House dust mitedriven neutrophilic airway inflammation in mice with TNFAIP3deficient myeloid cells is IL17independent

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ABSTRACT

Background: Asthma is a heterogeneous disease of the airways that involves several types of granulocytic inflammation. Recently, we have shown that the activation status of myeloid cells regulated by TNFAIP3/A20 is a crucial determinant of eosinophilic or neutrophilic airway inflammation. However, whether neutrophilic inflammation observed in this model is dependent on IL-17 remains unknown.

Objective: In this study, we investigated whether IL-17RA-signalling is essential for eosinophilic or neutrophilic inflammation in house dust mite (HDM)-driven airway inflammation.

Methods: *Tnfaip3*$^{fl/fl}$x*Lyz2*$^{+/cre}$ (*Tnfaip3*$^{LysM\text{-}KO}$) mice were crossed to *Il17ra*^{KO} mice, generating *Tnfaip3*^{LysM}*Il17ra*^{KO} mice and subjected to an HDM-driven airway inflammation model.

Results: Both eosinophilic and neutrophilic inflammation observed in HDM-exposed WT and *Tnfaip3*$^{LysM\text{-}KO}$ mice respectively, was unaltered in the absence of IL-17RA. Production of IL-5, IL-13 and IFNγ by CD4^{+} T cells was similar between WT, *Tnfaip3*$^{LysM\text{-}KO}$, and *Il17ra*^{KO} mice, whereas mucus-producing cells in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice were reduced compared to controls. Strikingly, spontaneous accumulation of pulmonary Th1, Th17 and $\gamma\delta$-17 T-cells was observed in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice, but not in the other genotypes. Th17 cell-associated cytokines such as GM-CSF and IL-22 were increased in the lungs of HDM-exposed *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice, compared to IL-17RA-sufficient controls. Moreover, neutrophilic chemo-attractants CXCL1, CXCL2, CXCL12, and Th17-promoting cytokines IL-1β and IL-6 were unaltered between *Tnfaip3*$^{LysM\text{-}KO}$ and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice.

Conclusion and Clinical Relevance: These findings show that neutrophilic airway inflammation induced by activated TNFAIP3/A20-deficient myeloid cells can develop in the absence of IL-17RA-signalling. Neutrophilic inflammation is likely maintained by similar quantities of pro-inflammatory cytokines IL-1β and IL-6, that can independently of IL-17 signalling, induce the expression of neutrophil chemo-attractants.

INTRODUCTION

Asthma is characterized by reversible airway obstruction, airway remodelling and mucus production, together with increased pulmonary inflammation[1]. Granulocytic cells observed in pulmonary inflammation of asthmatic patients can comprise eosinophils, neutrophils, or a mixture of both cell types[2]. Eosinophilic inflammation is induced by interleukin (IL)-5, a type 2 cytokine produced by both Th2 cells and innate lymphoid cells type 2 (ILC2s)[3]. Neutrophilic inflammation is triggered by IL-8 produced by airway epithelial cells after activation by IL-17[4]. IL-17 furthermore contributes to asthma symptoms, because (1) it induces airway remodelling by promoting fibroblast proliferation, (2) reduces apoptosis of smooth muscle cells, and (3) increases the expression of mucin genes in airway epithelial cells[5-7]. Th17 cells primarily produce IL-17 and Th17-associated neutrophilic inflammation is particularly found in late-onset asthma patients with a severe phenotype[8, 9]. Unfortunately, severe asthma patients are often unresponsive to corticosteroid treatment, leading to frequent asthma exacerbations and higher morbidity[10]. Neutrophils and Th17 cells are likely contributing to this phenotype, as both cell types are corticosteroid insensitive[11-13]. Therefore, it is imperative to investigate the contribution of IL-17-signalling to the development of neutrophilic asthma.

