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## Research paper

# Cylindromatosis (*Cyld*) gene mutation in T cells promotes the development of an IL-9-dependent allergic phenotype in experimental asthma

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## ABSTRACT

Cylindromatosis (CYLD) is a ubiquitously expressed deubiquitinating enzyme which removes activating ubiquitin residues from important signaling molecules of the NF- $\kappa$ B pathway. In CYLD<sup>ex7/8</sup> transgenic mice, a naturally occurring short isoform (sCYLD) is overexpressed in the absence of full length CYLD, leading to excessive NF- $\kappa$ B activity.

Herein, we investigated the impact of the CYLD<sup>ex7/8</sup> mutation selectively in T cells on the development of experimental allergic airway disease induced by sensitization and challenge with ovalbumin. Compared with their wildtype littermates, mice bearing the T cell-specific mutation (CD4<sup>+</sup>CYLD<sup>ex7/8</sup>) display stronger eosinophilia and mucus production in the lungs and higher IgE serum levels. The reason for these observations is excessive production of T cell-derived IL-9, a cytokine to whom allergy-promoting properties were ascribed. Consequently, blockade of IL-9 in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice alleviates the development of disease symptoms. Thus, by polarization of the T cell cytokine response, sCYLD can favor the development of allergic airway disease.

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## 1. Introduction

Allergic asthma is a chronic inflammatory disorder of the airways mainly characterized by airway hyperresponsiveness (AHR), overproduction of mucus and cellular infiltration of the lungs upon repeated exposure to the respective allergen. These symptoms are

based on a complex interplay of cells and cytokines that is still far from being comprehensively understood [1–3]. Besides environmental factors, genetic factors predispose to the development of the disease including loci on human chromosome 5q31–q33 that were shown to be associated with atopy and AHR [4]. Besides the genes encoding IL-4, IL-5 and IL-13, the IL-9 gene is also located in this region in humans and on the corresponding syntenic chromosome 13 in mice. A number of murine studies correlated the expression of IL-9 with the development of allergic airway disease [5–15]. However, the results of these publications are far from being consistent [16]. This is most likely due to the fact that the use of different mouse strains and of diverse protocols for airway disease makes comparison of data extremely difficult.

Initially, IL-9 was described as a Th2-type cytokine produced by murine CD4<sup>+</sup> T cells [17–22]. Since then, additional cell types have

**Abbreviations:** AHR, airway hyperreactivity; *Cyld*, Cylindromatosis (turban tumor syndrome) gene; fCYLD, full length CYLD; Mch, metacholine; sCYLD, short CYLD.

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been described as sources for IL-9, including mast cells and a subset of CD4<sup>+</sup> T cells termed Th9 cells according to their signature cytokine (see [23,24] for recent reviews). However, the cellular sources for IL-9 *in vivo* remained enigmatic. Th9 cells are distinct from Th2 cells and can be generated *in vitro* from TCR-activated naive CD4<sup>+</sup> T cells in the presence of IL-4 and TGF- $\beta$  [25–27]. Human Th9 cells were then found in the peripheral blood of allergic patients [28,29] and in mice with experimental allergic pulmonary disease [28,30]. In mice, adoptive transfer experiments revealed that transferred allergen-specific Th9 cells lead to allergic airway disease including AHR, mucus production and eosinophil recruitment upon provocation with the allergen [31]. Importantly, in the latter study it was also shown that treatment of mice which received Th9 cells with neutralizing anti-IL-9 blocks disease development.

Cylindromatosis (turban tumor syndrome) gene (*Cyld*) encodes a deubiquitinating enzyme removing activating lysine 63 (K63)-linked ubiquitin residues from important signaling molecules, including NEMO, TRAF6, TRAF-2 and Bcl-3, leading to downregulation of NF- $\kappa$ B activity [32–35]. Furthermore, experiments using CYLD knockout mice revealed important functions for CYLD in T cell development and tumorigenesis [36–38]. By deubiquitinating the Src kinase Lck, CYLD promotes the development of CD4 and CD8 positive T cells in thymus and periphery [36].

Previously, a CYLD<sup>ex7/8</sup> mouse strain was generated, lacking the full-length CYLD (FL-CYLD) but overexpressing the naturally occurring short CYLD (sCYLD) splice variant [39]. sCYLD is devoid of binding sites for TRAF2 and NEMO, yet it still retains the ability to bind to Bcl-3. The CYLD<sup>ex7/8</sup> mouse strain displays expanded mature B cell populations in secondary lymphoid organs based on enhanced NF- $\kappa$ B signaling and B cell survival [39]. Furthermore, hyperreactive bone marrow-derived dendritic cells from these mice produce increased levels of costimulatory molecules and pro-inflammatory cytokines due to elevated expression of NF- $\kappa$ B family members [40,41].

