrophils (Fig. 1B), an attenuated production of Th2-cytokines by allergen-restimulated mediastinal lymph node (MLN) cells (Fig. 1C), less peribronchial cell infiltration (Fig. 1D), and a decreased BHR (Fig. 1E) were observed in miR-155^{-/-} compared to WT

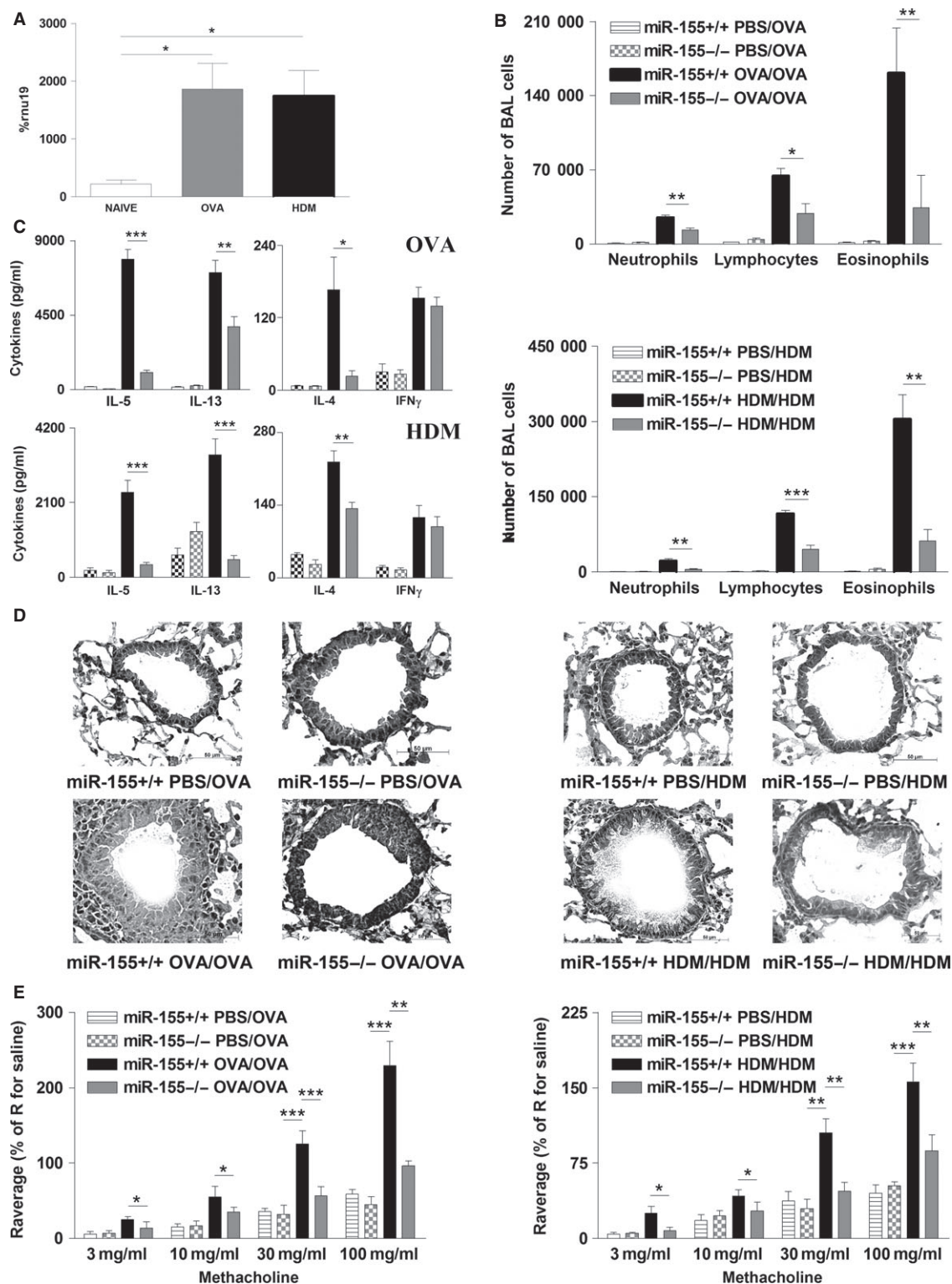


Figure 1 miR-155^{-/-} mice are partially protected against OVA- and HDM-induced AAI. (A) miR-155 expression in lungs of both OVA- and HDM-challenged mice. (B) BALF differential cell counts. (C) Cytokine concentration in supernatants of MLN cells restimulated with allergen cultured for 4 days. (D) Hematoxylin and eosin (H&E)-

stained lung sections of vehicle and allergen-challenged mice. Magnification: 40 $\times$ objective. (E) Changes in airway resistance [R] in response to increasing doses of methacholine were measured for analyzing bronchial hyper-responsiveness. Error bars represent SEM, $n = 5-6$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 1 Distorted expression of costimulatory molecules on miR-155^{-/-} BMDCs

	PBS-WT BMDCs	PBS-miR-155 ^{-/-} BMDCs	OVA-WT BMDCs	OVA-miR-155 ^{-/-} BMDCs
CD40	5.21 ± 2.1	4.05 ± 1.5	26.67 ± 3.5	18.14 ± 4.7
CD80	17.88 ± 4.6	13.91 ± 3.5	35.49 ± 4.3	23.66 ± 3.6*
CD83	3.38 ± 1.2	2.84 ± 0.6	9.53 ± 2.1	5.89 ± 1.4*
CD86	23.45 ± 5.9	18.46 ± 4.8	61.51 ± 5.5	44.17 ± 3.8*

OVA/PBS-pulsed WT and miR-155^{-/-} BMDCs were analyzed for their expression of CD40, CD80, CD83, and CD86. Expression is shown as median fluorescence intensity and presented as mean ± SEM from 1 representative experiment out of 3 (*n* = 5 mice per group).