Dendritic cell (DC) activation is essential for Th cell differentiation as antigen load, expression of costimulatory molecules, and DC-derived cytokines determine whether Th2 or Th17 cell differentiation is induced[14]. DC activation is controlled by TNFAIP3 (TNFα-induced protein 3, also known as A20), an ubiquitin modifying enzyme that deubiquitinates several key intermediate NF-κB signalling molecules, and thereby controls NF-κB-mediated cell activation[15]. TNFAIP3 is also implicated in Th2-associated disorders, as genetic polymorphisms in *TNFAIP3* and TNFAIP3 interacting protein (*TNIP*) have been associated with risk of developing allergies and asthma[16, 17]**.** Recently, we found that increasing the activation status of DCs by ablation of the *Tnfaip3* gene in myeloid cells induced a neutrophilic inflammation in house dust mite (HDM)-mediated asthma protocols, which was accompanied with enhanced number of IL-17-producing $CD4^+$ T cells[18].

To investigate whether the HDM-driven neutrophilic airway inflammation is dependent on IL-17, we crossed myeloid-specific *Tnfaip3* knockout mice (*Tnfaip3*$^{LysM\text{-}KO}$ mice)[19] to *Il17ra*^{KO} mice, generating *Tnfaip3*^{LysM}*Il17ra*^{KO} mice, in which IL-17A, IL-17E and IL-17F-signalling is disabled[20]. Absence of IL-17RA-signalling in *Tnfaip3*$^{LysM\text{-}KO}$ mice does not significantly affect neutrophilic inflammation, most likely due to enhanced amounts of IL-1β and IL-6 that can also promote the production of several neutrophil chemoattractants.

MATERIALS AND METHODS

Mice

Male and female C57BL/6 mice harbouring a conditional *Tnfaip3* allele between LoxP-flanked sites[21] were crossed to transgenic mice expressing the Cre recombinase under the LysM promotor[22], generating *Tnfaip3*$^{fl/fl}$x*Lyz2*$^{+/cre}$ mice, in which *Tnfaip3* will be deleted in cells that express or have expressed LysM[19] (*Tnfaip3*$^{LysM\text{-}KO}$ mice). *Tnfaip3*$^{fl/fl}$*Lyz2*$^{+/+}$ littermates (wild type (WT) mice) were used as controls. *Tnfaip3*^{LysM} mice were crossed with conventional *Il17ra*^{KO} mice[23], creating *Tnfaip3*$^{fl/fl}$x*Lyz2*$^{+/cre}$x*Il17ra*$^{-/-}$ mice (*Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice) and *Tnfaip3*$^{fl/fl}$x*Lyz2*$^{+/+}$x*Il17ra*$^{-/-}$ mice (*Il17ra*^{KO} mice). Mice were housed under specific pathogen-free conditions and were analysed at ~8 weeks (naïve and House Dust Mite (HDM) experiments) or at ~18 weeks (arthritis experiments). All experiments were approved by the animal ethical committee of the Erasmus MC, Rotterdam, The Netherlands (EMC3328 and EMC3333).

HDM-induced allergic airway inflammation

During intranasal (i.n.) exposures, mice were anesthetized using isofluorane. On day 0, mice were sensitized with 1 µg/40 µL HDM (Greer Laboratories Inc, Lenoir, NC, USA) i.n. or with 40 µL PBS (GIBCO Life Technologies, Carlsbad, CA, USA) as a control and challenged with 10 µg/40 µl HDM on days 7-11. Four days after the last challenge, bronchoalveolar lavage (BAL), lung, and mediastinal lymph node (MLN) were collected.

Cell suspension preparation

BAL was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA). The right lung was inflated with either 1:1 PBS/Tissue-TEK O.C.T. (VWR International, Darmstadt, Germany) solution, or snap-frozen in liquid nitrogen, and kept at −80°C until further processing for histology. The left lung was used for flow cytometry. Single-cell suspensions of the left lung were obtained by digesting using DNase (Sigma-Aldrich) and Liberase (Roche, Basel, Switzerland) for 30 min at 37°C. After digestion, the lungs were homogenized using a 100-µm cell strainer (Fischer Scientific, Waltham, MA, USA) and red blood cells were lysed using osmotic lysis buffer (8.3% NH_4CL, 1% $KHCO_3$, and 0.04% NA_2EDTA in Milli-Q). MLN and spleen were isolated for flow cytometry, for which they were homogenized through a 100-µm cell strainer.