Regarding its impact on T cells, CYLD<sup>ex7/8</sup> mice show reduced numbers of mature medullary thymic epithelial cells (mTECs) caused by a developmental block in mTEC maturation, leading to an impaired negative selection of T cells in the thymus [42]. Moreover, T cells from CYLD<sup>ex7/8</sup> mice have a hyperreactive phenotype characterized by increased production of pro-inflammatory cytokines and constitutive activation of the NF- $\kappa$ B pathway accompanied by impaired suppressive capacity of regulatory T cells [43].

Herein, we investigate the impact of T cell-specific expression of CYLD<sup>ex7/8</sup> in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice on the development of allergic airway disease. Disease symptoms including eosinophilia and mucus production in the lungs as well as allergen-specific IgE levels are aggravated, a consequence of excessive IL-9 production in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice.

## 2. Materials and methods

### 2.1. Mice

Mice with T cell-specific deletion of the exons 7 and 8 of CYLD were generated by crossing CD4-Cre to CYLD<sup>fl<sup>ex7/8</sup></sup> mice. CD4-Cre negative mice were regarded as congenic wildtype littermates. All mice are on a genetic C57BL/6 background, were bred in our own animal facility and used at the age of 8–12 weeks. Animal procedures were conducted in accordance with current federal, state, and institutional guidelines.

### 2.2. T cell isolation, differentiation and *in vitro* stimulation

For T cell isolation, spleens were mechanically disrupted and filtered from cell debris through 70  $\mu$ m cell strainer. T cells were

cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma-Aldrich), supplemented with 5% FCS, 1% penicillin-streptomycin, 1% L-glutamine, sodium pyruvate and 50  $\mu$ M  $\beta$ -mercaptoethanol. For Th2 differentiation, cells were stimulated with plate-bound anti-CD3 mAb (145-2C11; 3  $\mu$ g/ml) and anti-CD28 mAb (37.51; 5  $\mu$ g/ml) in the presence of 1000 U/ml IL-4, 100 U/ml IL-6 and anti-IFN- $\gamma$  (XMG1.2; 10  $\mu$ g/ml). For Th9 differentiation, cells were stimulated with plate-bound anti-CD3 mAb and anti-CD28 mAb in the presence of 300 U/ml IL-4, 20 mg/ml IFN- $\gamma$  mAb, and 4 ng/ml porcine TGF- $\beta$ .

On day 5, Th2 and Th9 cells were harvested, counted by exclusion of dead cells with trypan blue and re-stimulated with anti-CD3 mAb (5  $\mu$ g/ml) for 24 h.

To measure T cell-derived cytokine production *ex vivo*, lungs were digested with collagenase type I (0.5 mg/ml PBS, Sigma-Aldrich) and suspension was filtered through a 70  $\mu$ m cell strainer (BD Biosciences). Cells were incubated with or without 250  $\mu$ g/ml OVA in IMDM for 72 h. Cytokines in the supernatants were detected by sandwich ELISA using the following combinations of mAbs: IL-4, BVD4-1D11 and BVD6-24G2 [44]. IL-9, D9302C12 and 229.8 [25]. ELISA detection of IL-5 and IL-13 was performed according to manufacturer (R&D Systems).

### 2.3. Experimental protocols and measurement of penH by body plethysmography

To induce allergic airway disease, animals were sensitized intraperitoneal (i.p.) to the model antigen ovalbumin (OVA) and subsequently challenged by inhalation of the nebulized antigen as described elsewhere [45]. Briefly, the sensitization phase included two intraperitoneal injections of 20  $\mu$ g OVA (Grade V; Sigma-Aldrich) dissolved in 100  $\mu$ l phosphate buffered saline (PBS) on day 0 and 14. Challenges were performed on day 42, 43 and 44; animals were nebulized daily for 20 min with an OVA solution (1% in PBS) using an ultrasonic nebulizer (NE-U17, Omron, Hoofddorp, the Netherlands). Control mice received OVA challenge without prior sensitization. Thirty min before nebulization, 150  $\mu$ g blocking anti-IL-9 (MM9C1 [46]); or isotype control were applied intranasally. Experimental readouts were performed 48 h following the last challenge.

To evaluate airway responsiveness, the nondimensional parameter enhanced pause (penH) was assessed by using a non-invasive whole body plethysmograph [model PLY 3211; BUXCO Electronics, Inc.] as described [47]. Briefly, OVA-sensitized or non-sensitized control mice (described above) were nebulized for 3 min with saline and subsequently with rising doses (12.5, 25 and 50 mg/ml) of methacholine (Mch, Sigma-Aldrich). Following each nebulization step penH was recorded every 10 s for 5 min. Increase of penH was calculated by relating maximal penH of each Mch dose to the maximal penH of the saline baseline measurement. The non-invasive measurements were performed 24 h following the second challenge by inhalation.

