



Research Article

IL-17 regulates DC migration to the peribronchial LNs and allergen presentation in experimental allergic asthma

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IL-17 is associated with different phenotypes of asthma, however, it is not fully elucidated how it influences induction and maintenance of asthma and allergy. In order to determine the role of IL-17 in development of allergic asthma, we used IL-17A/F double KO (IL-17A/F KO) and WT mice with or without neutralization of IL-17 in an experimental allergic asthma model and analyzed airway hyperresponsiveness, lung inflammation, T helper cell polarization, and DCs influx and activation. We report that the absence of IL-17 reduced influx of DCs into lungs and lung draining LNs. Compared to WT mice, IL-17A/F KO mice or WT mice after neutralization of IL-17A showed reduced airway hyperresponsiveness, eosinophilia, mucus hypersecretion, and IgE levels. DCs from draining LNs of allergen-challenged IL-17A/F KO mice showed a reduction in expression of migratory and costimulatory molecules CCR7, CCR2, MHC-II, and CD40 compared to WT DCs. Moreover, in vivo stimulation of adoptively transferred antigen-specific cells was attenuated in lung-draining LNs in the absence of IL-17. Thus, we report that IL-17 enhances airway DC activation, migration, and function. Consequently, lack of IL-17 leads to reduced antigen-specific T cell priming and impaired development of experimental allergic asthma.

Keywords: airway hyperresponsiveness · bronchial LNs · DCs · IL-17A · experimental asthma · IL-17F · immunoglobulins · Th2 cells



Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

According to the statistics of both the World Health Organization and Global Asthma Reports, asthma affects more than 300 million people worldwide with an incidence of one in eight children and one in 12 adults [1, 2]. These statistics make it one of the most common chronic diseases [1, 2]. Asthma is clinically characterized by shortness of breath, wheeze, and cough due to bronchial obstruction caused by chronic inflammation of the airways with bronchial edema increased mucus secretion, and constriction of bronchial smooth muscle tissue. Airway inflammation can broadly be differentiated in type-2 high and type-2 low inflammation [3]. Both in humans and in experimental murine models, type-2 high asthma has been well defined and characterized based on presence of airway and blood eosinophilia, allergen-specific IgE, and other biomarkers that are driven by IL-13, IL-5, and IL-4, which are the signature cytokines associated with type 2 responses [3].

Type-2 low inflammation, on the other hand, is characterized by increased neutrophilic airway inflammation, sometimes in addition to eosinophilic inflammation. Th17 cells have been shown to play a determining role in this phenotype [3, 4]. Increased levels of IL-17 have been reported in lung specimens, BALF, sputum as well as in peripheral blood of patients with asthma [5–7]. High levels of IL-17 in severe asthma regulates the expression of inflammatory cytokines, chemokines, and cell adhesion molecules [8–11]. Through its influence on chemokines and growth factors such as G-CSF, GM-CSF, and TNF- α , IL-17 further promotes eosinophilia in airways in addition to causing asthma exacerbation and airway remodeling by triggering release of matrix metalloproteases (MMPs) or other inflammatory molecules such as IL-6, IL-8, IL-11, GM-CSF, and VEGF within the lung tissue [12, 13]. In genome-wide association studies, asthma development was linked to polymorphisms in IL-17 determining genes, and IL-17 secretion in cord blood correlated with the risk for later asthma development [14, 15].

Professional antigen-presenting cells and specifically DCs are required for the induction of asthma-associated features in murine models in sensitization and challenge phases [16, 17]. In humans, adaptive immunity and especially antigen-specific immune response against allergens is a hallmark of allergic asthma [3]. Furthermore, genetic polymorphisms at the 6p21, 32 locus encoding HLA-DRB1 and HLA-DQA1 antigen-presenting MHCII molecules are linked with asthma in humans [18]. DCs are central for priming and inducing efficient T helper cell responses in asthma and allergy [19–21]. Optimal activation of naive T cells in the draining LNs requires the presentation of processed antigen taken up in the peripheral tissues by migratory DCs [22, 23]. Thus, DCs ability to migrate is a critical pre-requisite for the priming of an effective immune response in the LN [16]. On its part, IL-17 affects DCs by enhancing functional differentiation of DC progenitors and, in a murine model for allograft rejection, it seems to modulate DC function and their Th1 priming capacity [24, 25]. In the skin, IL-17 induces Langerhans cell migration [26].

In this study, we investigate IL-17A/F KO mice and monoclonal antibodies to block IL-17A in an experimental allergic asthma

model. We show that in the absence of IL-17A and IL-17F, the allergic asthma phenotype is reduced and this attenuation correlates with reduced frequencies of infiltrating DCs, both in the lungs and draining LNs of allergic mice. Through in-depth proteome and transcriptome analyses, we furthermore demonstrate that the migration, antigen presentation, and activation of airway DCs are affected by IL-17. To our knowledge, this is the first study that links missing or low IL-17 in experimental asthma to reduced migration of DCs with a possible effect in attenuation of the severity of inflammation and AHR.

In summary, we propose that the presence of IL-17 in allergic asthma leads to enhanced activation, migration, and antigen presentation by DCs, which contributes to the development and course of the disease. Thus, a targeting approach aimed at attenuating DC migration and activation would be plausible in IL-17-dependent asthma/allergic conditions that are notoriously difficult to control.

Results

IL-17 expression enhances inflammation and airway hyperresponsiveness in experimental asthma

To explore how IL-17 influences the establishment and development of murine asthma, we subjected WT and IL-17A/F KO mice to an experimental allergic asthma model based on OVA immunization (Fig. 1A). Upon provocation with methacholine, allergic IL-17A/F KO mice showed reduced AHR as reflected by decreased resistance and increased compliance compared to WT mice (Fig. 1B). The inflammatory response in the airways was diminished in IL-17A/F KO compared to WT mice. Total cell numbers and numbers of eosinophils in the BALF of allergic WT mice were enhanced upon local administration of OVA. In contrast, in IL-17A/F KO mice, inflammation was lower as compared to allergic WT mice (Fig. 1C). In addition, H&E as well as Periodic-Acid Schiff (PAS) stained lung sections revealed reduced eosinophilic inflammation and mucus hypersecretion in OVA treated IL-17A/F KO compared to WT mice (Fig. 1D), which we further confirmed through analysis of pixel densities (Fig. 1E). In our setup, we did not observe neutrophilic influx in the allergic asthma model both in WT as well as in IL-17A/F KO mice. In OVA-re-stimulated cell culture supernatants of BLN cells, the levels of Th2 cytokines IL-5, IL-10, and IL-13 (Fig. 1F) were also lower in allergic IL-17A/F KO mice compared to their WT counterparts. Also, serum antigen-specific IgE was significantly lower in IL-17A/F KO than in WT mice (Fig. 1G). In summary, these data demonstrate that KO of IL-17A/F significantly impacts development of experimental allergic asthma in our model.

Neutralization of IL-17A impairs development of allergic asthma and ameliorates AHR

To assess the role of IL-17 in vivo and thereby determine its impact in our model, we neutralized IL-17A before we subjected mice to

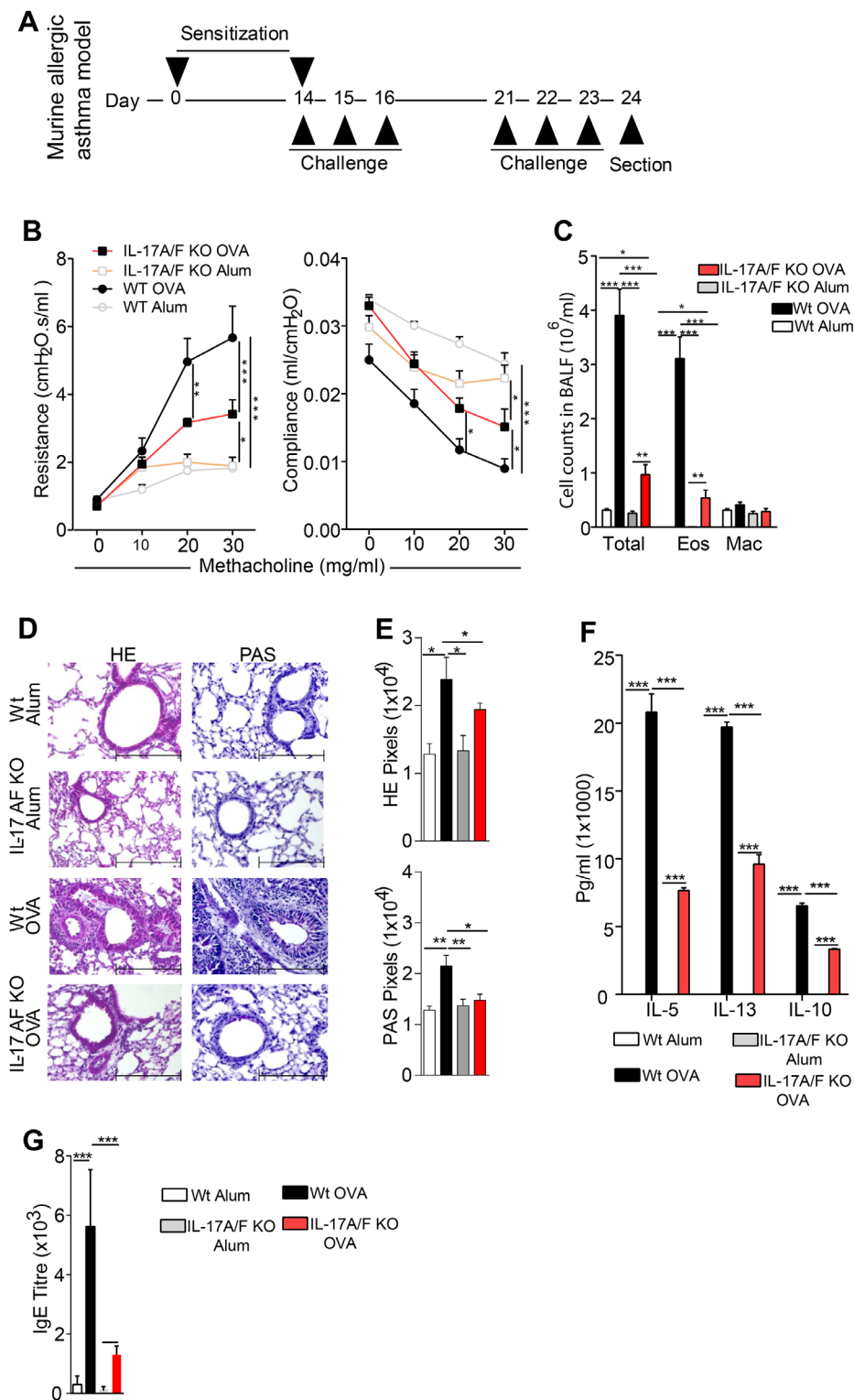


Figure 1. Attenuated airway hyper-responsiveness and eosinophilia in absence of IL-17A/F: Female WT C57BL/6J and IL-17A/F KO mice were subjected to the model of experimental allergic asthma (A). IL-17A/F KO mice show reduced airway hyperresponsiveness on challenge with increasing doses of methacholine, total cell counts in BALF determined using automated counter, eosinophilia (Eos), and macrophages (Mac) in cytopins as compared to WT animals (B and C). Lung inflammation and mucus production (H&E/ PAS) staining of paraffin-embedded lung-slices at magnification = $\times 200$, scale bar = 100 μ m and an objective quantification based on pixel densities (D and E). Th2 cytokines concentration in re-stimulated bronchial LN cells (F) and serum titres of allergen-specific Ig (G) were measured by ELISA. The error bars represent mean $\pm$ SEM and * <0.05 , ** <0.01 , and *** <0.001 and one-way ANOVA (C, E, F, and G) or two-way ANOVA (B) was used to compare differences in a representative experiment from two to three independent experiments with $n = 4$ –5 animals/group in each experiment.