**P* < 0.05; OVA-WT vs OVA-miR-155^{-/-}.

mice in both AAI models. These results suggest a strong proasthmatic effect of miR-155 in the lung.

miR-155 is crucial for allergen-induced maturation of BMDCs *in vitro*

To investigate whether miR-155 deficiency alters allergen-induced DC maturation, OVA-stimulated BMDCs were analyzed for CD40, CD80, CD83, and CD86 expression. CD80, CD83, and CD86 were decreased, whereas CD40 and MHCII expression were similar in miR-155^{-/-} BMDCs compared to WT BMDCs (Table 1; Fig. S2). Despite the altered maturation of allergen-challenged BMDCs associated with miR-155 deficiency, the release of IL-12p70, TNF-α, and IL-10 was similar compared to miR-155^{+/+} BMDCs (Fig. S3). Of note, allergen challenge of BMDCs was associated with increased miR-155 expression (Fig. S1).

miR-155-deficient BMDCs show reduced capacity to induce a Th2 response *in vivo*

To elucidate whether the distorted maturation of miR-155^{-/-} DCs is of functional relevance contributing to a milder phenotype in AAI, OVA-pulsed BMDCs from WT and miR-155^{-/-} mice were cocultured with purified naïve CD4⁺ OTII T cells. OTII CD4⁺ T cells cocultured with OVA-primed miR-155^{-/-} BMDCs produced lower amounts of IL-4, IL-5, and IL-13 compared to cocultures with WT BMDCs (Fig. 2A). Accordingly, allergen-restimulated MLN cells isolated from animals adoptively transferred with CD4⁺ OTII cells and OVA-primed miR-155^{-/-} BMDCs also produced less IL-4, IL-5, and IL-13 compared to mice adoptively transferred with CD4⁺ OTII T cells and WT BMDCs (Fig. 2B). Finally, for better defining the relevance of miR-155 on DCs function in the context of lung inflammation, we used a DCs-driven model of AAI, where BMDCs got instilled i.t. before allergen challenge. As expected, OVA-challenged WT mice receiving OVA-pulsed miR-155^{-/-} BMDCs developed milder BAL eosinophilia, peribronchial and perivascular inflammation, as well as a decreased production of Th2-cytokines by allergen-restimulated MLN cells compared to murine recipients of OVA-pulsed WT BMDCs (Fig. 2C–E).

However, BAL eosinophilia was partially restored by applying OVA-pulsed WT BMDCs to miR-155^{-/-} animals (Fig. 2F). Similar results were observed using HDM instead of OVA (Fig. S4).

miR-155 influences ATP homeostasis of DCs

Database screening (miRWalk, microRNA.org, Targetscan, and miRTarBase) for potential targets of miR-155 revealed ENTPD1 and ENTPD3, members of the ectonucleotidase family responsible for ATP degradation (Fig. 3A). Accordingly, lung MNCs isolated from miR-155^{-/-} mice showed elevated *Entpd1* and *Entpd3* mRNA expression compared to WT (Fig. 3B). To elucidate whether the upregulation of *Entpd1* and *Entpd3* is of functional relevance, ATPase activity in lung sections from naïve mice was determined via enzyme histochemistry. As expected, lungs from naïve miR-155^{-/-} mice showed an increased ATPase activity compared to WT (Fig. 3C). Additionally, immature BMDCs as well as OVA- and HDM-stimulated miR-155^{-/-} BMDCs showed higher expression levels of *Entpd1* and *Entpd3* (Fig. 3D). This observation was further ascertained by bioluminescent data on significantly higher ATP-scavenging capacity of miR-155^{-/-} BMDCs in comparison with WT (Fig. 3E).

miR-155 deficiency disturbs ATP-induced migration and activation of DCs

Previously, we demonstrated that ATP is important for DCs activation (5). ATP-induced P2RY2 signaling is important for DCs recruitment into the lung, whereas ATP-induced P2RX7 signaling plays a role in the production and release of mature IL-1β. Thus, we suggested that the enhanced ectonucleotidase activity on miR-155^{-/-} DCs might interfere with ATP-induced P2RY2 and P2RX7 signaling, thereby disturbing DCs activation. *In vitro* transwell assays revealed miR-155^{-/-} BMDCs showing a reduced migration capacity toward ATP compared to WT (Fig. 4A). Furthermore, ATP-induced recruitment of endogenous blood DCs to the lung of OVA-challenged miR-155^{-/-} mice was decreased (Fig. 4B). Addressing IL-1β secretion of DCs, miR-155^{-/-} and WT BMDCs were treated with OVA, HDM, or vehicle with or without additional ATP for 24 h *in vitro*. Indeed, miR-155^{-/-} BMDCs secreted lower amounts of IL-1β upon ATP/OVA or ATP/HDM treatment compared to WT (Fig. 4C). A prominent difference in neither *P2ry2* nor *P2rx7* expression was observed between miR-155^{-/-} and WT DCs excluding decreased *P2ry2* and *P2rx7* levels being the reason for the attenuated DCs activation associated with miR-155 deficiency (Fig. 4D).

Discussion

miR-155 is an important determinant of adaptive and innate immune responses by posttranscriptional regulation of gene expression in activated B and T cells as well as in macrophages and DCs (11). Although previous studies suggest a role of miR-155 in the pathogenesis of allergic diseases such as atopic dermatitis and AAI, the precise role of miR-155 on DCs