### 2.4. Broncho-alveolar lavage (BAL)

BAL was taken via the tracheal tube. 1 ml PBS was slowly filled into the lungs and recovered. Following centrifugation, supernatants were transferred in a new tube and stored at –20 °C. Cell pellets were dispensed in PBS and cell counts were determined by using trypan blue exclusion. Differential cell counts were made from cytocentrifuged preparations which were fixed and stained with the Microscopy Hemacolor®-Set (Merck, Darmstadt, Germany). At least 200 cells were counted and percentage and absolute numbers of each cell type were calculated.

## 2.5. Histology

Histology was performed as described [45]. In brief, lungs were fixed following inflation of 10% formalin (1 ml) via the tracheal tube and were immersed in a tube filled with 10% formalin for further fixation. Finally, lungs were embedded in paraffin and 4  $\mu$ m tissue sections of the central lung region were cut. Lung slides were stained with hematoxylin/eosin (HE) and periodic acid-Schiff (PAS), respectively. To analyze inflammation, slides were scored in a qualitative measurement as described [48]. Development of goblet cell metaplasia was assessed on PAS-stained slides by counting PAS positive cells per mm basement membrane using imaging software (Analysis, Soft Imaging Systems, Stuttgart, Germany).

## 2.6. Determination of antigen-specific immunoglobulines by ELISA

Serum was obtained 48 h following the last challenge. OVA-specific-IgG1 and -IgG2 titers were determined according to protocols provided by the manufacturer (BD Pharmingen, Heidelberg, Germany). To determine OVA-specific IgE, a protocol was used as described in [49]. Briefly, plates were coated with rat anti-mouse IgE (R35-72, BD-Pharmingen) over night. Following blocking with 3% BSA in PBS, serial dilutions of sera were added and incubated for 2 h. After washing, biotin-labeled OVA was added and incubated for 1 h. Absorption was read after addition of streptavidin-horseradish peroxidase and o-phenylenediamine. By adding sulfuric acid color reaction was stopped and optical density was measured at 450 nm.

The antibody titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis.

## 3. Results

### 3.1. Production of IL-9 is elevated in T cells derived from CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice

Previously, it has been shown that sCYLD acts as an additional coactivator of NF- $\kappa$ B signaling. One of the target genes of NF- $\kappa$ B is Interleukin-9 (IL-9), the signature cytokine of recently described Th9 cells, which has been linked to the development of allergic asthma.

In order to achieve T cell-specific depletion of exons 7 and 8 of the *Cyld* gene as described recently [43], mice expressing Cre

recombinase under the control of the CD4 promoter were crossed to animals carrying a conditional allele of *Cyld*. As congenic littermate controls, mice with the floxed *Cyld* gene but lacking Cre were used throughout the studies outlined below.

CD4<sup>+</sup> T cells were isolated from the spleen and differentiated *in vitro*. In order to achieve Th9 polarization, cells were cultured in the presence of TGF- $\beta$ , IL-4, IL-6 and anti-IFN- $\gamma$ .

To serve as internal reference, Th2 cells were generated by using a combination of IL-4, IL-6 and anti-IFN- $\gamma$ . For secondary stimulation, cells were harvested on day 5 and activated with anti-CD3. After 24 h, cytokine levels in the supernatants were measured by ELISA.

As depicted in Fig. 1A, Th9 cells derived from T cells expressing solely sCYLD produce excessive amounts of IL-9 upon polyclonal activation *in vitro*. Mutant Th9 cells exceed the IL-9 production of their wildtype littermates more than 20 fold. In contrast, the production of the signature cytokine IL-4 by Th2 cells is comparable between both genotypes (Fig. 1B).

### 3.2. Symptoms of allergic airway disease are aggravated in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice by overexpression of IL-9

IL-9 has been implicated in the development of allergic airway disease. In order to investigate whether our finding of increased IL-9 expression in CD4 T cells has also relevant effects *in vivo*, we employed a model for acute murine allergic airway disease, as outlined in Fig. 2A. Following sensitization to the model antigen OVA and challenge, single cell suspensions from the lungs were prepared and T cell-derived cytokines were measured by ELISA following restimulation with OVA *in vitro*.

As depicted in Fig. 2B, the production of IL-4, IL-5 and IL-13 following allergen stimulation is comparable between both strains. Yet, in accordance with our findings *in vitro*, the expression of IL-9 in lungs derived from mice expressing sCYLD solely and excessively in T cells far exceeds the respective control. These observations led us to the conclusion that our model is suitable to investigate the impact of excessive IL-9 production by T cells.