the asthma protocol (Fig. 2A). Neutralization of IL-17A led to reduced symptoms in the murine asthma model compared to the control group. Lung function parameters were improved (Fig. 2B) and BALF total cell counts as well as airway eosinophilia were lower after neutralization of IL-17A (Fig. 2C). Furthermore, less lung inflammation was observed (Fig. 2D–E) and Th2 associated

cytokines after re-stimulation of LN cells were reduced while IFN- γ was elevated (Fig. 2F). We furthered this observation through establishment of a model we called “therapeutic” (Fig. 3A) in which we assessed whether neutralization of IL-17 in already sensitized mice would ameliorate experimental asthma through blocking IL-17A before mice were re-exposed to the sensitizing

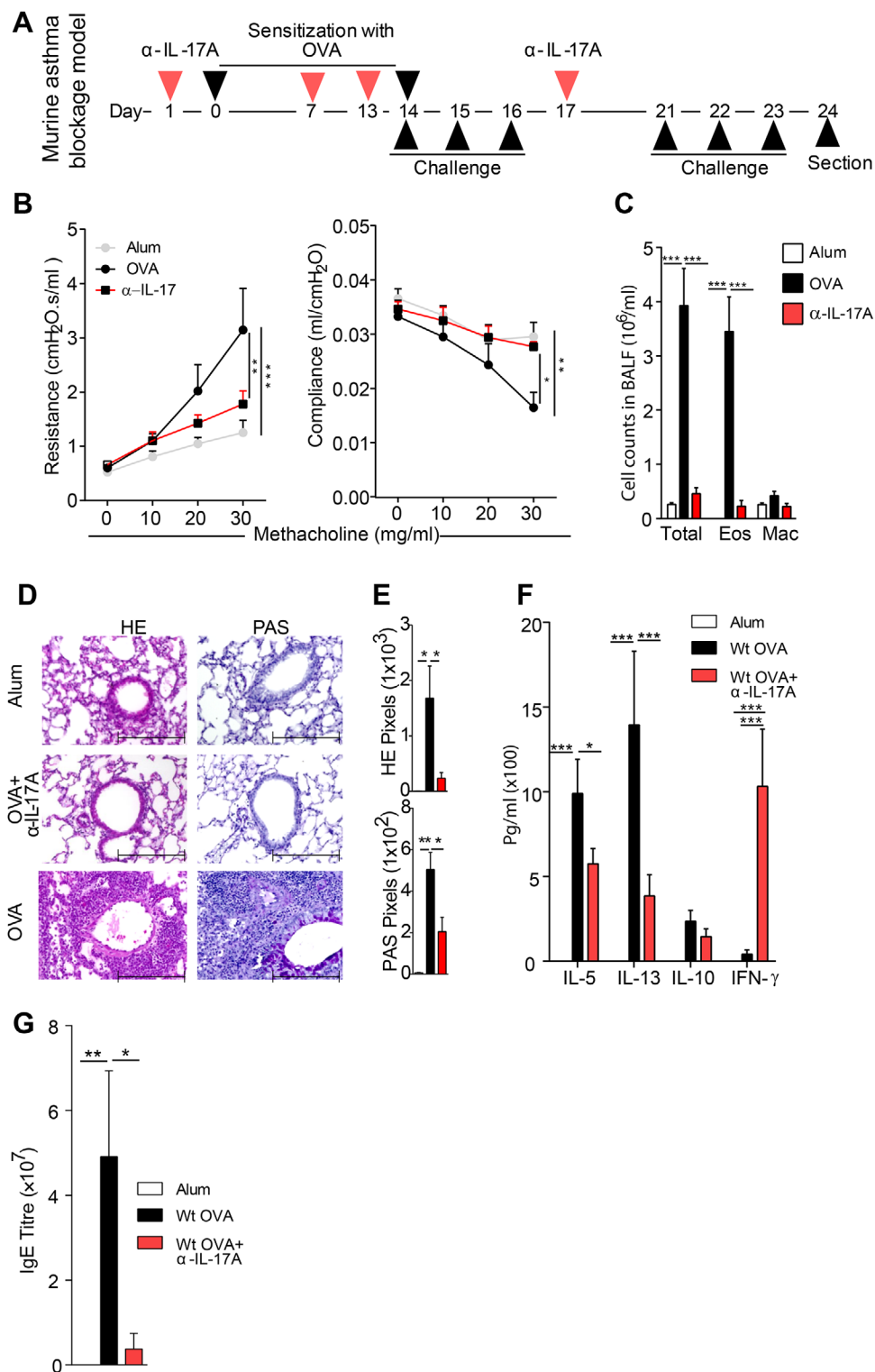


Figure 2. Blockage of IL-17A impairs development of experimental allergic asthma: IL-17A was blocked in WT mice before starting and throughout asthma protocol (A). Airway hyper responsiveness after methacholine challenge (B), BALF total cell count determined using automated counter, eosinophils (Eos), and macrophages (Mac) in cytopins (C). Lung inflammation and mucus production (H&E/PAS) staining of paraffin-embedded lung-slices at magnification = $\times 200$, scale bar = 100 μ m, and pixel quantifications (D and E). Th2 and Th1 cytokine concentrations in re-stimulated bronchial LN cells using ELISAs (F) and serum titres of allergen-specific immunoglobulin measured using ELISA (G) comparing influence of blocking IL-17A on experimental asthma. The error bars represent mean $\pm$ SEM and * <0.05 , ** <0.01 , and *** <0.001 . One-way ANOVA (C, E, F, and G) or two-way ANOVA (B) was used to compare differences in a representative experiment from two to three independent experiments with $n = 4$ –5 animals/group in each experiment. The red filled arrow heads show the days on which α -IL-17A was administered.