Flow cytometry procedures

Flow cytometry surface and intracellular staining procedures have been described previously[24]. Monoclonal antibodies used for flow cytometric analyses are listed in **Supplementary table 1**. For all experiments, dead cells were excluded using fixable viability

dye (eBioscience, San diego, CA, USA). For measuring cytokine production, cells were stimulated with 10 ng/mL PMA (Sigma-Aldrich), 250 ng/mL ionomycin (Sigma-Aldrich), and GolgiStop (BD Biosciences, San Jose, CA, USA) for 4 h at 37°C. Data were acquired using a LSR II flow cytometer (BD Biosciences) with FACS DivaTM software and analysed with FlowJo version 9 (Tree Star Inc software, Ashland, OR, USA).

Lung histology

Six-µm-thick paraffin embedded lung sections were stained with periodic acid-Schiff (PAS) to visualize goblet cell hyperplasia.

Cytokine mRNA assessment by Quantitative Real-Time PCR

Homogenized left lower lung lobe was used to isolate and purify total RNA using the GeneElute mammalian total RNA miniprep system (Sigma-Aldrich) and RNA quantity was determined using a NanoDrop 1000 (VWR International). Up to 0.5 µg of total RNA was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen). Gene expression was analysed for *Gapdh, Cxcl1, Cxcl2, Cxcl12, Il1b, Il6, Il22, Il23*, *Csf2,* and *Muc5a* in SYBR Green Master Mixes (Qiagen, Hilden, Germany) using an ABI Prism 7300 Sequence Detector and ABI Prism Sequence Detection Software version 1.4 (Applied Biosystems, Foster City, CA, USA). Forward and reverse primers for each gene are listed in **Supplementary table 2**. Samples were analysed simultaneously for *Gapdh* mRNA as internal control. Each sample was assayed in duplicate and relative expression was calculated as 2^(-ΔCt), where ΔCt is the difference between Ct of the gene of interest and GAPDH.

Statistical analysis

All data was presented as means ± SEM. Mann–Whitney *U*-tests were used for comparison between two groups, and a *P*-value of <0.05 was considered statistically significant. All analyses were performed using Prism (Version 5, GraphPad Software, La Jolla, CA, USA).

RESULTS

Loss of IL-17RA-signalling combined with myeloid TNFAIP3 deficiency increases splenic monocytes, neutrophils and γδ T cells with progressing age.

To investigate the role of IL-17RA-signalling in HDM-driven neutrophilic airway inflammation responses, we crossed *Tnfaip3*^{LysM} mice[18, 19] with conventional *Il17ra*^{KO} mice[23]. It has been demonstrated that aged *Tnfaip3*$^{LysM\text{-}KO}$ mice develop arthritis[19] and that *Il17ra*^{KO} mice have altered monocyte[25] and neutrophil[26, 27] homeostasis. We therefore first examined whether abrogation of IL-17RA-signalling in *Tnfaip3*$^{LysM\text{-}KO}$ mice induces additional

alterations in the immune system. We assessed spleens of 8 and 18-week-old mice, as a representation of the systemic immune state. Both 8 and 18-week-old *Tnfaip3*$^{LysM\text{-}KO}$ and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice showed splenomegaly in comparison to WT and *Il17ra*^{KO} control mice (**Figure 1A**), whereas total splenic cell counts were only increased in 8 and 18-week old *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice (**Figure 1B**). Monocytes and neutrophils (gated as shown in **Supplementary Figure 1**) were significantly increased in 8-week-old *Tnfaip3*$^{LysM\text{-}KO}$ mice in comparison to WT mice (**Figure 1C-D**), however only neutrophils were significantly increased in 18-week-old *Tnfaip3*$^{LysM\text{-}KO}$ mice compared to WT mice (**Figure 1D**), confirming previous findings[19]. Interestingly, both neutrophils and monocytes were significantly increased in 18-week-old *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 1C-D**). Despite elevated monocyte and neutrophil numbers in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice, the macroscopic and microscopic arthritis phenotype was similar between *Tnfaip3*$^{LysM\text{-}KO}$ mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice (**Supplementary Figure 2**).