Typically, the sensitization and challenge procedure leads to recruitment and extravasation of eosinophils which can be partly recovered by performing bronchoalveolar lavage (Fig. 3A). On an average, eosinophil numbers recovered from the lungs of CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice were more than 25 fold increased compared to their littermates. To neutralize IL-9 activity *in vivo*, we applied a monoclonal antibody against IL-9 (MM9C1) or an isotype control intra-nasally thirty minutes before each challenge (Fig. 2A).

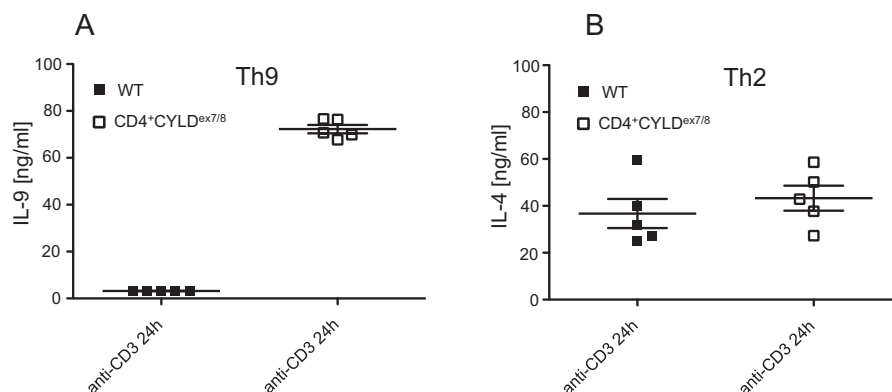


**Fig. 1.** IL-9 production of Th9 cells from CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice is strongly elevated. Naive CD4<sup>+</sup> T cells from either CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice or their wildtype littermates were stimulated and cultured under Th2- or Th9-promoting conditions. On day five, 1x10<sup>6</sup> cells of each cell type were restimulated with anti CD3 for 24 h. After stimulation, supernatants were collected and analyzed for the presence of IL-9 produced by Th9 cells (A) and IL-4 produced by Th2 cells (B). Shown are 5 biological replicates (mean  $\pm$  SEM).