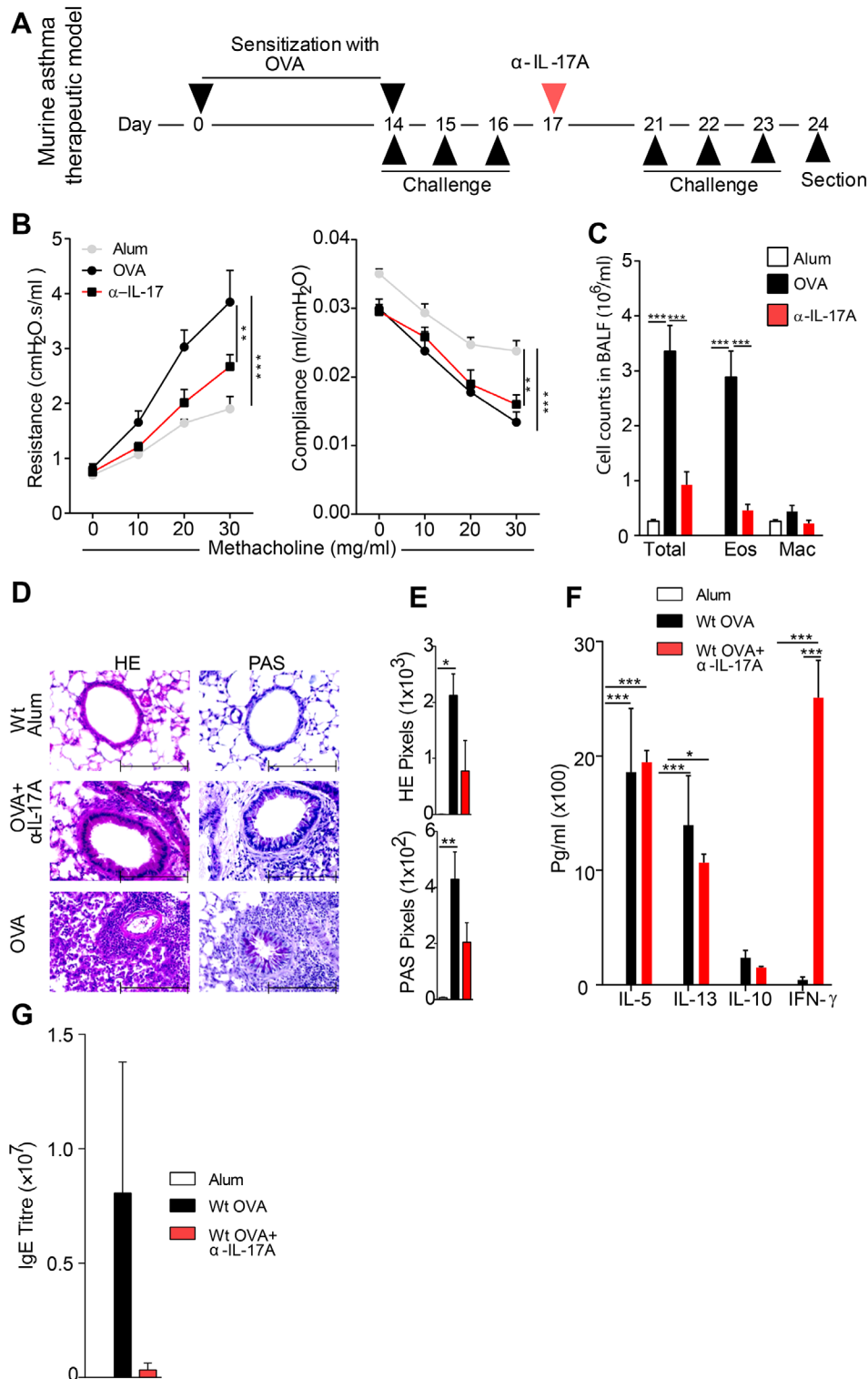


Figure 3. Therapeutic application of anti-IL-17A ameliorates symptoms of allergic asthma: IL-17A was blocked before re-exposure of WT mice to the allergen through challenge (A). Airway hyperresponsiveness after methacholine challenge (B), BALF total cell count determined using automated counter, eosinophils (Eos), and macrophages (Mac) in cytopins (C). Lung inflammation and mucus production (H&E/PAS) staining of paraffin-embedded lung-slices at magnification = $\times 200$, scale bar = 100 μm , and quantification of pixel densities (D and E). ELISA measurements of Th2 and Th1 cytokine concentrations in re-stimulated bronchial LN cells (F) and serum titres of allergen-specific Ig (G) comparing influence of IL-17A neutralization before challenge on experimental asthma. The error bars represent mean $\pm$ SEM and * < 0.05 , ** < 0.01 , and *** < 0.001 and one-way ANOVA (C, E, F, and G) or two-way ANOVA (B) was used to compare differences in a representative experiment from two to three independent experiments with $n = 4$ –5 animals/group in each experiment. The red filled arrow head shows the day on which α -IL-17A was administered

antigen. Blocking IL-17A before challenge led to reduced AHR, eosinophilic inflammation, and mucus hypersecretion in allergic mice (Fig. 3B–E). However, this later neutralization of IL-17A did not lead to attenuation of Th2 helper cell-associated cytokines or reduction in IgE but correlated with elevated levels of IFN- γ (Fig. 3F). Interestingly, we observed a significant and very strong

increase of IFN- γ in supernatants of OVA-restimulated LN cells of OVA-immunized WT mice that were treated with a monoclonal anti-IL17 antibody. This strong increase of IFN- γ production might be due to a compensatory upregulation of IFN- γ producing Th1 cells after inhibition of IL-17 via a monoclonal antibody. It has been observed previously that IL-17 and IFN- γ have compensatory

effects on each other. For instance, in tuberculosis infection model increase of IL-17 diminished IFN- γ production [27]. In contrast to our observation in OVA-immunized WT mice that were treated with anti-IL-17A antibody, we did not detect IFN- γ in IL-17A/F KO mice after OVA immunization (see Fig. 1F). This observation is in line with previous reports in which constitutive deficiency of IL-17 in mice is associated with an altered microbiota as well as an impaired Th1 response due to reduced IFN- γ positive CD4⁺ tissue-resident memory T cells [28]. Taken together, neutralization of IL-17 before sensitization or re-exposure during challenge attenuates allergic airway inflammation and Th2 polarization.

Absence of IL-17 attenuates DC frequencies and antigen responses in draining LNs

Antigen uptake and processing by professional antigen-presenting cells such as DCs are required for an effective priming of naïve T cells. To elucidate the role of IL-17 in airway inflammation, we analyzed immune responses in lung draining LNs. DCs sample antigen in the periphery and migrate to local LNs where they present to T cells and prime immune response [16, 29, 30]. We hypothesized that reduced airway inflammation in IL-17A/F KO mice was due to attenuated DC-T cell interaction and Th2 priming in LNs. To address this hypothesis, we adoptively transferred OT-II cells intravenously via the tail vein followed by intranasal application of OVA (Fig. 4A). In the absence of IL-17, we observed a reduction in vivo proliferation of OT-II cells in lung draining LNs (Fig. 4B and C and Supporting information Fig. 1). In addition, we also observed significantly fewer cells in lung draining LNs of IL-17A/F KO mice as compared to their WT counterpart after treatment (Fig. 4D). More so, in the lungs and draining LNs of IL-17A/F KO mice, frequencies of DCs after intranasal challenge were much lower than in WT animals (Fig. 4E and F and Supporting Information S1). However, frequencies of myeloid progenitor cells in NM were not affected in IL-17A/F KO mice (Fig. 4G-H).

Impaired DC activation and migration in IL-17A/F KO mice

Our finding that T cell responses are attenuated in mediastinal LNs of IL-17A/F KO mice could partly be explained by reduced frequencies of DCs in the LNs as compared to WT mice. Furthermore, IL-17 could also affect DC functions such as their ability to take up antigen. To address this, we analyzed in vivo antigen uptake by pulmonary DCs through intranasal administration of APC-conjugated OVA to WT and IL-17A/F KO mice (Fig. 5A and Supporting Information Fig. S2). Subsequent ex vivo analysis revealed a significant reduction in frequencies of antigen-positive DCs in IL-17A/F KO mice as compared to their WT counterparts (Fig. 5B and C). In order to further understand impact of IL-17 on activation and migration potential of DCs, we applied chip cytometry for identification of pulmonary DCs (Fig. 5D), a novel technique that is suitable for in-depth single-cell characterization [31].

We have shown in our previous analysis that chip cytometry measurements correlate very well with flow cytometry observations [32, 33]. In addition, chip cytometry allows comprehensive phenotyping of single cells and small cell populations thereby increasing the analytical power of samples with few cells such as DCs in LNs. Based on CD11c and MHC-II expression, we thus analyzed phenotypic characteristics of DCs in lung draining LNs from both WT and IL-17A/F KO mice after asthma protocol (Fig. 1A) using chip cytometry. As shown in Fig. 5E, unbiased hierarchical clustering of single cells based on surface expression of CCR7, MHC-II, CD40, CCR2, and CD62L on DCs derived from lung draining LNs shows that whereas WT cells have an enhanced expression of these molecules and can be segregated based on whether they are from antigen exposed animals or not, this unbiased separation does not occur in cells derived from IL-17A/F KO mice (Fig. 5E). We confirmed this lack of hierarchical clustering of DCs isolated from IL-17A/F KO mice through further unbiased analytical approaches involving linear discriminant analysis (LDA), which revealed that no significant changes occur on DCs from IL-17A/F KO mice after OVA challenge (Supporting Information Fig. S3). Further quantitative analysis revealed substantial reduction in expression of MHC-II, CD40, CCR7, and CCR2 in DCs isolated from IL-17A/F KO mice when compared to the DCs from WT mice (Fig. 5F). To test differences in activation or functional capacities of DCs derived from IL-17AF KO compared to WT mice, we co-cultured splenic and lung derived DCs with T cells and compared IL-2 levels in the supernatants 2 days after co-culture with similar results (Supporting Information Fig. E2). This finding supports a comparable stimulatory capacity of WT and IL-17AF KO DCs in vitro. However, when we performed a migration assay toward Ccl19, the number of DCs migrating toward Ccl19 in absence of IL-17 was significantly reduced (see Supporting Information Fig. E2). Our data further support our finding that IL-17 attenuates the migratory potential of DCs. Thus, deficiency of IL-17A/F in our model correlated with lower expression levels of activation and migration associated molecules on DCs derived from lung draining LNs.

Transcriptional signature of lung draining LN DCs in the absence of IL-17A/F

The above observations led us to investigate if the differences between WT and IL-17A/F KO mice exist already at transcriptional level. Thus, we carried out transcriptomic analysis of DCs sorted from lung-draining LNs of either naïve or mice subjected to intranasal allergen challenge (Fig. 6A). Using a well-established protocol [34,35] for preparation of single cells from LNs without having to digest using collagenase and DNase, we isolated lung draining LNs from both WT and IL-17A/F KO mice after exposure to OVA via intranasal challenge (Fig. 5A). We then analyzed transcriptomic patterns of a set of genes involved in migration and function of non-lymphoid-tissue DCs into the draining LNs in response to tissue injury or immunization that were previously described [36]. Analysis of the 321 genes that were described as determinants of inflammatory DCs (Supporting Information