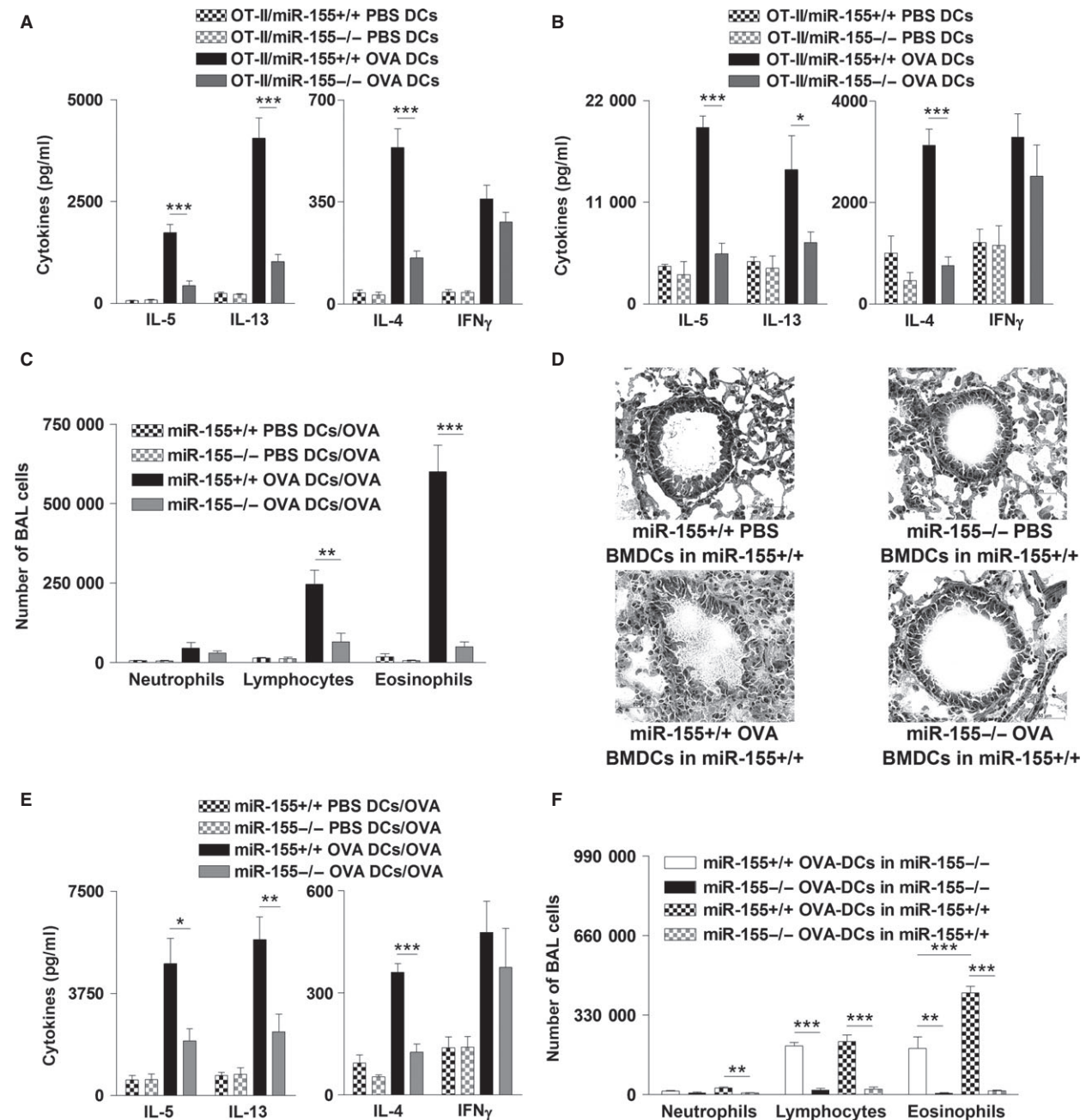


Figure 2 miR-155^{-/-} murine BMDCs show an impaired CD4⁺ T-cell priming capacity and fail to induce AAI in a DC-driven OVA model. (A) Cytokine content in supernatants of CD4⁺ OTII T cells/OVA-BMDCs cocultures and (B) OVA-restimulated MLN cells isolated from mice adoptively transferred with OT-II T cells and OVA-primed BMDCs before OVA challenge after 4 days of culturing. (C) Differential BALF cell count, (D) cytokine concentration in supernatants of OVA-restimulated MLN cells, and (E) H&E-stained lung sections (magnification: 40 $\times$ objective) of miR-155^{+/+} mice adoptively transferred with vehicle/OVA-primed BMDCs before allergen challenge. (F) Differential BALF cell count of miR-155^{+/+} and miR-155^{-/-} mice receiving OVA-primed miR-155^{+/+} or miR-155^{-/-} BMDCs before OVA challenge. Error bars represent SEM, $n = 4-5$. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

biology in AAI remains unclear (22, 24). In this study, we show that DCs require miR-155 to orchestrate a complete Th2 response in both OVA- and HDM-driven models of AAI. Mechanistically, we provide evidence for a novel cross talk of

miR-155 and ATP-P2R signaling in DCs upon allergen challenge. Examining miR-155^{-/-} mice in both models, we could show that all cardinal features of AAI, including eosinophilic airway inflammation, peribronchial inflammation,