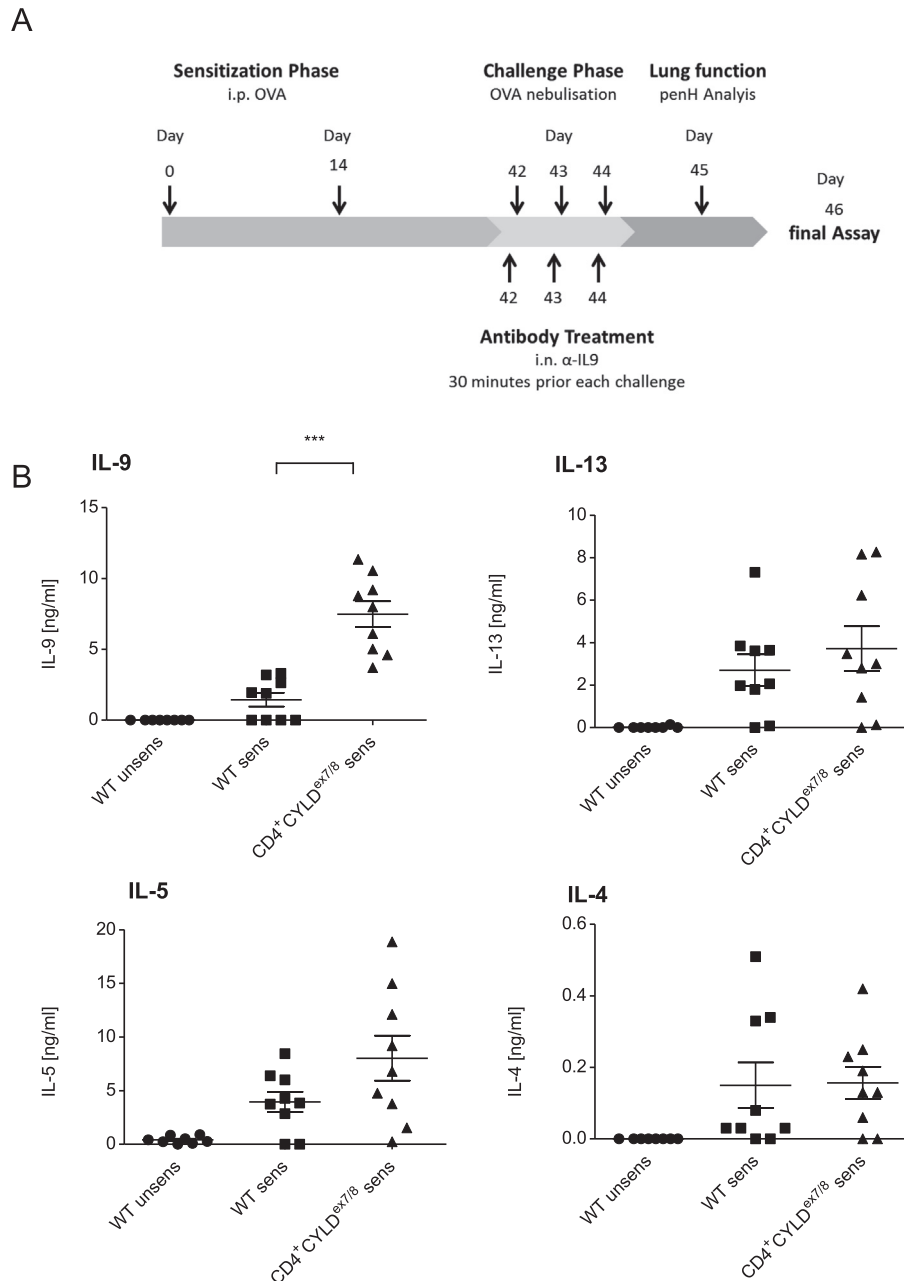


**Fig. 2.** Lung T cells from sensitized and challenged CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice express higher levels of IL-9 after restimulation with OVA. (A) CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice and their wildtype littermates were sensitized by two injections (i.p.) of OVA (20 µg) and challenged. Thirty minutes before nebulization anti-IL-9 or isotype control (150 µg) was applied intranasally. PenH was measured after 24 h and the assays were performed 48 h after the last challenge. (B) For restimulation of lung T cells, CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice or their wildtype littermates were sensitized and challenged as outlined in (A), yet without injection of anti-IL-9 or isotype control prior to nebulization. Lung cells were isolated from mice 48 h following the last challenge (day 46) and stimulated ( $5 \times 10^5/100 \mu\text{l}$ ) with 250 µg/ml OVA for 72 h. After stimulation, supernatants were collected and the production of the indicated cytokines was measured by ELISA (means  $\pm$  SEM).  $n = 8-9$  mice per group. \*\*\* $p \leq 0.001$ .

Strikingly, eosinophil numbers in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice were highly susceptible to anti-IL-9 treatment (Fig. 3A). In the presence of neutralizing antibody, eosinophil counts in the BAL of CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice were reduced to the level of their congenic littermates which themselves remained insensitive to anti-IL-9. This finding indicates that, beyond quantitative consequences of the CYLD mutation on the recruitment of eosinophils, also qualitative alterations occur because in the current disease setting an IL-9 independent parameter becomes IL-9 dependent.

This finding was corroborated by analyzing HE-stained sections of lung tissue for cellular infiltration 48 h after the last challenge. Peribronchial and perivascular inflammation was graded using a

qualitative measurement and Fig. 3B demonstrates excessive cellular infiltration in the lung tissue from CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice. Yet, in contrast to the littermates, tissue infiltration in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> lungs can be significantly reduced by neutralizing IL-9.

Besides cellular infiltration, enhanced mucus production due to goblet cell metaplasia is another pathogenic parameter contributing to the asthmatic phenotype. Consequently, we analyzed the numbers of goblet cells in PAS-stained lung sections (Fig. 3C). Sensitization and antigen challenge elicits metaplasia of PAS-positive goblet cells lining the airways which is even more pronounced in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice. Application of anti-IL-9 antibody had no effect on reduction of goblet cell numbers in sensitized and challenged