As IL-17 controls its own expression in CD4$^+$ T cells[26], we assessed conventional TCRαβ T cells and γδ T cells in the spleen (gating shown in **Figure 1E**). Total CD4$^+$ T helper (Th) cell numbers were not different between the genotypes in 8-week old mice, but were significantly increased in 18-week-old *Il17ra*^{KO} mice compared to WT mice (**Figure 1F**). Splenic RORγt$^+$ Th17 cells were elevated in 8-week-old *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *Tnfaip3*$^{LysM\text{-}KO}$ mice, but this was no longer seen in 18-week-old mice (**Figure 1G**). Only 18-week-old *Il17ra*^{KO} mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice had increased splenic γδ T cell numbers compared to respective *Il17ra*^{WT} controls (**Figure 1H**). Splenic CD8$^+$ T cells were reduced in *Tnfaip3*$^{LysM\text{-}KO}$ mice and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to respective *Tnfaip3*$^{LysM\text{-}WT}$ littermate controls at both ages (**Figure 1I**). Splenic B cell numbers did not differ between genotypes in both 8-week-old and 18-week-old mice (**Figure 1J**).

Taken together, these data show that myeloid TNFAIP3 deficiency with additional loss of IL-17RA-signalling induces minimal systemic immune changes at a young age, as only splenic Th17 cells are increased and CD8$^+$ T cells are decreased. In contrast, with progressing age myeloid TNFAIP3 deficient mice with abrogated IL-17RA-signalling accumulate splenic monocytes, neutrophils and γδ T cells.

House dust mite-induced eosinophilic and neutrophilic airway inflammation is unaltered in the absence of IL-17RA-signalling.

To investigate the requirement of IL-17RA-signalling on neutrophilic airway inflammation, we exposed young *Tnfaip3*^{LysM}*Il17ra* mice to an HDM-driven airway inflammation model (**Figure 2A**). As previously shown[18], HDM-sensitization and challenge induced a predominant eosinophilic inflammation in WT mice compared to PBS-sensitization, whereas *Tnfaip3*$^{LysM\text{-}KO}$ mice developed a primarily neutrophilic inflammation in the bronchoalveolar lavage (BAL) (**Figure 2B**). Absence of IL-17RA-signalling did not significantly