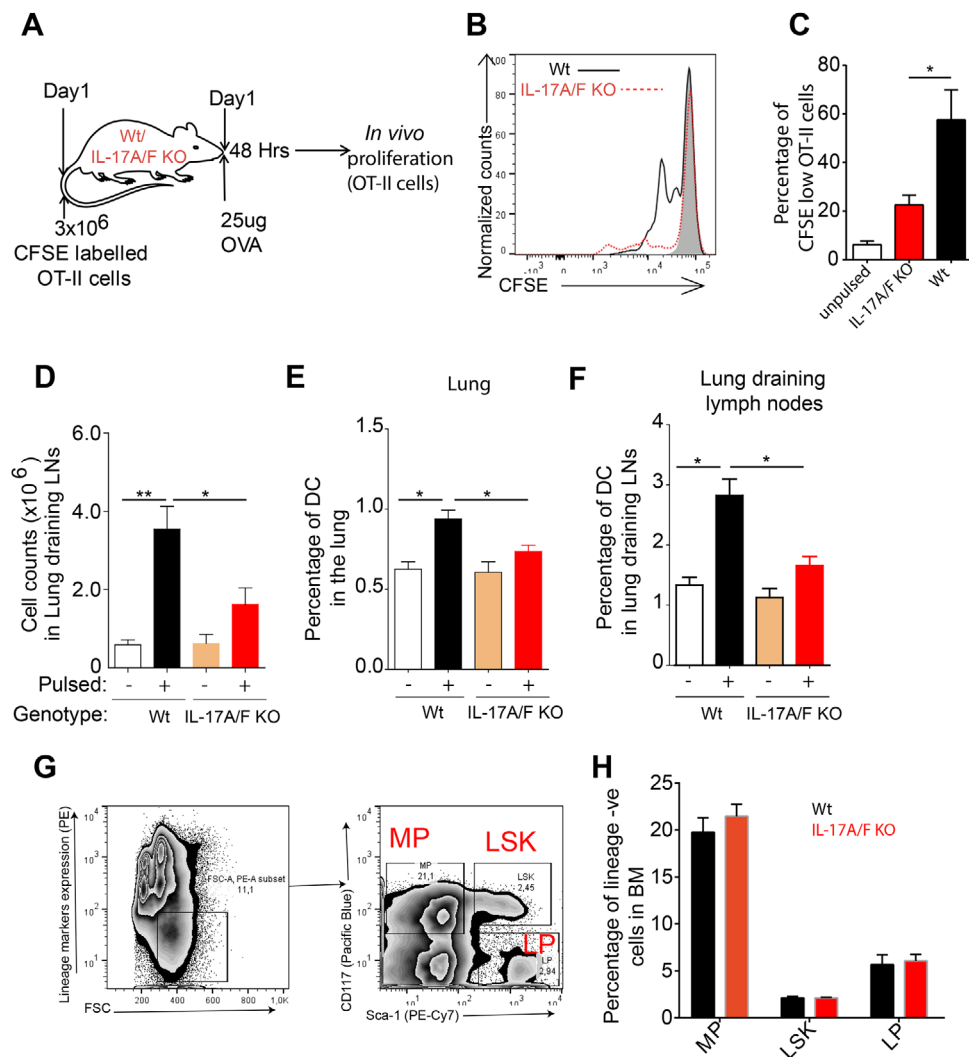


Figure 4. Impaired *in vivo* proliferation of antigen-specific T cells in lung draining LNs and reduced influx of DCs in absence of IL-17: CFSE-labeled OT-II cells were adoptively transferred into either WT or IL-17A/F KO mice followed by challenge with OVA and subsequent analysis of *in vivo* proliferation (A). Identification of transferred cells and comparative analysis of proliferation and total cell counts in mediastinal lung draining LNs was determined by flow cytometry (B–D). Influx of DC into the lungs and lung draining LNs was analyzed after intranasal application of OVA (E and F). Quantification of various progenitor cell populations in BM of either WT or IL-17A/F KO mice (G and H). All bars display mean $\pm$ SEM with * <0.05 , and ** <0.01 . One-way ANOVA (C–F) and unpaired Student's *t*-test (H) was used to compare differences in data pulled from —two to three independent experiments. MP (myeloid Progenitors), LP (Lymphoid Progenitors), LSK (Lineage[−], Sca-1⁺, cKit⁺).

Table 2) as reported by Miller and colleagues [36] revealed that they are significantly upregulated in a challenged WT animals compared to their unchallenged WT counterparts, which is not seen in IL-17A/F KO mice after challenge (Fig. 6B and C). A closer look into the gene sets revealed that these changes occurred in genes that are involved in antigen presentation, co-stimulation, and cytokine production by DCs (Fig. 6D). These patterns were confirmed when looking at individual genes involved in the processes of antigen presentation, co-stimulation, and cytokine production (Fig. 7A and B). Importantly, these transcriptomic changes were not significant between the WT and IL-17A/F KO mice in those gene sets that are reported to be associated with the expression of chemokine receptors or involved in DC lineage commitment [36] (Fig. 7C and D). Due to a reduction in several genes

that have been shown to be associated with inflammatory DCs ability to present antigen, provide co-stimulation and also migration to peripheral LNs in IL-17A/F KO mice, we think IL-17A/F is necessary for an efficient DC stimulation in an inflammatory environment.

Discussion

In our study, we aimed to characterize the role of IL-17 in initiation and establishment of airway inflammation and AHR using a well-established experimental mouse model of allergic asthma. We found that absence of IL-17A/F or neutralization of IL-17A alone led to attenuation of severity of experimental allergic asthma, either before sensitization or challenge. These findings are in

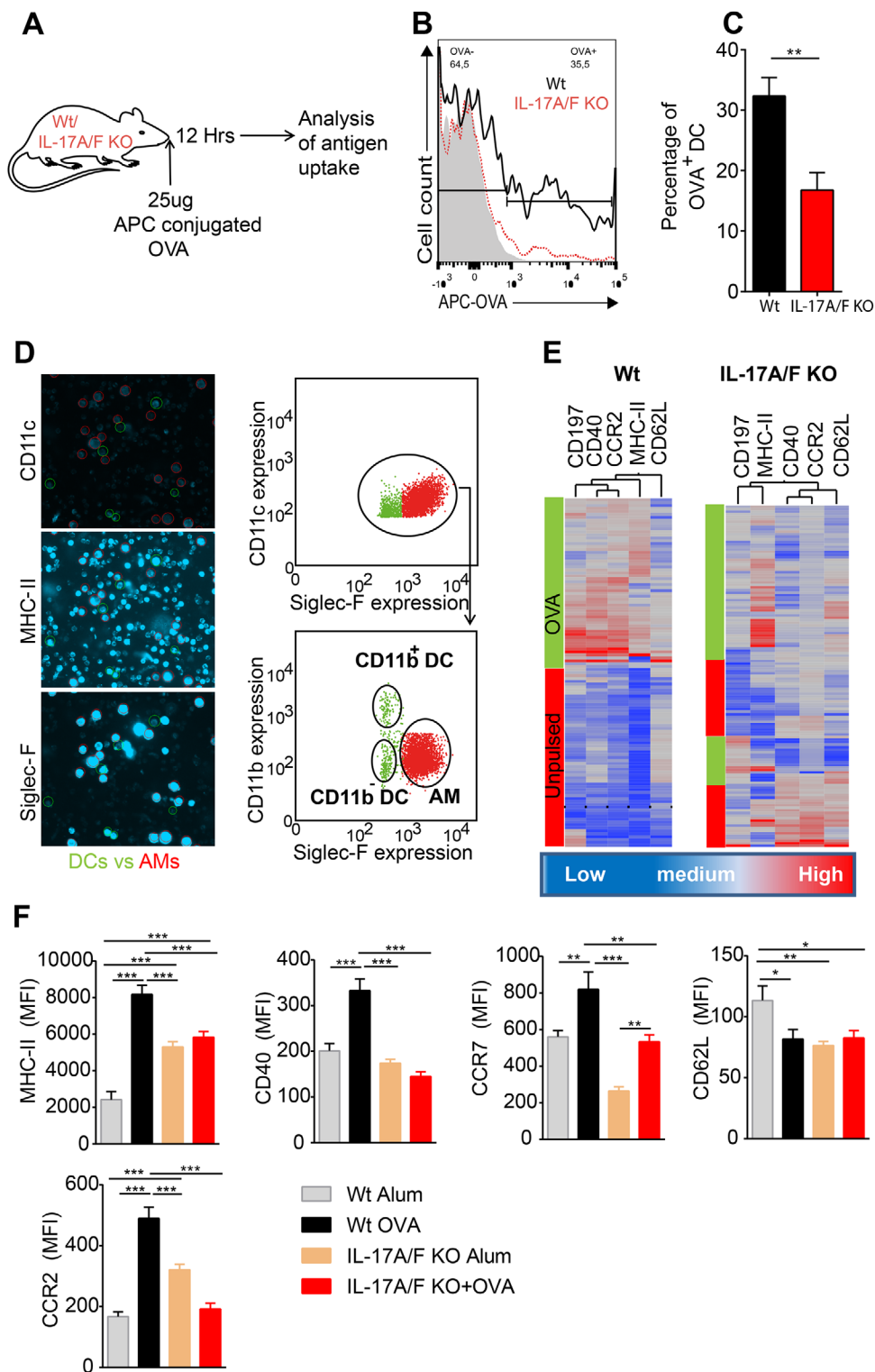
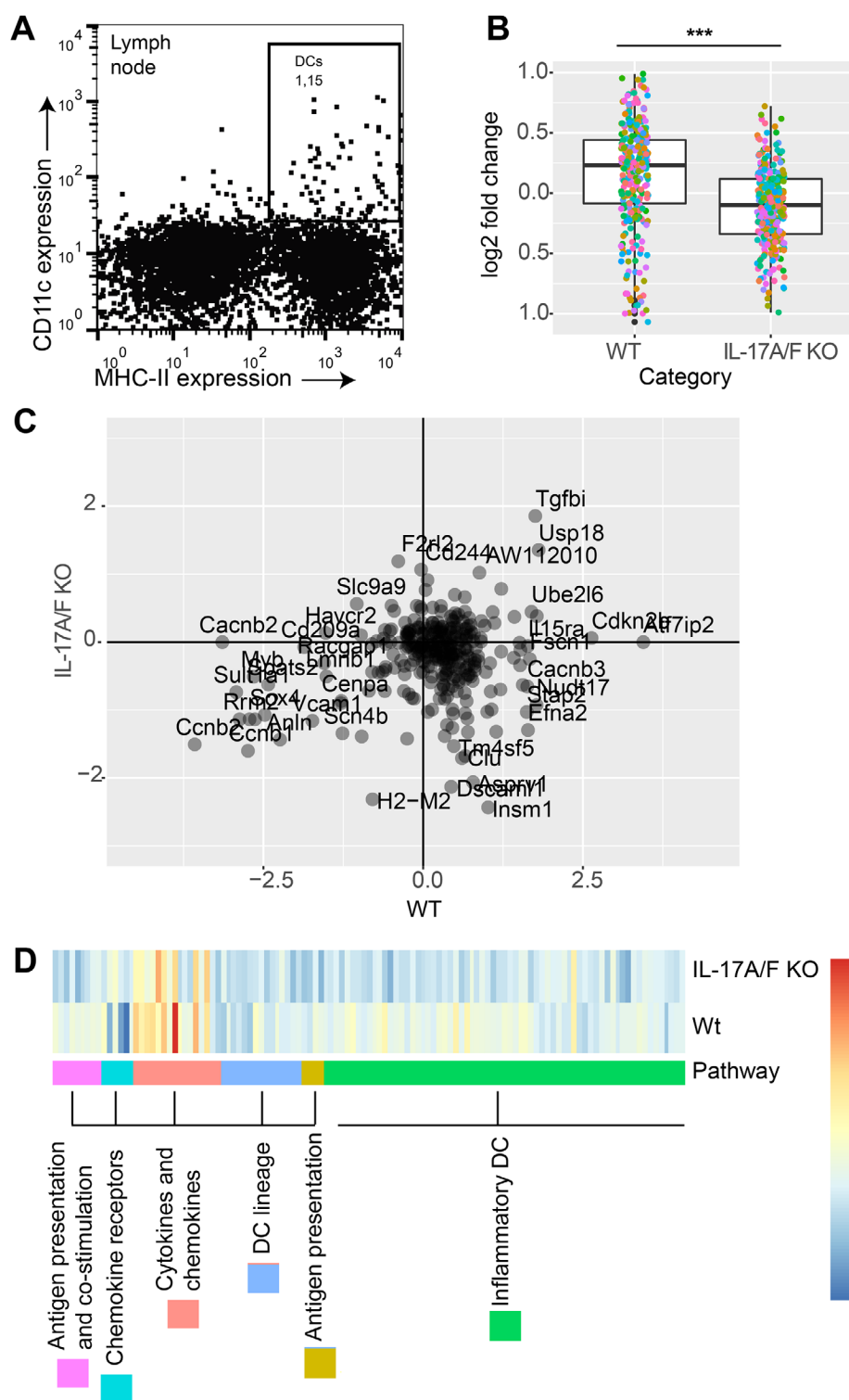