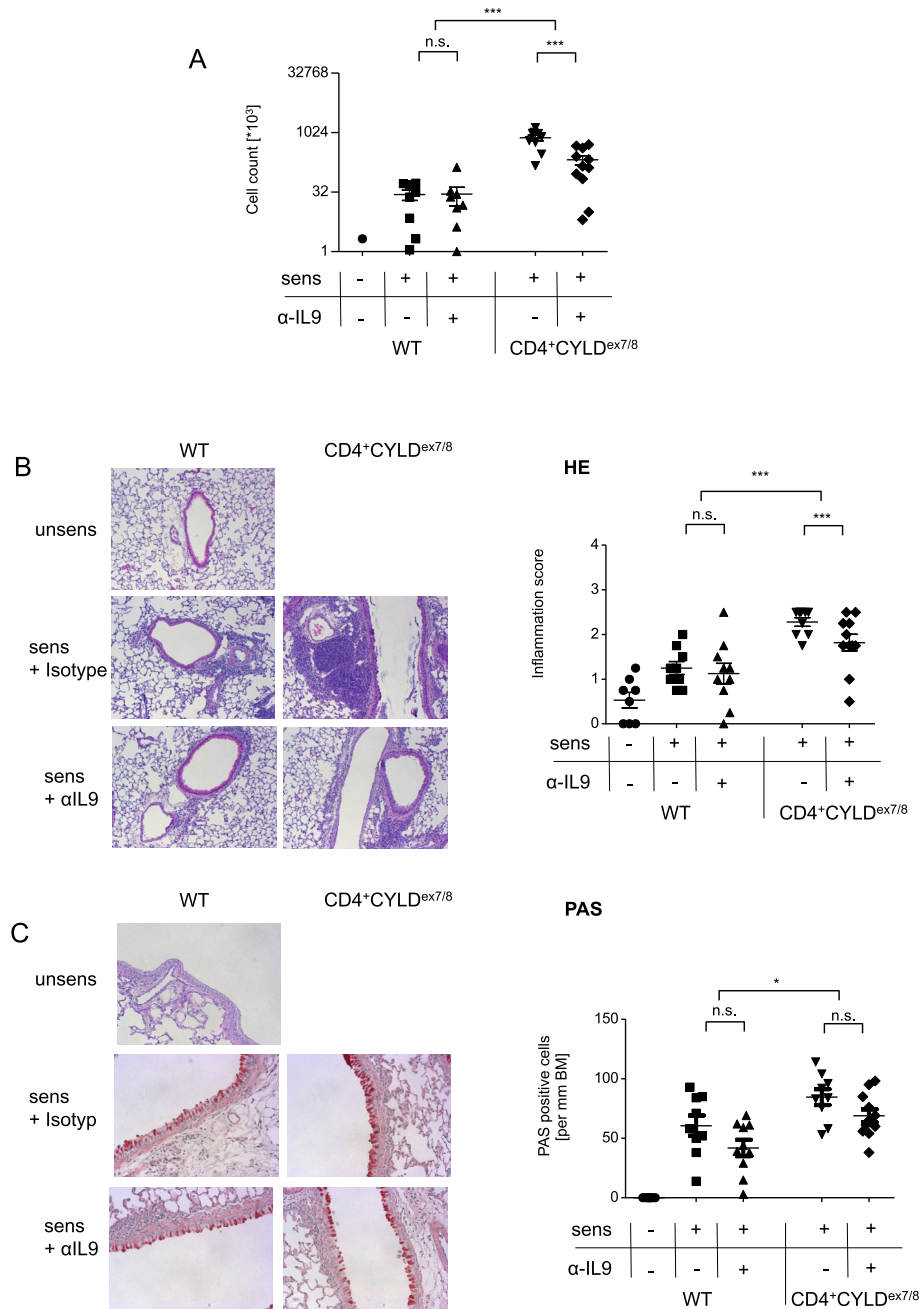


**Fig. 3.** OVA-induced allergic airway disease leads to enhanced eosinophilia, inflammatory infiltrations and increased mucin levels in goblet cells in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice. (A) The indicated mouse strains were sensitized by two injections (i.p.) of OVA (20  $\mu$ g) or received PBS as negative controls and were then challenged three times with nebulized OVA. Thirty minutes before each challenge mice were treated intranasally with 150  $\mu$ g anti-IL-9 or isotype control respectively. Forty-eight hours after the last challenge eosinophils from wild-type mice and CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice were counted in the BAL fluid (means  $\pm$  SEM).  $n = 8$ –11 mice per group. \*\*\* $p \leq 0.001$ . (B) After the indicated treatment, one lung lobe from each mouse was isolated, fixed and embedded in paraffin. Tissue sections were stained with H&E and qualitatively scored for inflammatory infiltrations. Bar graphs demonstrate perivascular and peribronchial score by mean  $\pm$  SEM. (C) The numbers of mucus-producing goblet cells were determined by PAS-staining of lung tissue sections and indicated as PAS<sup>+</sup> cells per mm basement membrane (mean  $\pm$  SEM).  $n = 7$ –13 mice per group. Original magnification  $\times 100$  (HE) and  $\times 200$  (PAS), respectively.

WT animals. In the sensitized and challenged CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice a numerical reduction in goblet cell count was detectable, which however failed to reach statistical significance ( $p = 0.089$ ).