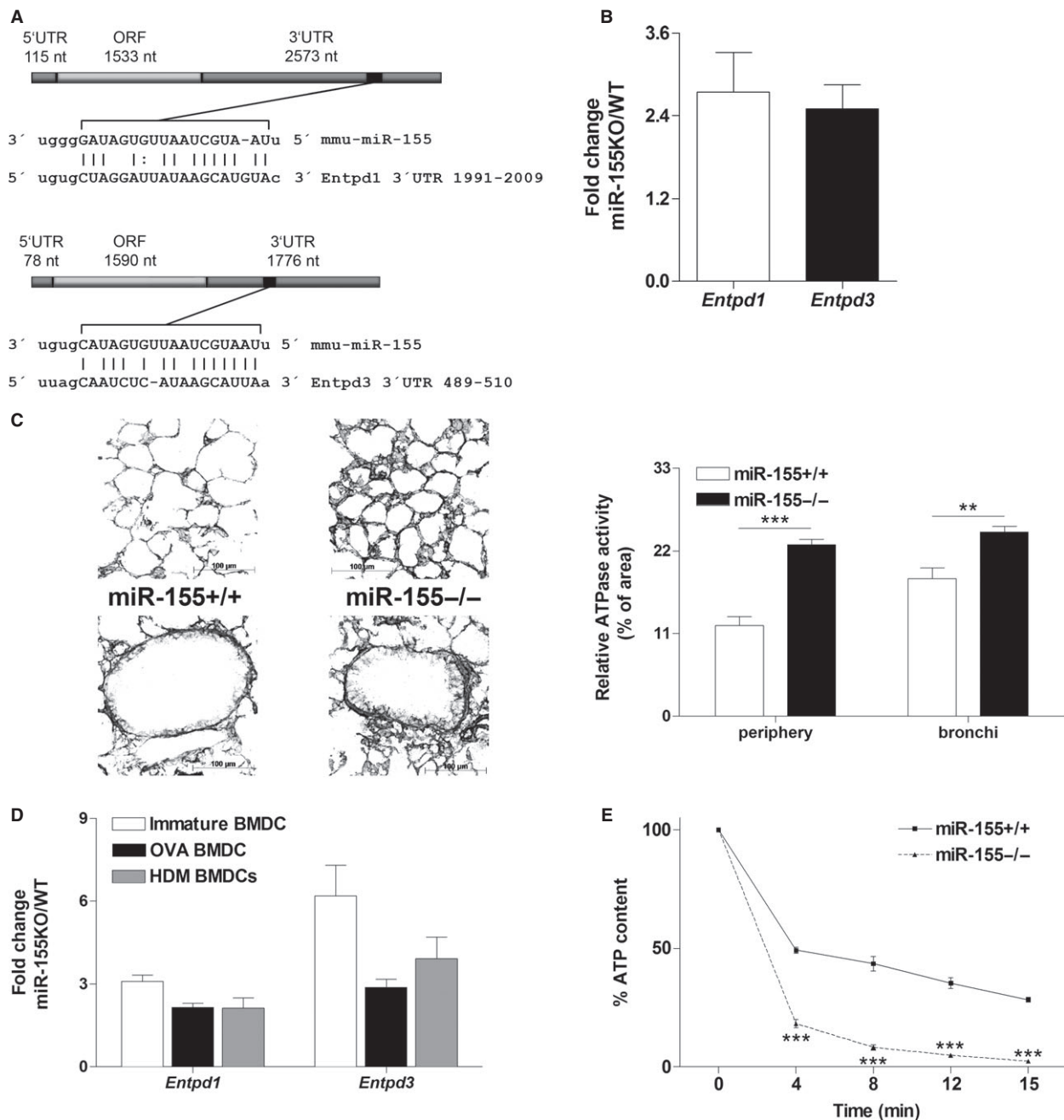


Figure 3 Targeting of ectonucleotidases by miR-155 leads to an increased ATP degradation in miR-155^{-/-} mice. (A) Scheme of predicted miR-155-binding sites on *Entpd1* and *Entpd3* mRNA UTR. (B) Relative expression of *Entpd1* and *Entpd3* in lungs and (D) vehicle/allergen-stimulated BMDCs of miR-155^{-/-} mice compared to miR-155^{+/+} mice. (C) Enzyme histochemistry showing ATPase activity in bronchus

and periphery of naïve miR-155^{+/+} and miR-155^{-/-} lungs. Magnification: 20× objective. Relative ATPase activity determined as percentage of brown-dyed area in stained sections. (E) Percentage of ATP content left in supernatants of mature miR-155^{+/+} and miR-155^{-/-} BMDCs at different time points after adding 10 μM ATP. Error bars represent SEM, $n = 4-6$. ** $P < 0.01$ and *** $P < 0.001$.

Th2-cytokine production of allergen-restimulated mediastinal lymph node cells, and bronchial hyper-reactivity to inhaled methacholine, are remarkably decreased compared to WT animals. The reduced levels of BAL eosinophils and peribronchial inflammation as well as IL-13 production of

allergen-restimulated MLN cells are again in accordance with recent studies, where the importance of miR-155 in T cells for the regulation of the Th2 response in AAI is reported (22, 23). In contrast to those reports focusing on T cells, our current findings show that miR-155 expression in DCs is also a

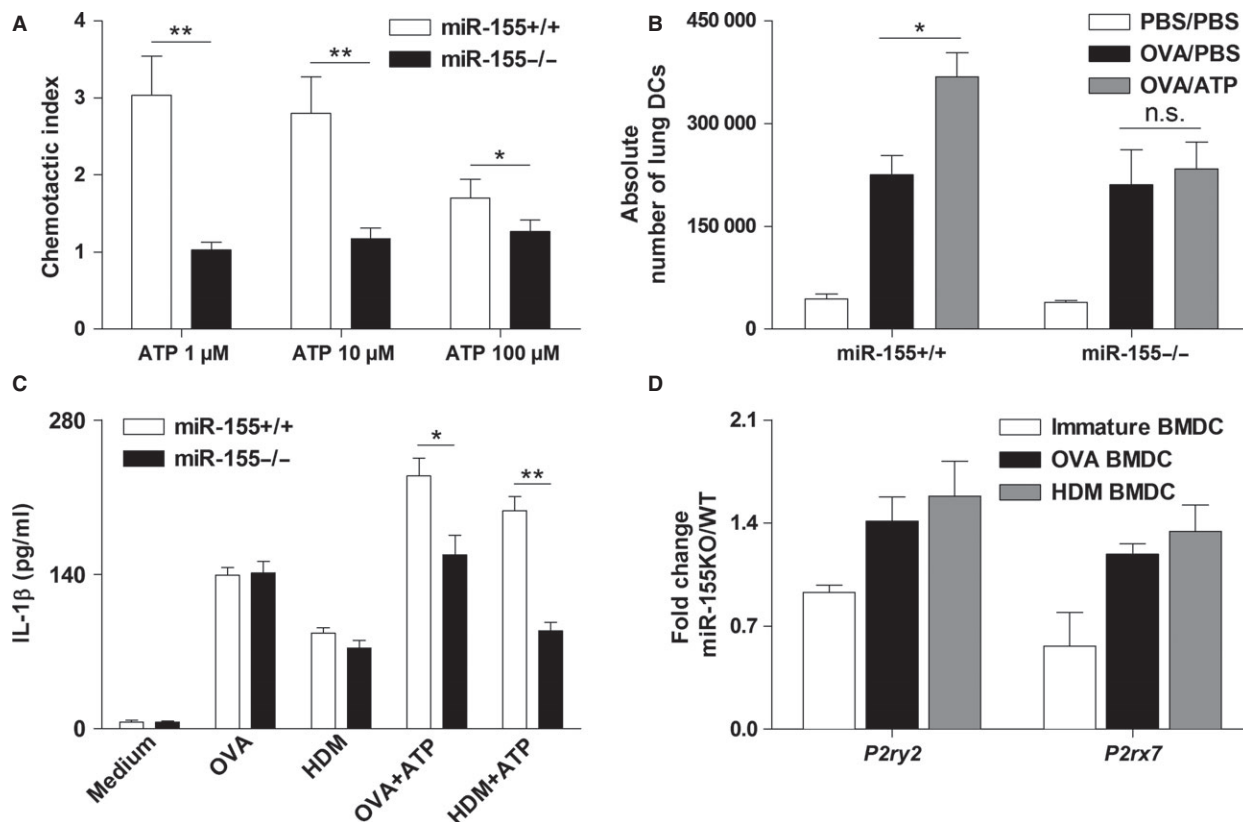


Figure 4 miR-155 deficiency decreases ATP-induced cell recruitment in mice. (A) *In vitro* chemotaxis of miR-155^{+/+} and miR-155^{-/-} BMDCs toward ATP relative to PBS. (B) Number of lung DCs in miR-155^{+/+} and miR-155^{-/-} mice treated with OVA-vehicle/ATP. (C) Concentration of IL-1 β in supernatants of miR-155^{+/+} and

miR-155^{-/-} BMDCs stimulated with vehicle or OVA/HDM $\pm$ ATP. (D) Fold regulation of *P2ry2* and *P2rx7* expression in immature or OVA/HDM-stimulated miR-155^{-/-} BMDCs relative to miR-155^{+/+} BMDCs. Error bars represent SEM, $n = 4-6$. * $P < 0.05$ and ** $P < 0.01$.

necessary prerequisite for airway immunity in mice. Hereby, we provide a new insight on the role of miR-155 in DCs recruitment to site of inflammation and in T-cell priming during AAI. Our data underline the importance of miR-155 for the initiation and maintenance of AAI driven by DCs, thus ensuring an unimpaired Th2 response.