Figure 5. Lack of IL-17 leads to reduced frequencies and activation of conventional DCs: WT or IL-17A/F KO mice were intranasally inoculated with fluorescence conjugated OVA (A). Flow cytometry analysis of antigen positive DCs (see supplementary information regarding gating strategy for lung DC) in the lung after 12 h (B and C). Chip cytometry based single cell identification of DC and alveolar macrophages (AM) in the lung (D) followed by single cell analysis of various co-stimulatory and migratory molecules on the surface of lung draining LN DCs using chip cytometry in WT and IL-17A/F KO mice (E and F). The error bars represent mean $\pm$ SEM and * <0.05 , ** <0.01 , and *** <0.001 and unpaired two-tailed Student's t-test (C) or one-way ANOVA (F) was used to compare differences in data pulled from—two to three independent experiments.



line with the results of other studies using related mouse models [37, 38]. Moreover, we report that the observed reduction in pulmonary eosinophilic inflammation, AHR and goblet cell hyperplasia correlated with reduced conventional DC frequencies both in the lung and lung draining mediastinal LNs of IL-17A/F KO allergic animals. In concomitance with reduced DC frequencies, we observed diminished T cell responses to cognate antigen in

lung draining LNs in absence of IL-17; probably an effect that resulted from the dearth of DCs and thereby reduced antigen presentation in LNs [21, 39, 40]. To our knowledge, this is the first evidence that links availability of IL-17 in an experimental asthma model to reduced migration of DCs with a possible effect in attenuation of severity of inflammation and AHR. Given that IL-17 and Th17 cells are involved in asthma development and shown to be

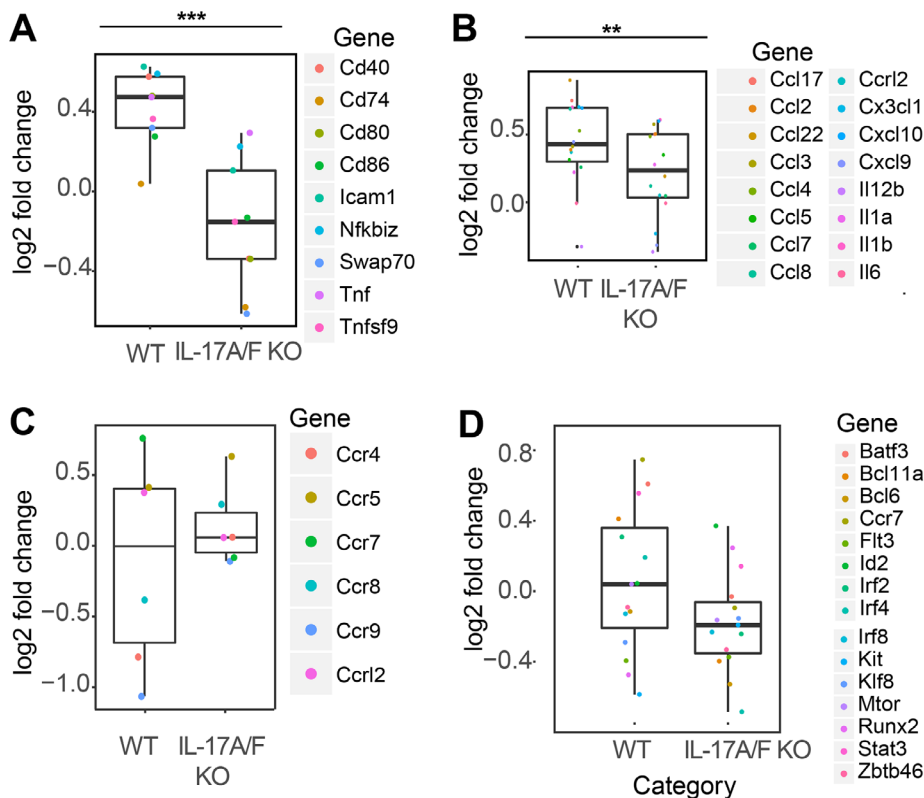


Figure 7. Expression of genes associated with antigen presentation and co-stimulation in conventional DCs is reduced in absence of IL-17. Representation of selected transcripts associated with antigen presentation and co-stimulation of DCs (A), cytokines and chemokines (B), chemokine receptors (C), and DC lineage (D) as either box plots or in form of scatter graphs (Supporting Information Fig. S3) showing log₂ fold changes in expression of genes after exposure to antigen in both WT and IL-17A/F KO mice. RNA used in this analysis was extracted from sorted DCs from two different intranasal challenge experiments with *n* = 10 animals/group in total. Wilcoxon Ranked test was used to calculate significance between fold change in WT and IL-17A/F KO mice.

associated with severity and course of disease [7–9, 37, 38], we believe this is an important mechanism by which this cytokine enhances inflammation and associated pathologies such as those in asthma and allergy.

Induction of immunologically protective as well as pathogenic responses are a product of a cascade of events involving antigen sampling by professional antigen presenting cells, their activation, migration and eventually presentation to naïve or memory effector cells such as T cells. Being the most important APC when it comes to activating naïve T cells, DCs are indispensable during induction of immune responses to foreign antigens or in maintaining tolerance to self [41]. For immature DCs to effectively traffic from the peripheral to the LNs, where antigen presentation and priming takes place, specific adhesion molecules and maturation-dependent chemo-attractant cues such as CCR2–CCL2, CCR5–CCL20, or CCR5–CCL5 chemokine receptor–ligand pathways have been conclusively shown to be necessary [42,43]. In addition, to migrate to draining LNs, mature DCs upregulate CCR7 [44–46]. Thus, paucity of antigen-specific OT-II cells in lung draining LNs in our study is likely a result of impaired functional and migratory potential of DCs. Indeed, our single-cell analysis of surface molecules expressed on DCs showed that, levels of MHC-II, CD40, CCR7, and CCR2 were reduced in IL-17A/F KO mice as compared to their WT counterparts after exposure to antigen. However, to track the fate of DCs, we used a cell transfer model (see Figs. 4 and 5) while we used a sensitization model with systemic antigen application to test the effect of IL-17 deficiency on the asthma phenotype (see Figs. 1–3). This is a limitation of our study since we cannot directly translate the information on DCs

gained in the cell transfer experiments to the immunization model. Nevertheless, the aim of our cell transfer experiments was to compare the impact of IL-17 on the antigen-presenting potency of DCs. By applying the same number of antigen-specific OT-II cells in vivo, we standardized the effector cell population to selectively focus on differences in DC function between WT and IL-17 KO mice in response to OVA stimulation. By this means, we avoided different T cell frequencies and differences in T cell activation as possible confounders during the sensitization phase, which depends on the frequency and functional potential of antigen-presenting DCs. In addition, the DCs used in our analysis were not isolated from animals that were sensitized via peritoneal cavity but were from intranasally challenged animals using a second model, which we believe is more suited to clarify the impact of IL-17 on DCs.

Indeed, the involvement of IL-17 in the migration of various immune cells has been shown in several other studies *albeit* using different models. Notably, IL-17 directly enhances B cell migration both in viral infection and asthmatic conditions through its action on structural lung cells that led to the production of migration influencing chemokines such as Cxcl12 and Cxcl13 [8, 47]. Here, we provide evidence that IL-17 impacts on the functional and migratory potential of DCs and thereby directly influences severity of experimental allergic asthma. A direct link of how DCs affect Th17 cells and thereby maintain inflammation in the lung was recently shown by Shalaby and colleagues using the house dust extract augmented OVA model [48]. According to Shalaby and colleagues, activation of DCs via TLR4 after exposure to common house dust extracts in a murine model of allergic asthma applying OVA/HDE led to revival and maintenance of effector/memory

Th17 cells in the lung. This observation augments previous work by Lajoie and colleagues showing that presence of IL-17 leads to the production of IL-23 by DCs and thereby to the sustenance of Th17 cells in a model of severe asthma [49].

An important observation in our current study is the attenuation of DC frequencies both in the lungs and lung draining LNs in the absence of IL-17. Although we show that CCR7 expression at transcript and protein level is reduced in absence of IL-17, nevertheless, we are still searching for more molecular partners that are involved in this impaired influx into the lung and lung draining LNs. Our current tenet is that reduced DC frequencies coupled with impaired antigen presentation potential and co-stimulation in absence of IL-17 explain observed ameliorative effects in our asthma model. Since it is well established that DCs are the main leukocyte sub-population that primes Th cell subset differentiation [50], our study adds to understanding possible cellular aspects of IL-17 driven asthma and allergic diseases. Although we do not have a mechanistic explanation of molecular pathways, IL-17A has been shown to enhance motility and invasion of cells through increasing expression of PI3K, Akt, and MMP-2/9 in glioblastomas and human cervical and prostate cancer cells in recent reports [51,52]. In our model, apart from the reduction in frequencies of DCs in lung draining LNs, we also observed attenuated expression of transcripts associated with cytokines and chemokines involved in migration such as Ccl17 and Ccl22, although their common ligand Ccr4 was not affected in IL-17A/F deficient animals. Both Ccl17 and Ccl22 have been shown to be involved in allergy and inflammatory diseases such as atopic dermatitis and directly impact the migratory potential of DCs [53–56].

In conclusion, we propose that the presence of IL-17 in asthma and allergy leads to enhanced activation, migration, and antigen presentation by DCs, which contributes to the development and course of the disease.

Materials and methods

Animals

The Committee on Animal Welfare in the State of Lower Saxony (LAVES) approved all animal protocols that were used in this study. All mice used in this study had C57BL/6 background. IL-17A and IL-17F deficient mice *B6.IL17a^{flm1Prz}/J* (IL-17A/F KO) mice were published before [57]. Female age-matched C57BL/6J (WT) mice as controls were obtained from Charles River Laboratories (Sulzfeld, Germany) and OT-II [58] transgenic mice with B6xB6.C-H-2^{bm1} genetic background were provided by Georg Behrens, Hannover Medical School. Mice were maintained in the animal facility at Hannover Medical School, Hannover, Germany.