Allergic airway disease is furthermore characterized by airway hyperreactivity to inhaled bronchoconstrictors such as methacholine. However, in pilot experiments, invasive measurements of airway hyperresponsiveness using an established system of invasive airway function assessment (flexiVent, Scireq, Canada) showed highly increased baseline resistance in sensitized and challenged CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice and only little increase in airway

resistance following exposure to methacholine (data not shown). This is most likely explained by the excessive inflammatory response in these mice. We therefore measured the nondimensional parameter enhanced pause (penH) using a non-invasive whole body plethysmograph 24 h after the last antigenic challenge. Response curves to increasing doses of methacholine are depicted in Fig. 4. Indeed, sensitized and challenged mice showed increased response in penH following methacholine exposure. A similar response was detectable in sensitized and challenged CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice. Following application of anti-IL-9 a decrease was

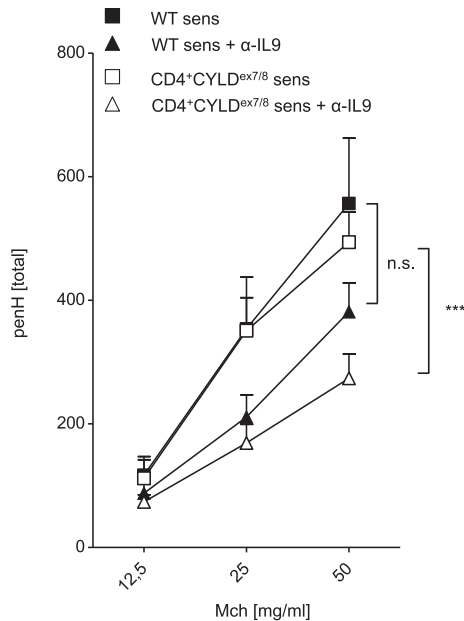


**Fig. 4.** In sensitized CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice, AHR is alleviated by intranasal treatment with anti-IL-9. To induce experimental airway hyperresponsiveness mice were treated as outlined in Fig. 2A. Twenty-four hours after the last challenge airway resistance was measured by using non-invasive whole body plethysmography and fold increase of PenH was calculated to increasing doses of metacholine. Bars indicate mean ± SEM. n = 8–11 mice per group.

detectable in the sensitized and challenged animals. However, in wild-type mice, this decrease was small and not statistically significant. In contrast, sensitized and challenged CD4<sup>+</sup>CYLD<sup>ex7/8</sup> animals

showed a stronger and also statistical significant decrease in response to methacholine following anti-IL-9 treatment.

Typically, the development of allergic airway disease is accompanied by the production of IgE and IgG1 as dominant antibody isotypes. Previously it was reported that IL-9 potentiates the IL-4 induced release of IgE and IgG1 from B cells [50,51]. In CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice, an exaggerated humoral response regarding the production of antigen-specific IgE and IgG1 can be measured (Fig. 5). In contrast, levels of IgG2b do not differ significantly between both strains. Regarding the effects of anti-IL-9 treatment, only IgE production in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice is significantly reduced.

Taken together, overexpression of sCYLD in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice leads to the development of Th9-polarized immunity. Thus in our model, disease parameters which appear to be independent of IL-9 in wild-type C57BL/6 mice including eosinophilia, mucus production, AHR and production of IgE become largely IL-9-dependent in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice.

#### 4. Discussion

NF-κB is involved in differentiation of Th9 cells and in the acute activation of the IL-9 promoter (see [24,52] for recent reviews). Thus, it was tempting to speculate that enhanced activation of the NF-κB pathway, which was repeatedly and consistently observed in cells carrying the CYLD<sup>ex7/8</sup> mutation [39–41,43], has an impact on the development of Th9 cells. Indeed, *in vitro* polarization of naive CD4<sup>+</sup>CYLD<sup>ex7/8</sup> T cells leads to massive overproduction of IL-9, whereas cytokine production of corresponding Th2 cells is unaffected.

Enhanced production of IL-9 was also seen following antigen-specific restimulation of lung T cells *ex vivo*, indicating a bias towards Th9 development in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice.