Our study is based on the fact that miR-155 plays an important role in DCs biology (25), and DCs are of critical importance for the initiation and maintenance of AAI (3-6, 26). miR-155 has been related to pathogen-associated molecular pattern (PAMP)-induced maturation of DCs (27-29). We found that pulsing BMDCs with either OVA or HDM is associated with an upregulation of miR-155 expression. Furthermore, miR-155 deficiency on OVA-pulsed BMDCs was accompanied by decreased expression of CD80, CD83, and CD86, pointing out to a direct effect of miR-155 on DCs maturation in AAI. Of note, while Dunan-Sauthier et al. described a decreased expression of CD40 and CD86 associated with reduced mRNA expression of IL-12p35, IL-12p40, and TNF- α by miR-155^{-/-} BMDCs following LPS-induced maturation (20), other groups reported no differences in the expression of costimulatory molecules between LPS-treated WT BMDCs and miR-155^{-/-} BMDCs (11, 29). It has been shown previ-

ously that the CD80-CD86 complex as well as CD83 on DCs is important for the stimulation and differentiation of allergen-specific Th2 cells (3, 26, 30-32). Thus, we hypothesized that miR-155-deficient DCs might have a 'disturbed' Th2-cell priming capacity in the context of AAI. Indeed, Th2-cell differentiation of adoptively transferred OVA-specific CD4⁺ OTII T cells was reduced in animals receiving OVA-pulsed miR-155^{-/-} BMDCs compared to animals receiving OVA-pulsed WT BMDCs by means of IL-4, IL-5, and IL-13 production by MLN cells upon OVA restimulation *in vitro*. The same effect could be seen when OVA-pulsed miR-155^{-/-} DCs were cocultured with CD4⁺ OTII T cells. These observations are supported by previous studies demonstrating that miR-155 deficiency on mature BMDCs is accompanied by an attenuated capacity to activate CD4⁺ T cells (OTII) regarding T-cell proliferation, IL-2 production, and CD69 expression in cocultures (11, 20). This effect might be due to miR-155-silencing c-Fos, an inhibitor of pro-inflammatory cytokine production in DCs (20). To further elucidate a potential involvement of miR-155 on DCs in Th2-cell priming in AAI, we used a DCs-driven model where AAI is induced by adoptive transfer of OVA- or HDM-pulsed BMDCs to the lungs of naïve animals before OVA or HDM challenge, respectively. As expected, WT

animals receiving allergen-pulsed miR-155^{-/-} DCs showed a very strong reduction in cardinal features of AAI, including BAL eosinophilia, peribronchial inflammation, and Th2-cytokine production by allergen-restimulated MLN cells compared with WT mice receiving allergen-pulsed WT DCs. Interestingly, the i.t. application of allergen-pulsed WT BMDCs to miR-155^{-/-} mice also induced slightly reduced BALF eosinophilia compared with WT recipient animals receiving allergen-pulsed WT BMDCs. Nevertheless, this reduction is not as pronounced as the one in miR-155^{-/-} animals adoptively transferred with allergen-pulsed miR-155^{-/-} BMDCs, suggesting a pivotal role of miR-155 expression on DCs in the sensitization phase of AAI. The decrease in eosinophils in the miR-155^{-/-} mice receiving allergen-pulsed WT BMDCs could be explained by the attenuated CD4⁺ T-cell-mediated response in miR-155-deficient animals reported in recent publications (11, 22, 23, 33).

Interestingly, bioinformatic target predication algorithms revealed that *Entpd1* and *Entpd3*—two members of the ectonucleotidase gene family which tightly regulate extracellular ATP concentration under physiological and pathophysiological conditions (34)—harbor potential miR-155-binding sites in their 3'-UTR, suggesting them to be potential targets. Indeed, the *Entpd1* expression and *Entpd3* expression in lung tissue and BMDCs of miR-155-deficient mice were upregulated compared to WT, which was of functional relevance, as both miR-155^{-/-} lungs and BMDCs showed an increased ATPase activity. Keeping in mind that allergen challenge leads to accumulation of endogenous ATP in the airways, which plays a crucial role in AAI by inducing P2R signaling on DCs (3, 34), we suggest a 'distorted' ATP-P2R signaling on DCs in miR-155^{-/-} mice. This concept is supported by the observation that miR-155^{-/-} DCs failed to migrate toward ATP *in vivo* and *in vitro*. We previously showed that ATP-induced P2RY2 activation drives the lung recruitment of DCs after allergen challenge (35). Additionally, ATP is contributing to AAI via cross-linking P2RX7, which promotes maturation and release of IL-1 β , a pro-inflammatory cytokine involved in DC-mediated Th2-cell activation and proliferation (26, 36). Indeed, miR-155^{-/-} BMDCs treated with OVA/HDM and ATP produced lower amounts of IL-1 β compared to WT BMDCs. Ceppi et al. reported that miR-155 knockdown in human blood-derived DCs leads to an increased IL-1 β production after LPS-induced maturation (27). However, we could not observe an altered IL-1 β production by murine miR-155^{-/-} BMDCs after OVA- or HDM-induced maturation.

Taken together, we demonstrate that miR-155 contributes to pathogenesis of AAI in mice. Although there is a growing body of evidence suggesting miR-155 playing a crucial role on T cells in allergic diseases, we show, for the first time, that miR-155 is required for allergen-induced maturation and T-cell priming of DCs in AAI. Thereby, we provide evidence that miR-155 plays a crucial role during allergen encounter by APCs and not only during the actual adaptive immune response. Mechanistically, we show that miR-155 modulates DCs lung recruitment and T-cell priming by regulating ATPase activity and the corresponding ATP-induced P2RY2 and P2RX7 signaling. Inhibiting miR-155 might be a promis-

ing option for treating allergic asthma and other DCs-driven Th2-cell-mediated diseases.

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Conflicts of interest

The authors declare that they have no relevant conflict of interests.

Author contributions

Andreas Zech performed and designed most of the experiments, analyzed the data, and wrote the manuscript. C. Korcan Ayata performed gene expression assay design and analysis, did the *in silico* profiling of miR-155 targets, and contributed to the manuscript by proofreading. Franziska Pankratz performed miR-155 qPCR experiments. Anja Meyer assisted in animal experiments and performed ELISA. Kristin Baudiß assisted in animal experiments and gave intellectual input for the preparation of the manuscript. Sanja Cicko assisted in animal experiments and gave intellectual input for the preparation of the manuscript. Gennady G. Yegutkin helped in designing and performing the enzyme histology and gave intellectual input for the preparation of the manuscript. Sebastian Grundmann helped in designing the miR-155 qPCR experiments and establishing the miR-155 mice breeding, gave intellectual input, and helped in finalizing the manuscript. Marco Idzko formulated the hypothesis, supervised the study and design of the experiments, and did the final editing of the manuscript.