Induction of allergic asthma

We have established an experimental asthma protocol in which mice are sensitized intraperitoneally (i.p.) with Polymyxin-treated

Grade V OVA (20 µg) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Alum; Fischer Scientific International) followed by repeated intranasal (i.n.) challenges (20 µg OVA in 40 µL normal saline). Control mice received Alum without OVA i.p. and 0.9 % NaCl i.n. instead of OVA [35, 59, 60].

In vivo antibody treatments

C57BL/6J WT mice were injected i.p. with 100 µg anti-IL-17A mAb (clone eBioMM17F3, eBioscience Inc., San Diego, USA) or with isotype control mouse IgG1 κ mAb (eBioscience, Inc., San Diego, USA) as outlined in Figs. 2A and 3A.

Measurement of airway responsiveness

Invasive lung function was assessed as described previously [35, 60, 61]. Briefly, 24 h following the last allergen challenge anesthetized tracheotomized mice were connected to the flex-iVent system (SCIREQ, Montreal, Canada) and exposed to different concentrations of aerosolized methacholine (0, 10, 20, and 30 mg/mL) in 0.9% NaCl.

Analysis of BALF

BALF was performed by flushing the right lung with PBS/2 mM EDTA. Total number of BALF cells was measured by automated cell counting (CedexHiRes, Innovatis, Bielefeld, Germany). BALF differential cell counts were performed by staining cytospin preparations with Diff-Quick (Medion Diagnostics, Duedingen, Switzerland).

Lung histology

The left lungs were inflated with PBS and fixed in 4% formalin before embedding in paraffin as previously described [35]. 4 µm sections were stained with H&E (Merck, Germany) or with periodic acid–Schiff reagent (PAS, Sigma, Taufkirchen, Germany) and scanned using Keyence BZ-9000 microscope (Keyence, Osaka, Japan) at 200× magnification. Inflammation and mucus production was further analyzed objectively using computer-based pixel densities determination as previously done in our laboratory [34, 59, 60].

Cytokine production

Splenic and lung draining bronchial LNs cells (BLN) were re-stimulated in vitro (5×10^6 cells/mL) with OVA (200 µg/mL) in RPMI (supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin). Cytokines were measured in supernatants using DuoSet ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

OVA-specific IgE and IgG1 assay

Serum levels of OVA-specific IgE were measured by ELISA according to a standard protocol as described previously [59].

CFSE-labeling of OT-II cells and in vivo proliferation assay

Ovalbumin specific T helper (OT-II) cells were purified from LNs and spleens of OT-II mice using CD4⁺ T cells enrichment kit (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by labeling with 1 μ M CFSE (Life Technologies, ThermoFisher Scientific, Waltham, MA, USA). A total of 3×10^6 CFSE-labeled OT-II cells were intravenously injected into recipient mice. Forty-eight hours later, mice were sacrificed and lung draining LNs cells were analyzed by flow cytometry using anti-CD3, anti-V α 2, anti-CD4, and CFSE on a FACS Canto flow cytometer (BD Biosciences, Mountain View, CA, USA). FlowJo software (Treestar Inc., Ashland, OR, USA) was used to analyze data.

Flow cytometry-based characterization of pulmonary and LNs APCs

Lung and lung-draining LN DCs and alveolar macrophages (AM) were sorted as previously described [62–64] following flowcytometry guidelines [65]. Briefly, single cells from lung tissue after digestion of the lungs using a mixture of collagenase and DNase (Miltenyi) were stained with CD11c, MHC-II, and siglec.F and MHC-II^{hi} CD11c⁺Siglec-F[−] cells in the lung were sorted as lung DC. For isolation of DC from lung draining LN, single cells were prepared through mashing LNs and separation using 70 μ M nylon mesh followed by sorting of CD11c⁺MHC-II⁺ cells as LN DCs. Antibodies used for identification and sorting of DCs and AM; CD11c, MHC-II, CD11b, CD103, Siglec-F while CD197, CD40, CCR2, and CD62L were used to analyze DCs and AM using chip cytometry (see Supporting Information Table 1 for clones, fluorophore, and manufacturer information).

Chip cytometry

Chip cytometry was performed as described previously [66, 67]. Briefly, cells were fixed on cell-adhesive microfluidic-chips according to company instructions (ZellSafe Chips, Zellkraftwerk GmbH, Leipzig, Germany). ZellSafe chips were then subsequently subjected to an iterative staining/bleaching cycles using ZellScannerONE (Zellkraftwerk GmbH, Leipzig, Germany). Data acquisition and analysis of fluorescence intensities was accomplished using the ZellExplorer App (Zellkraftwerk GmbH, Leipzig, Germany). SAS software JMP version 12.2.0 was used to create heatmaps based on fluorescence intensities on individual cells.

RNA-Sequencing and raw data processing

Total cellular RNA was extracted using the RNeasy Micro Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions and as we had previously done [68].

Library generation

Libraries were generated as previously described [68]. Briefly, 5 ng of total RNA per sample was used for library preparation with the SMARTer Stranded Total RNA-Seq Kit – Pico Input Mammalian (#635006; Takara/Clontech) according to conditions recommended in user manual #101215.

Sequencing run

Sequencing was performed as in our previous work [68]. Equal molar amounts of four individual libraries were pooled, denatured with NaOH and finally diluted to 1.5 pM according to the Denature and Dilute Libraries Guide (Document # 15048776 v02; Illumina). A total of 1.3 mL of the denatured pool was run on an Illumina NextSeq 500 sequencer using a High Output Flowcell for 75 bp single reads (#FC-404-2005; Illumina).

Raw data processing and quality control

The FASTQ files were adapter and quality trimmed using Trim Galore! (version 0.4.1) with default settings as described in the User Guide except for the setting of the quality cutoff (−q/−quality) that was set to a Phred score of 15. Trim Galore! used Cutadapt (version 1.9.1) as a subroutine.

Mapping

After trimming, FASTQ files were mapped against a reference genome with the splice-aware aligner STAR (version 2.5.0c) to generate BAM files. The BAM files were built in a 2-pass Mapping (−twopassMode Basic) and were finally sorted (−outSAMtype BAM SortedByCoordinate). The genome index files were created by STAR with default settings using *Mus musculus* sequence and annotation data (Ensembl, build GRCm38) available on Illumina's iGenome site (http://support.illumina.com/sequencing/sequencing_software/igenome.html).

Quantification, final data analysis, and visualization

Generated BAM files were imported to StrandNGS software (version 2.8; built 230243) according to the following settings: Organism: Mouse; Build: mm10 (UCSC); Transcript Annotation: Ensembl Transcripts (2013.04.03); Library layout: Directional Single End. Quantification was performed using RPKM normalization

algorithm (including partial reads). Normalized read counts were thresholded to 1 and were log (base 2) transformed.

Statistical analysis

GraphPad Prism software version 6 was used for statistical analysis (GraphPad Software, La Jolla, CA, USA). Unless otherwise indicated, one-way or two-way ANOVA was used to compare differences between more than two groups and Wilcoxon Ranked or Student's *t*-test was used to determine the statistical significance of differences between two groups. *p*-Values less than 0.05 (*), *p* < 0.01 (**), and *p* < 0.001 (***) were considered as significant.

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Abbreviations: AHR: airway hyperresponsiveness · AM: alveolar macrophages · BLN: bronchial LN · Th: T helper

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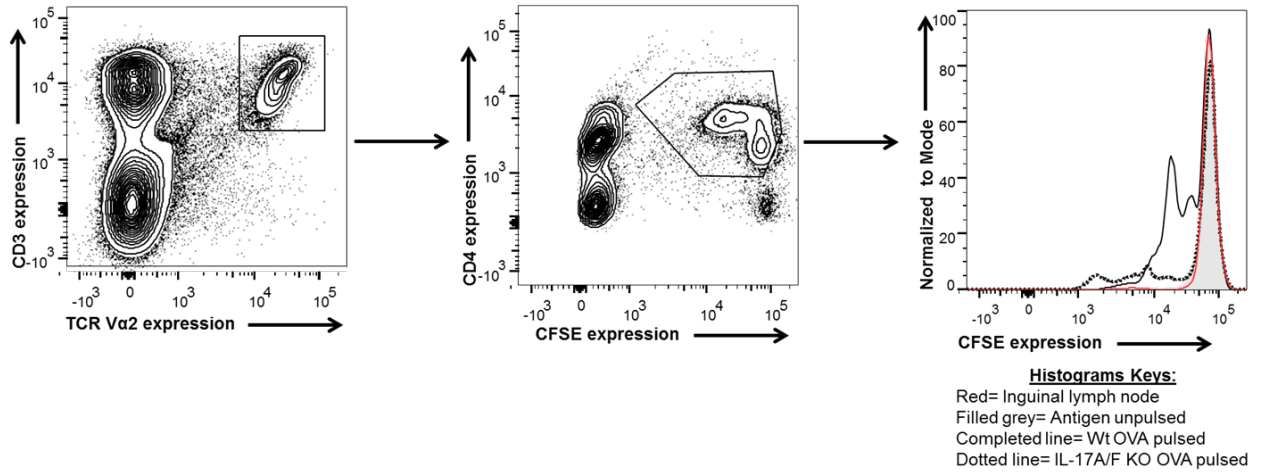
Revised: 30/1/2020

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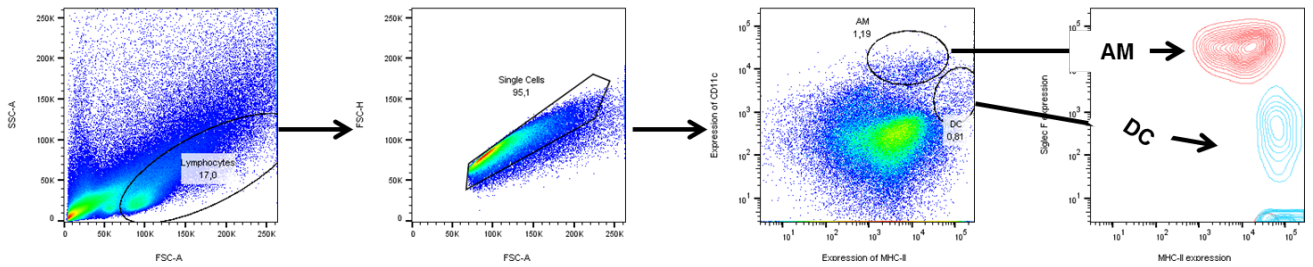
Accepted article online: 6/3/2020