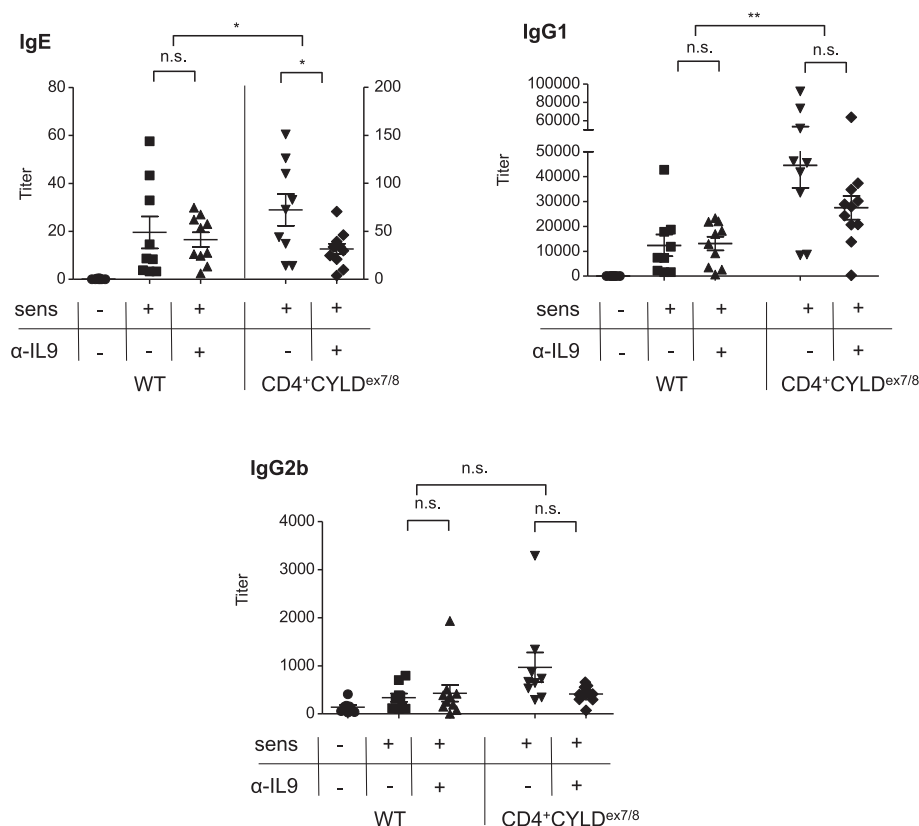


**Fig. 5.** CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice develop higher levels of Ag-specific IgE and IgG1. Forty-eight hours after the last OVA-challenge of the mice described in Fig. 3, blood was collected and titers of Ag-specific IgE, IgG1 and IgG2b were assessed in the sera via ELISA. Bars indicate mean ± SEM. n = 8–11 mice per group. n.s., not significant.

Regarding the contribution of IL-9 to the development of experimental allergic airway disease, a consistent picture does not yet exist. Intratracheal application of IL-9 [7,6] as well as lung-specific overexpression of IL-9 [8,12] promote eosinophilia, mucus production, and AHR. Production of mucus appears to depend mainly on IL-9-induced IL-13 [12]. However, in IL-9 knockout mice, the development of pulmonary inflammation and AHR is unaffected, implying compensatory roles of additional cytokines [16]. Comparability of these studies is hampered by several factors, notably the genetic background of mice [53–55] and the experimental asthma protocol used [56].

In our adjuvant-free sensitization model, blockade of IL-9 during the challenge phase using a neutralizing antibody has virtually no effect on lung eosinophilia, mucus production, AHR and production of IgE/IgG1 in C57BL/6 wildtype littermates. However, in CD4<sup>+</sup>CYLD<sup>ex7/8</sup> mice, eosinophilia, mucus production as well as IgE and IgG1 levels are all increased. In these mice, eosinophilia, AHR and IgE/IgG1 levels are significantly sensitive to anti-IL-9 treatment. Thus, overexpression of sCYLD changes the asthmatic phenotype, generating a model that allows to investigate the influence of IL-9 on different disease parameters.

In humans, therapeutic targeting of single cytokines in asthmatic patients in general reveals responders and nonresponders, leading to low overall clinical efficacies of such treatments. These findings reflect the complexity of asthma pathophysiology and emphasize the need of disease substratification in order to identify patients who will benefit from the neutralization of cytokines including IL-13, IL-5, IL-9 and TNF [1]. Without the possibility to preselect patients according to distinct asthma phenotypes [57], negative outcomes of such trials have to be expected.

Concerning IL-9, only descriptive human data are available, yet an IL-9-dependent asthma phenotype has not been described so far. However, a humanized monoclonal antibody against IL-9 has been used in two phase II studies with evidence of clinical activity in asthmatics [58]. In this context, it is feasible that IL-9 is expressed at an early stage in the pathogenesis of the disease, before its chronic manifestation. This assumption is supported by the recent observation that IL-9 plays an important role in the early type 2 response against *Nippostrongylus brasiliensis*. In this model, the early and transient expression of IL-9 from CD4<sup>+</sup> T cells preceded the production of IL-4, IL-5 and IL-13 and was critical for their expression [59]. This finding was further corroborated by using IL-9 fate reporter mice, which display early and transient reporter expression in papain-induced lung inflammation [30]. In the latter study, innate lymphoid cells were found as an innate source for IL-9. Thus, it should be taken into account that early IL-9 production sets the stage for the development of both detrimental and beneficial immune responses.

However, our data presented herein allow us to conclude that T cells are the most important source for IL-9 in our model.

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