Supporting Information

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

Figure S1. Fold regulation in miR-155 expression in OVA- or HDM-stimulated miR-155^{+/+} BMDCs relative to naïve miR-155^{+/+} BMDCs.

Figure S2. DC maturation of OVA-pulsed BMDCs isolated from miR-155^{+/+} (shaded histogram) or miR-155^{-/-} (open histogram) mice were determined by surface expression of CD40, CD80, CD86 or MHCII of live cell gated and CD11c⁺ cells shown as histograms.

Figure S3. A and B, IL-10, IL-12p70 and TNF- α concentration in supernatants of OVA- or PBS- (Supp. Fig. 3A) and HDM- or PBS- (Supp. Fig. 3B) pulsed BMDCs isolated from miR-155^{+/+} or miR-155^{-/-} mice. Error bars represent SEM, *n* = 3. n.d. = not detectable.

Figure S4. A and B, (Supp. Fig. 2A) Number of differential BAL cells and (Supp. Fig. 2B) cytokine concentration in supernatants of HDM re-stimulated MLN cells of miR-155^{+/+} mice adoptively transferred with vehicle/HDM-primed miR-155^{+/+} or miR-155^{-/-} BMDCs before allergen challenge.

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Supplements

OVA-alum model of allergic airway inflammation (AAI)

C57BL/6 and miR-155^{-/-} mice were sensitized to ovalbumin (OVA) via intraperitoneal (i.p.) injection (10µg OVA grade V, Worthington Biochemical, Lakewood, NJ, adsorbed to 1mg aluminum hydroxide; Thermo Scientific Pierce Inject Alum) on days 0 and 7, and were challenged with OVA aerosols on days 17-19, delivered from a nebulizer delivering 1% OVA in PBS for 30 minutes. Control mice received PBS/alum i.p.. Twenty-four hours after the last OVA aerosol exposure, bronchoalveolar lavage (BAL) was performed with 3x 1ml PBS (Life Technologies, Darmstadt, Germany) supplemented with 0.1mM sodium EDTA, followed by lung resection and storage in OCT freezing medium for histological assays. For cytokine measurements mediastinal lymph node (MLN) cells were plated in 96 well round U-bottom plates (2x10⁵ cells/well), re-stimulated with OVA (25µg/ml) and incubated for 5 days at 37°C with 5% CO₂. The amount of IL-4, IL-5, IL-13, and IFN-γ in supernatants was assayed by ELISA DuoSet (R&D Systems, Minneapolis, MN).

Induction of AAI with house dust mite extract

Deeply anesthetized C57BL/6 and miR-155^{-/-} mice were sensitized on days 0 and 7 and challenged on day 14 via intra-tracheal (i.t.) injection of 100µg *dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenior, NC) in 80µl PBS. Control mice received PBS. BAL, histology and cytokine measurements were performed as described above. HDM (40µg/ml) was used for MLN cell re-stimulation.

Flow cytometry

BAL Cells were incubated with an unlabeled anti-CD16/32 (1:100) antibody to avoid unspecific antibody binding. Afterwards they were stained with anti-Gr-1 FITC- (1:50), anti-CCR3 PE- (1:50), anti-CD3- and anti-CD19 Pe-Cy7- (both 1:100) and anti-CD11c APC-conjugated (1:400) antibodies. For BMDC maturation measurement cells were incubated with FITC-labeled I-Ad/I-Ed (anti-Rat used 1:200 and anti-armerian hamster used 1:100), anti-CD40 (1:50), anti-CD80 (1:200), anti-CD83 (1:200) and anti-CD86 (1:400), anti-MHCII (1:400) as well as APC-labeled anti-CD11c (1:400) antibodies. All antibodies were purchased from eBiosciences (Frankfurt am Main, Germany). Flow cytometry analyses were performed with a FacsCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR) as previously described (1).

Invasive lung function measurement

Mice were anesthetized, intubated with an 18-gauge catheter and mechanically ventilated (Minivent 485, Hugo-Sachs, March, Germany) during exposure to increasing concentrations of nebulized methacholine (0mg/ml, 3mg/ml, 10mg/ml, 30mg/ml and 100mg/ml). Respiratory sequence was set to 200 breaths per minute with a tidal volume of 200µl and a positive end expiratory pressure of 2ml H₂O. Resistance was recorded with a Resistance Plethysmograph (EMMS, Bordon, UK), as previously described (1). Baseline resistance was restored before administering the subsequent doses of methacholine.

OTII CD4⁺ T cell and BMDC Co-Culture

Naïve CD4⁺ T cells were magnetically isolated (Miltenyi, Bergisch Gladbach, Germany) from spleen of C57BL/6 OTII mice expressing an OVA-specific transgenic

T cell receptor. 1×10^5 CD4⁺ T cells and 2×10^4 OVA/vehicle-pulsed BMDCs were co-cultured for 5 days in 200 μ l RPMI 1640 medium (Life Technologies, Darmstadt, Germany) containing 10% FCS, 5% penicillin/ streptomycin and 50 μ M β -mercaptoethanol in a U-bottom 96-well plate (Thermo Scientific) in a 5% CO₂ incubator. The amount of IL-4, IL-5, IL-13, and IFN- γ in supernatants was assayed by ELISA DuoSet (R&D Systems, Minneapolis, MN).

Adoptive transfer of CD4⁺ OTII T cells and BMDCs to mice

10×10^6 CD4⁺ OTII-cells were injected into the tail vein of naïve C57BL/6 mice 2 days before i.t. application of either OVA or vehicle-pulsed BMDCs. After 4 days MLN cells were isolated, re-stimulated with OVA (25 μ g/ml) and incubated at 37°C in a 5% CO₂ incubator for 5 days. IL-4, IL-5, IL-13, and IFN- γ content was assayed by ELISA (R&D Systems, Minneapolis, MN).

OVA/HDM-pulsed BMDCs-induced Th2-sensitization in AAI

1×10^6 vehicle/allergen-pulsed WT and miR-155^{-/-} DCs, were administered i.t. in C57BL/6 and miR-155^{-/-} mice on day 0. At day 10 mice were exposed to 1% OVA aerosol for 30min for three consecutive days. Asthma parameters were addressed 24 hours after the last aerosol application as described before.