Supplementary Fig. S1

(A)

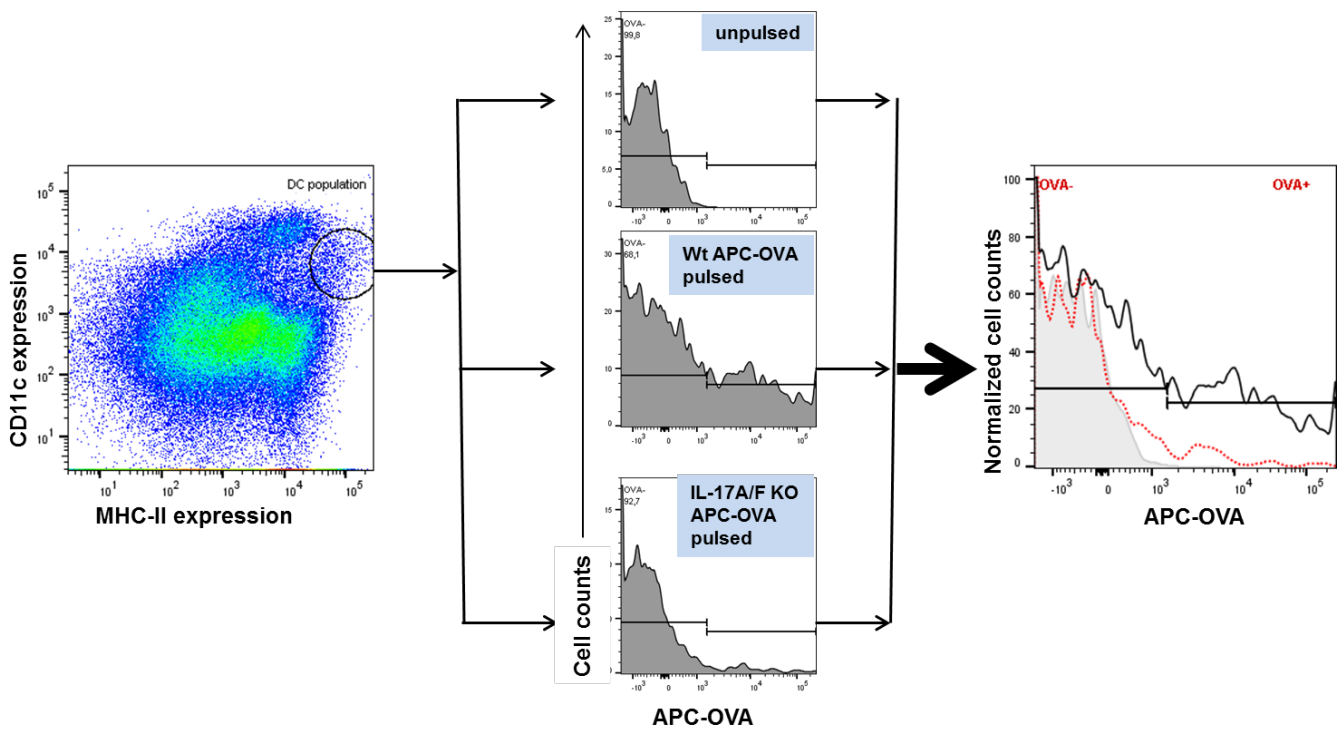


(B)



S1. Gating strategies applied for identification of adoptively transferred OT-II cells (A) and conventional DC and AMs in the lungs (B): Adoptively transferred OT-II cells are identified based on CD3, TCR Va2, CD4 and CFSE expression **(A)**. Conventional pulmonary DC and AMs are identified based on expression of CD11c, MHC-II and Siglec F expression **(B)**.

Supplementary Fig. S2

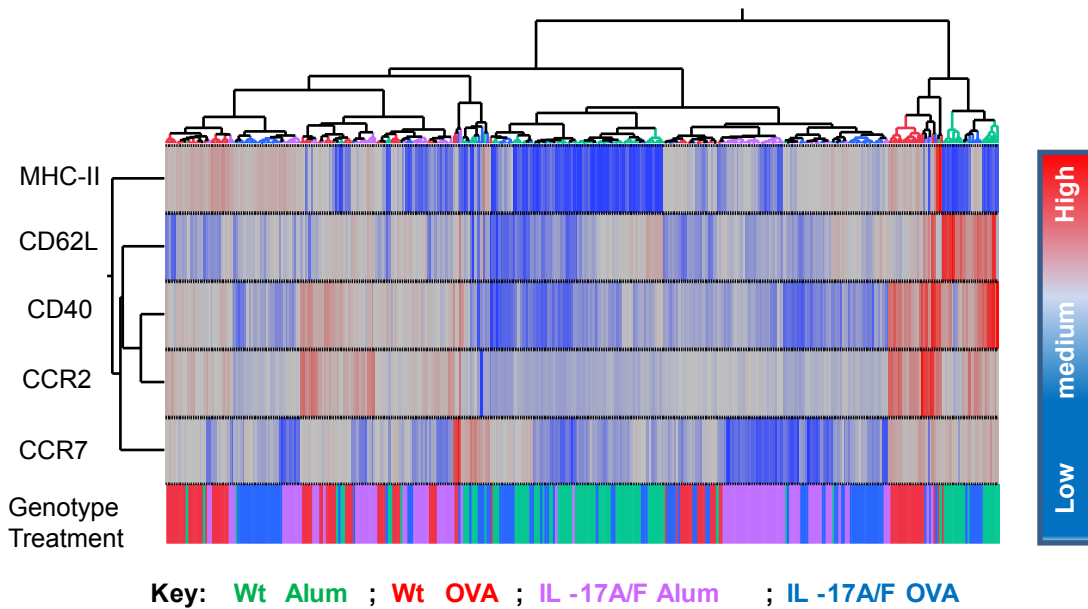


S2. Gating strategy applied in ex vivo analysis of *in vivo* uptake of APC-Conjugated OVA:

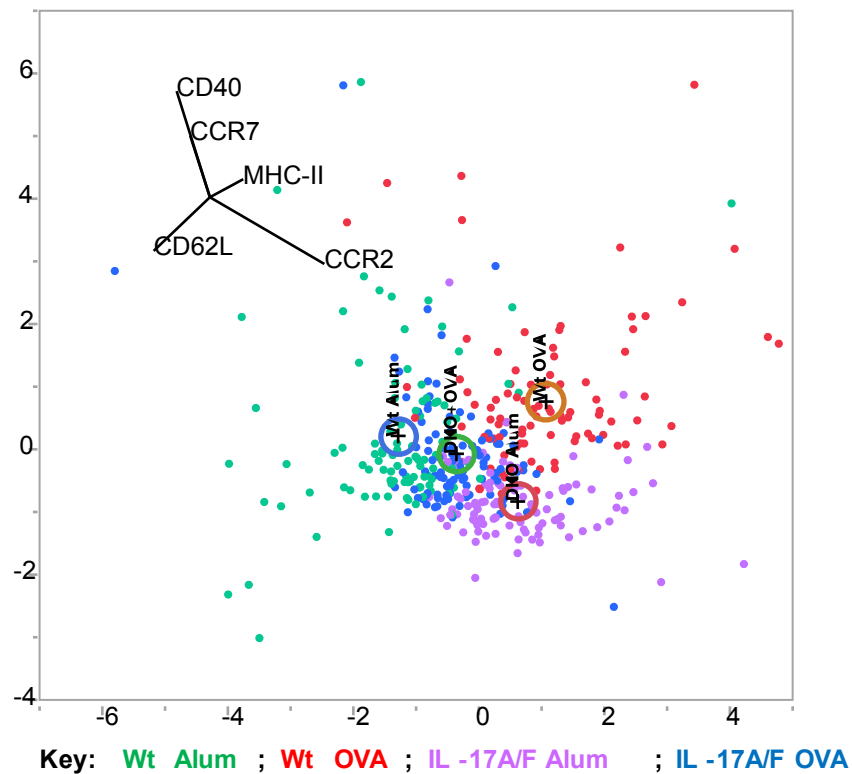
Conventional pulmonary DC are identified based on expression of CD11c and MHC-II and later analyzed for APC-OVA⁺ and APC-OVA⁻ subpopulations compared to DC from mice that were not pulsed with APC-OVA. Overlaid histograms show relations of *in vivo* uptake between the Wt (black complete line) and IL-17A/F KO (red dotted line) against unpulsed (grey filled).

Supplementary Fig. S3

A



B



S3. Unbiased single cells analysis: All groups into a single heatmap (A) and Linear Discriminant Analysis (LDA) (B) showing how various activation markers influence distribution of both Wt and IL-17A/F KO cells either after Alum or OVA challenge.

Legend for A and B respectively: Wt Alum (Green lines or dots); Wt OVA (Red lines or dots); IL-17A/F KO (Purple lines or dots) and IL-17A/F KO OVA (Blue lines or dots)

Extra Figures E1 and E2

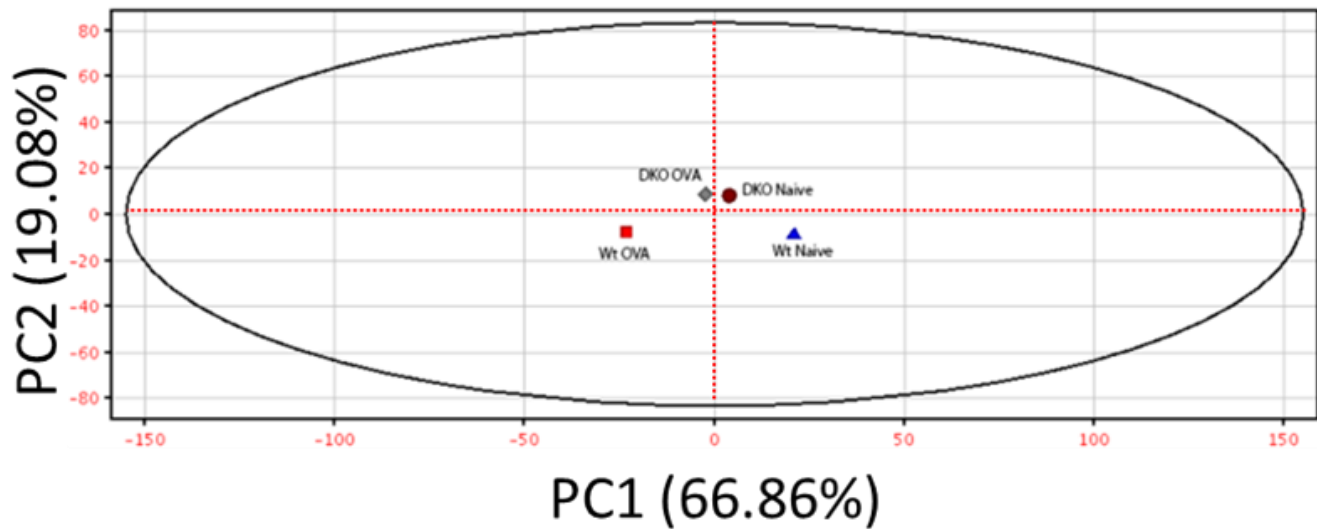


Fig.E1. Principle Component Analysis based on transcriptomes of DCs derived from lymph nodes of OVA challenged vs unchallenged wt or IL-17AF KO (DKO) mice.