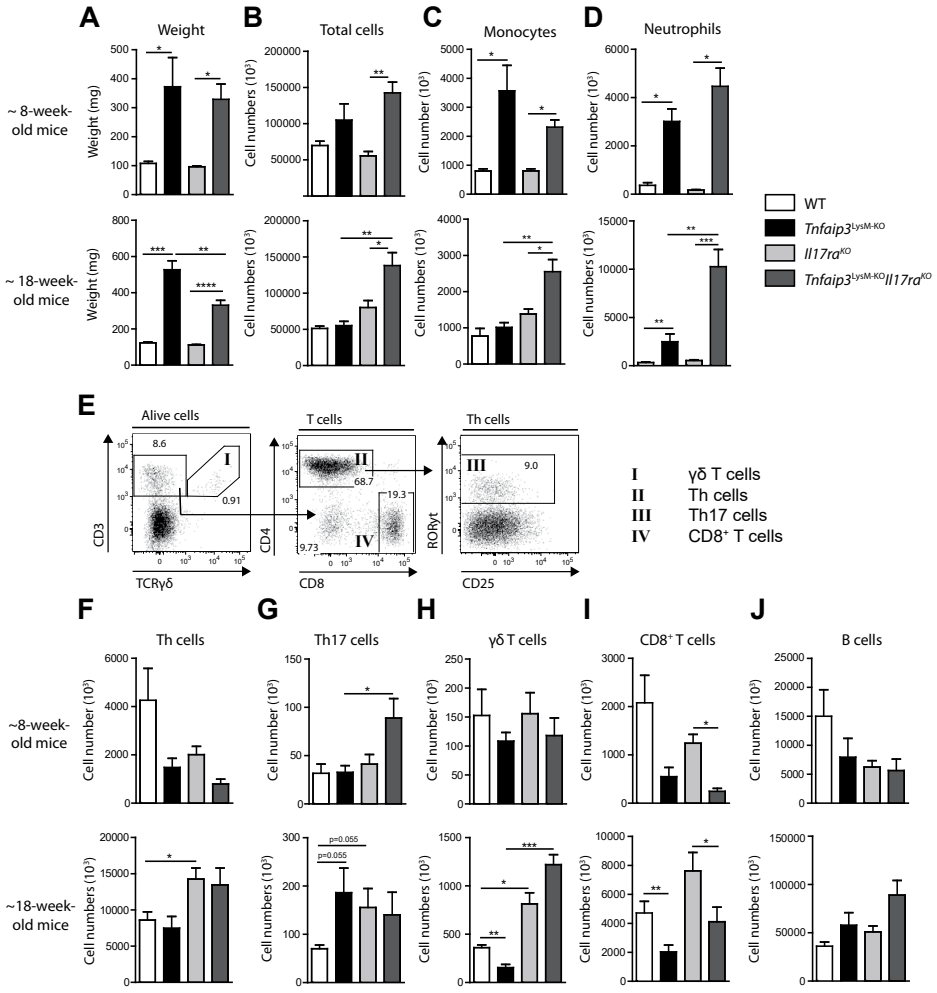

Figure 1: Loss of IL-17RA-signalling combined with myeloid TNFAIP3 deficiency increases splenic monocytes, neutrophils and γδ T cells with progressing age.

Tnfaip3^{LysM}*Il17ra* mice were analysed at 8 weeks and 18 weeks of age. (**A-B**) Quantification of spleen weight (**A**) and total cell numbers (**B**). (**C-D**) Enumeration of monocytes (**C**) and neutrophils (**C**) analysed in spleen cell suspensions by flow cytometry. (**E**) Flow cytometric gating strategy of T cells and γδ T cells. Example is shown from a spleen obtained from a WT mouse. (**F-H**) Cell numbers are depicted of Th cells (**F**), Th17 cells (**G**), γδ T cells (**H**), CD8$^+$ T cells (**I**) and B cells (**J**) in spleen cell suspensions by flow cytometry. Results are presented as mean ± SEM of n = 4-10 per group and are pooled from several experiments. *$P < 0.05$, **$P <$ 0.01, ***$P < 0.001$.

alter eosinophilic or neutrophilic inflammation in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 2B**). BAL DCs were increased in both HDM-sensitized WT mice and *Il17ra*^{KO} mice compared to their respective PBS-sensitized littermates (**Figure 2B**). However, in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice,

DC numbers were reduced compared to HDM-sensitized WT mice and were increased in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 2B**). The absence of IL-17RA did not significantly alter the number of BAL macrophages in comparison to IL-17RA sufficient controls (**Figure 2B**).

HDM-sensitized WT and *Il17ra*^{KO} mice exhibited enhanced small airway mucus-producing goblet cells and inflammatory cells compared to their PBS-sensitized controls (**Figure 2C**). HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice had similar numbers of mucus-positive cells in both small and large airways compared to HDM-sensitized WT mice (**Figure 2C**). Remarkably, with additional loss of IL-17RA-signalling, the amount of goblet cells in small and large airways and lung *Muc5a* mRNA levels were severely reduced in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Il17ra*^{KO} mice (**Figure 2C-D**).

In HDM-sensitized WT mice, the numbers of total T cells and CD4^{+} T cells in BAL fluid increased compared to PBS-sensitized WT mice (**Figure 2E**). Total BAL T cells, Th cells and γδ T cells were prominently elevated in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 2E**). HDM-sensitized *Il17ra*^{KO} mice had a slight increase in γδ T cells compared to HDM-sensitized WT mice (**Figure 2E**). Differences in total T cells and γδ T cells were not observed in the MLN (**Supplementary Figure 3**).