Quantitative PCR

RNA was isolated with Qiazol (Qiagen, Hilden, Germany). miR-155 expression was validated by quantitative stem-loop PCR technology (TaqMan MicroRNA Assays, Life technologies, Darmstadt, Germany) as previously described (2). The use of target-specific reverse transcription primers and TaqMan hybridization probes allows the specific detection of mature microRNAs. MicroRNA expression was normalized to the small RNA rnu19. For mRNA quantification, cDNA synthesis was performed using

the First Strand cDNA synthesis kit (Fisher Scientific, Schwerte, Germany). QPCR was performed on a 480 Lightcycler (Roche, Mannheim, Germany), with Takyon Probe Mastermix (Eurogentec, Cologne, Germany). Primer/dual-labeled probe design and relative quantification were performed as previously described (3).

Preparation of Lung Single Cell Suspensions

Enzymatic digestion of lungs and isolation of single cells were performed as previously described (4). Briefly, isolated lungs were cleaned from thymic and cardiovascular residues; the main bronchi and trachea were removed. Lung lobes were minced with scissors and incubated for 30 min in digestion medium (RPMI 1640 with 5% FCS, 1mg/ml collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) and 0.02mg/ml DNase I (Sigma-Aldrich, Munich, Germany) in a humidified incubator at 37°C and 5% CO₂. Organ fragments were washed and resuspended in fresh digestion medium and incubated for another 15 min. Samples were centrifuged and resuspended in calcium and magnesium-free PBS containing 10mM EDTA for 5 min at room temperature on a shaker. Finally, the cells were subjected to red blood cell lysis, washed in FACS buffer (PBS without Ca²⁺ Mg²⁺, 0.1% azide, 5% EDTA-treated FCS, and 5mM EDTA), passed through a 50µm cell strainer, and kept on ice until antibody labeling. EDTA-treated FCS was prepared by passing FCS through a 0.2-µm filter and mixing 1ml of 0.1M disodium EDTA solution for every 10ml of FCS.

Enzyme-histochemistry

Adenosine triphosphatase (ATPase) activity was visualized using a Gomori lead nitrate (Pb(NO₃)₂) method (5) adapted from Vongtau *et al.* (6). Briefly, lung cryo-sections were incubated with Trizma-maleate buffer (TMB: Trizma-maleate 9,4g/l) pH

7,4 for 10min. Enzyme reaction was performed by incubating the sections in TMB containing sucrose (80g/l), 0,25mM CaCl_2 , 2mM lead nitrate and 0,3mM ATP for 1 hour at 37°C. Reaction product was visualized by incubating the sections in 0.5% Ammonium sulfide $((\text{NH}_4)_2\text{S})$ solution. Same procedure was done without ATP-substrate as control and no signal was obtained. Pictures were acquired on an Axio Lab.A1 microscope with an Axio Cam ICc 1 using Axio V4.8.2.0 (Zeiss, Jena, Germany). Signal quantification was performed using ImageJ.

ATP degradation assay

10^5 BMDCs were washed and placed into 96-well V-bottom plate in 100 μl serum free RPMI medium. 100 μl of ATP ($2 \times 10^{-5}\text{M}$) in PBS were added to the wells on different time points. Plate was centrifuged for 4 min with 300g and 100 μl supernatant was transferred into a white 96-well flat plate with an ATP standard (10^{-5} to $0,36 \times 10^{-9}\text{M}$) prepared. 50 μl ATPlite solution (PerkinElmer, Rodgau, Germany) was added to each well. Luminescence was measured for 5000ms using an Infinite 200 PRO (Tecan, Crailsheim, Germany).

Supplementary Literature

1. Muller T, Robaye B, Vieira RP, Ferrari D, Grimm M, Jakob T, et al. The purinergic receptor P2Y₂ receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation. *Allergy* 2010;**65**(12):1545-1553.
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Supplementary Figure E1:

Fold regulation in miR-155 expression in OVA- or HDM-stimulated miR-155^{+/+} BMDCs relative to naïve miR-155^{+/+} BMDCs.

Supplementary Figure E2:

DC maturation of OVA-pulsed BMDCs isolated from miR-155^{+/+} (shaded histogram) or miR-155^{-/-} (open histogram) mice were determined by surface expression of CD40, CD80, CD86 or MHCII of live cell gated and CD11c⁺ cells shown as histograms.

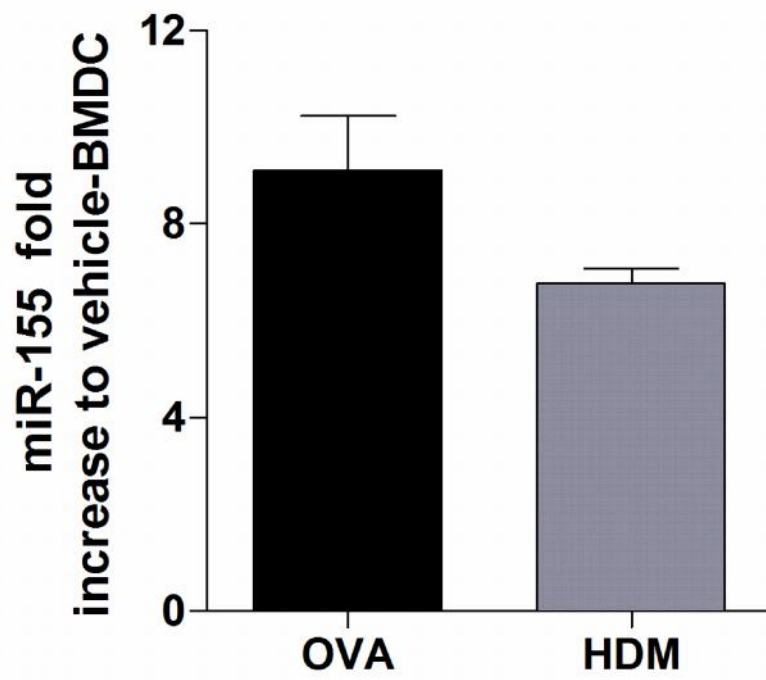
Supplementary Figure E3:

A and B, IL-10, IL-12p70 and TNF- α concentration in supernatants of OVA- or PBS- (Supp. Fig. 3A) and HDM- or PBS- (Supp. Fig. 3B) pulsed BMDCs isolated from miR-155^{+/+} or miR-155^{-/-} mice. Error bars represent SEM, n=3. n.d. = not detectable