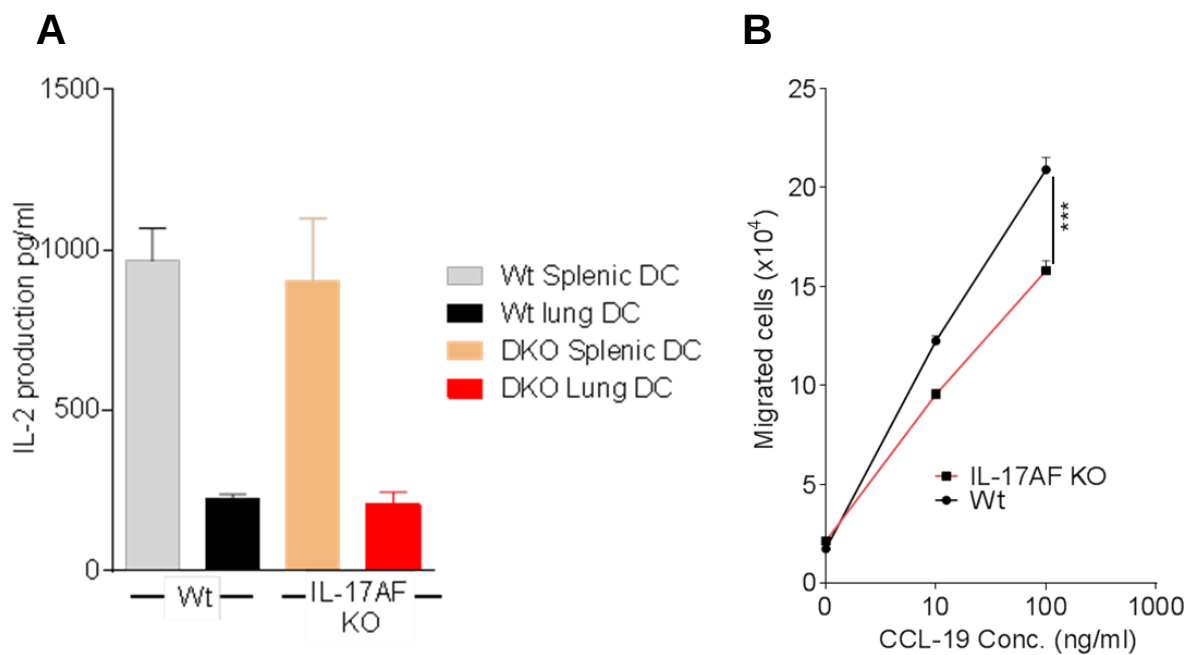


Fig.E2. Measurement of IL-2 in a co-culture experiment of T cells and DCs derived from spleens and lungs of WT and IL-17 AF KO mice **(A)** and Ccl19 migration of splenic DC of WT and IL-17AF KO mice **(B)**.

Supplementary Table 1

<i>Epitope</i>	<i>Fluorophore</i>	<i>Clone</i>	<i>Manufacturer</i>
CD11c	FITC;APC-Cy7	HL3	BD
CD11b	PerCP-Cy5.5	M1/70	BD
CD40	Pacific Blue;PE	3/23	Biolegend
Siglec-F	PE	E50-2440	BD
CD103	FITC;PE	M290	BD
CD197	PE	4B12	BD
CD62L	PE	MEL-14	BD
MHC-II	APC	M5/114.15.2	eBioscience
CD3	APC;PE	17A2	eBioscience
CD4	APC-H7	GK1.5	BD
TCR α 2	PE	B20.1	BD
CD117	Pacific Blue	2B8	Biolegend
Sca-1	PE-Cy7	E13-161.7	Biolegend

Supplementary Table 2

Gene symbol	Gene symbol	Gene symbol	Gene symbol
Plcl1	Samsn1	Mical3	Slc35c2
Sh3bp4	Zfp160	Eno2	Nfatc2
Gal3st2	Zfp677	Tapbp1	Il6ra
Gal3st2	Tmem8	Fam60a	BC028528
Tnfrsf11a	Abcg1	4933426I21Rik	Cd53
Lad1	H2-gs10	Sdhaf1	Stmn1
Slamf1	H2-Q8	Il4i1	Eif4g3
Slco5a1	H2-Q6	Mex3b	Sigmar1
Ogfrl1	Mmp25	Ccdc90b	Stmn1
Idh1	H2-T22	Rab30	Ccnb1
Mreg	H2-M2	Pgap2	Cenpa
Rasal2	Foxp4	Il21r	Alox5ap
Rabgap1l	Rftn1	Prr14	Sh3tc1
Gpr52	Pot1b	6030429G01Rik	Naaa
Zbtb37	Stap2	Relb	Unc119b
Rcsd1	Arhgap28	Kcnk6	Lat2
Herc4	Cdc42ep3	1700067C01Rik	Hk2
Adora2a	Gypc	Sdhaf1	Frmd4b
Efna2	Fam13b	Lrrk1	Plbd1
9030607L17Rik	Pla2g16	Fah	Tgfb1
Pctk2	Iscu	Zfand6	Rasgrp4
Tmcc3	BC016495	St5	Napsa
Gm10752	Papss2	Fam53b	Tm6sf1
Plekhg1	Pcgf5	Atp11a	Pak1
Stx11	Stxbp3b	Lamp1	Nucb2
Myb	AW112010	Casp3	Itgax
1110038D17Rik	Frmd4a	D130040H23Rik	Ptpre
Nuak1	Gm10851	Hsh2d	Fosb
Plxnc1	Il15ra	2510049I19Rik	Ccnb1
Socs2	Nostrin	Nlrc5	E2f8
Tmtc2	Ube2l6	Nlrc5	Fes
Tmem19	Phf21a	Gins3	Sult1a1
Rassf3	Sema6d	Atmin	Stk32c
Dtx3	Stard7	Ap3m2	Jund
Pisd-ps1	Insm1	D030016E14Rik	Man2b1
Pus10	Stk4	Il15	Adcy7
Gfpt2	Pfkfb3	Tmem123	Cd209a
Irf1	Ehmt1	Zfp809	Dpep2
Zmynd15	Stxbp1	Scn4b	Slc9a9
Tm4sf5	Traf1	Dscaml1	Anln
Eno3	Strbp	Stoml1	Plekho2
Arl5c	Gtdc1	4921528I07Rik	Ccnb2
Cacnb1	Arl5a	Ube1l	Cfp
Ccr7	Vps39	C330006D17Rik	
Fam49a	Rassf2	Gramd1b	
Socs2	Fam164a	Sik2	
Tmem18	Rap2b	Tspan3	
1700047I17Rik1	Pias3	C230081A13Rik	
Daam1	Rnf115	Mpi	

Gm10432	Gbp5	4922501C03Rik
Actn1	Gbp2	4930579C12Rik
Gtf2a1	Gyg	Rasa2
Ptpn21	Gnb4	E030011O05Rik
Eml5	E130311K13Rik	Mtmr1
Itgb8	Slc33a1	Tbc1d8b
Serpinb6b	Nudt17	Enox2
Hivep1	Polr3c	Arhgef9
Aof1	BC037703	Zmat1
Serinc5	Stxbp3a	Inpp5d
Net1	Vcam1	Cd244
Sox4	Synpo2	5033414K04Rik
Slc22a23	N28178	Fuca2
Cdc14b	Npr2	Arhgap18
Gm10021	Nans	Mical1
B930046C15Rik	Galnt12	Itgb2
3830406C13Rik	Ttc39a	Gna15
E130112L23Rik	Pik3r3	Havcr2
Zmym2	Rnf19b	Gm2a
Clu	Cdkn2b	Plek
Ccdc122	Eif2c1	Anxa6
Epsti1	Oprd1	Cd68
544988	Extl1	Rrm2
Gm3264	Spsb1	Plekhg3
D830030K20Rik	Mmp23	Jarid2
Hn1l	Cc2d2a	Tgfb1
ENSMUSG00000079376	Anxa3	Dapk1
Hn1l	Bmp2k	Mctp1
D830030K20Rik	Iscu	F2rl2
D830030K20Rik	BC023744	Gmcs
Gcap14	BC023744	Fgd3
Psme2	BC023744	Wdfy2
Tbc1d4	BC023744	Gng2
Dap	Gatsl2	Grap2
Laptm4b	Gatsl2	Parvg
Apol7e	Fscn1	Nr4a1
Gtpbp1	Sepsecs	Sla
Cacnb3	BC062109	5031439G07Rik
Spats2	5830443L24Rik	Slc38a1
Slc4a8	BC057170	Racgap1
Ankrd33b	Gbp4	Itgb7
Ankrd33b	Mpa2l	Ciita
Ankrd33b	Ssh1	Tfrc
Rnf19a	Mtif3	Hlcs
Arc	N4bp2l1	Mapk14
Adcy6	Fam40b	Cyp4f16
Lima1	Trim24	Myo1f
Atf7ip2	Gimap4	H2-DMa
Snn	Herc5	H2-DMb2
Ift57	A930038C07Rik	H2-Ob

Cblb	Asprv1	Zfp36l2
Fam60a	Vhl	Lmnb1
Rogdi	Rasgef1a	Lpxn
Gm9861	Usp18	Entpd1
4921513D23Rik	Clec2d	Pik3ap1
Dnm1l	Crebl2	Cacnb2
1700021K19Rik	Aebp2	Gsn
Ktelc1	B630005N14Rik	Arhgap15
Cdgap	Dok1	Camk1d
Dzip3	Htra2	Plcb2
	Mxd1	Ehd4