In conclusion, absence of IL-17RA-signalling did not significantly alter eosinophilic or neutrophilic airway inflammation in respectively HDM-treated *Il17ra*^{KO} and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. In contrast, abrogated IL-17RA-signalling in combination with *Tnfaip3*-deficient myeloid cells hampered goblet cell hyperplasia. While Th cells and γδ T cells increase equally in *Tnfaip3*$^{LysM\text{-}KO}$ and WT mice upon HDM sensitisation, these populations remarkably increase with loss of IL-17RA-signalling.

Loss of IL-17RA-signalling does not reduce lung Th2 cytokines in an HDM-sensitized model, but increases IL-17 production.

The effects of IL-17 on Th2 differentiation in allergic asthma models depend on the allergen used and the timing of IL-17 exposure[28-30]. As eosinophilia and neutrophilia were only moderately affected by the loss of IL-17RA in HDM-sensitized *Tnfaip3*$^{LysM\text{-}WT}$ and *Tnfaip3*$^{LysM\text{-}KO}$ mice, we determined the effects of IL-17RA-signalling on cytokine secretion by T cells upon HDM-provoked airway inflammation. As expected, IL-13 and IL-5-expressing Th cells were increased within the BAL of HDM-sensitized WT mice compared to PBS-sensitized WT mice (**Figure 3A-B**). IL-13^{+} and IL-5^{+} Th cells were unaltered in HDM-sensitized *Il17ra*^{KO} and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to their respective controls with functional IL-17RA-signalling (**Figure 3B**). HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il-17ra*^{KO} mice had reduced IL-13^{+} and IL-5^{+} Th cells compared to HDM-sensitized *Il17ra*^{KO} mice (**Figure 3B**). As previously shown[18], BAL IL-17^{+} Th cells increased in HDM-sensitized