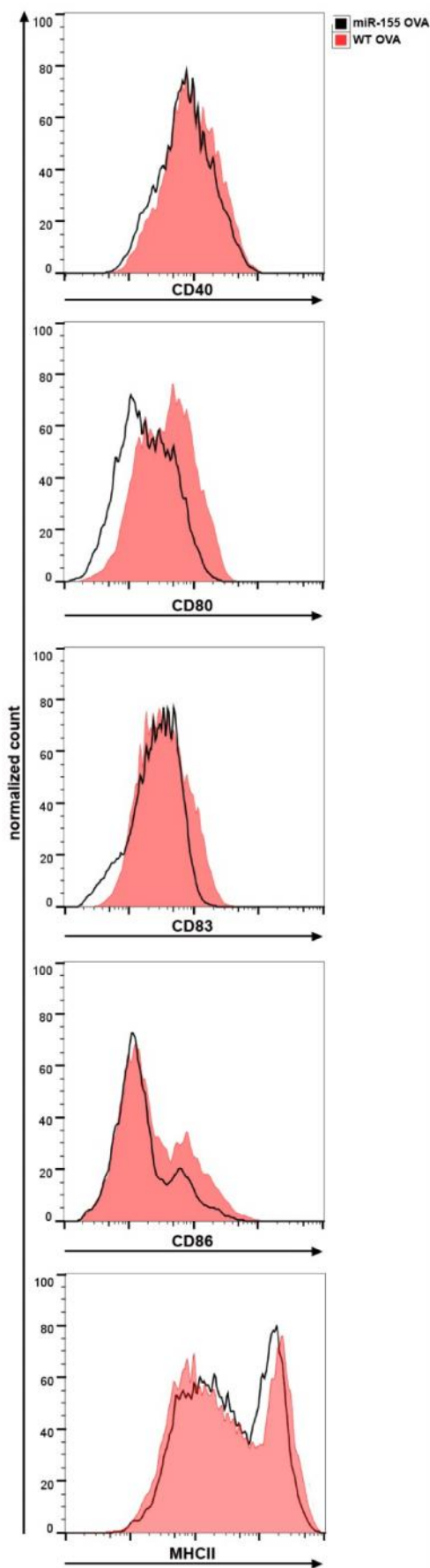
Supplementary Figure E4:

A and B, (Supp. Fig. 2A) Number of differential BAL cells and (Supp. Fig. 2B) cytokine concentration in supernatants of HDM re-stimulated MLN cells of miR-155^{+/+} mice adoptively transferred with vehicle/HDM-primed miR-155^{+/+} or miR-155^{-/-} BMDCs before allergen challenge. **C**, Number of differential BAL cells of either miR-155^{+/+} or miR-155^{-/-} mice adoptively transferred with HDM-primed miR-155^{+/+} or miR-155^{-/-} BMDCs before allergen challenge. Error bars represent SEM, n=4-5. * P<0.05, ** P<0.01 and *** P<0.001.

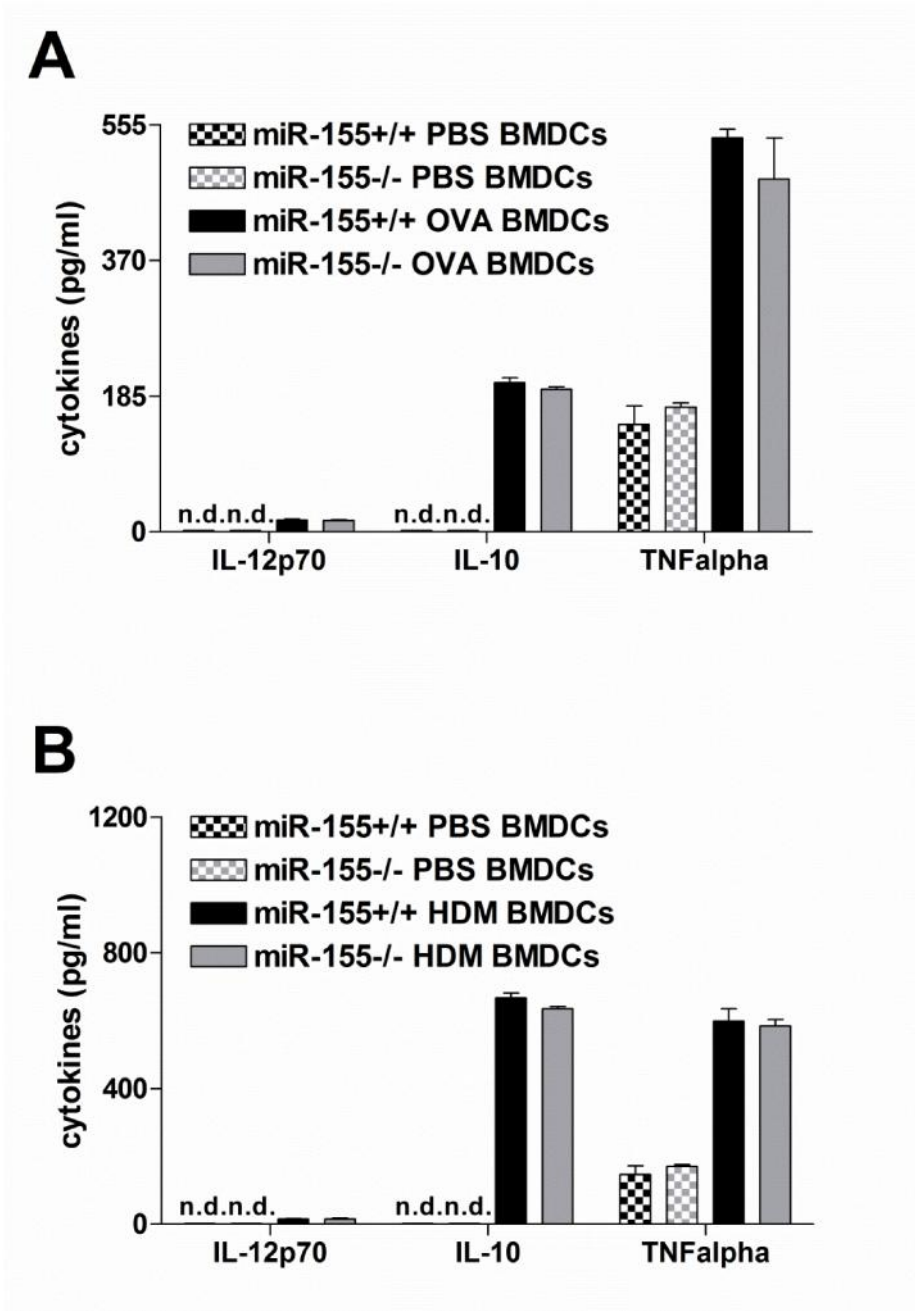
Supplementary Figure E1



Supplementary Figure E2



Supplementary Figure E3



Supplementary Figure E4