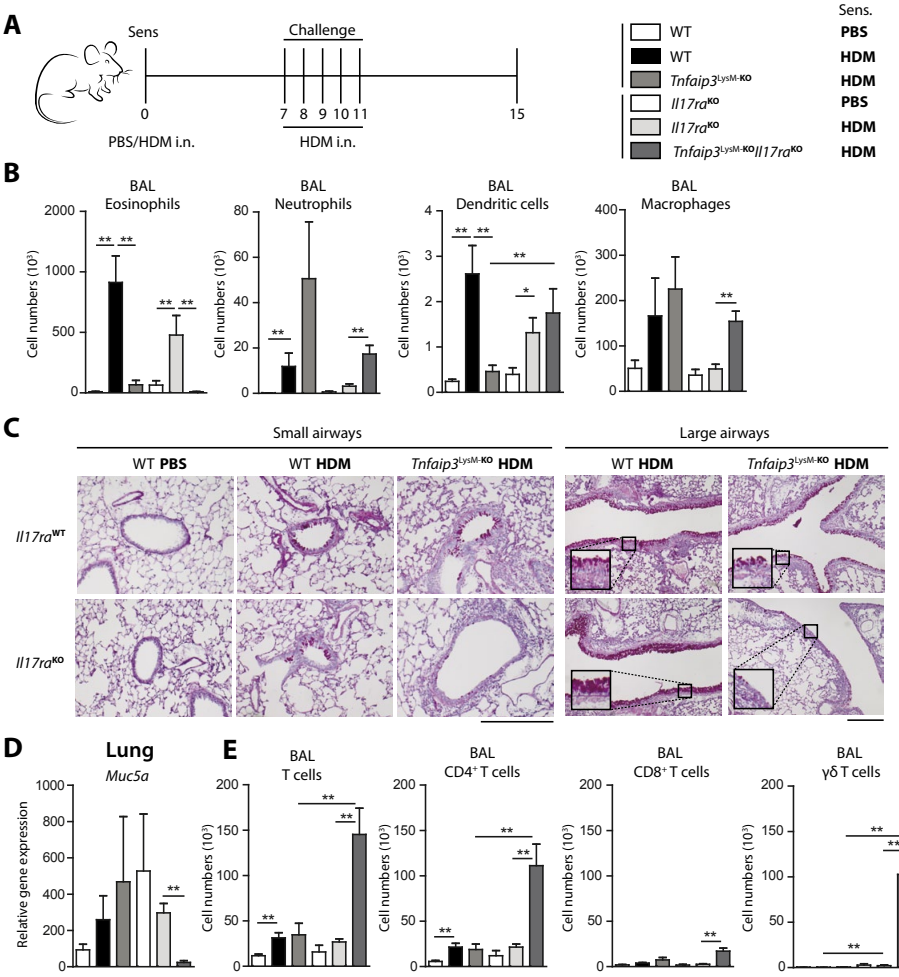

Figure 2: House dust mite induced eosinophilic and neutrophilic airway inflammation is unaltered in the absence of IL-17RA-signalling.

(**A**) Mice were sensitized with PBS or HDM (1 µg) on day 0 and challenged with 10 µg HDM from day 7-11. Analysis was performed at day 15. (**B**) Quantification of bronchoalveolar lavage (BAL) fluid eosinophils, neutrophils, dendritic cells and macrophages by flow cytometry. (**C**) Periodic Acid Schiff (PAS) stained lung small airway and large airway histology of *Tnfaip3*^{LysM}*Il17ra* mice after HDM exposure. Scale bar indicates 200µM. (**D**) *Muc5a* mRNA levels within lung homogenates of PBS and HDM challenged *Tnfaip3*^{LysM}*Il17ra* mice. (**E**) Enumeration of total CD3$^+$ T cells, CD4$^+$ Th cells, CD8$^+$ T cells and γδ T cells in BAL by flow cytometry. Results are presented as mean ± SEM of $n = 6$ per group and are representative of two independent experiments. *$P < 0.05$, **$P < 0.01$.

Tnfaip3$^{LysM\text{-}KO}$ mice compared to HDM-sensitized WT controls. Already in PBS-sensitized *Il17ra*^{KO} mice, an increase of BAL IL-17$^+$ Th cells was observed compared to PBS-sensitized WT mice, which was even more enhanced in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice (**Figure 3B**). BAL IFNγ-producing Th cells were only increased in HDM-sensitized

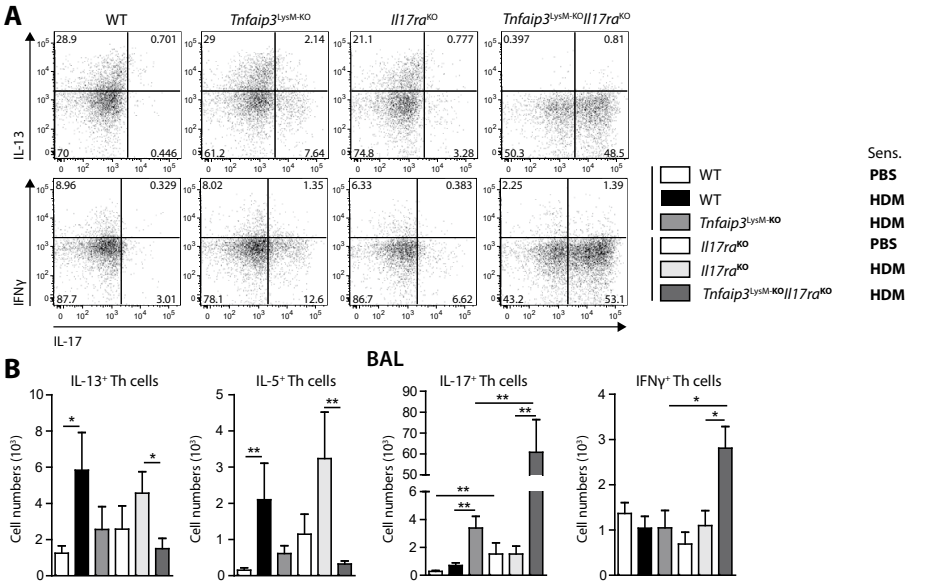

Figure 3: Loss of IL-17RA-signalling does not affect lung Th2 cytokines in a HDM-sensitized model, but increase IL-17 production.

Tnfaip3^{LysM}*Il17ra* mice were analysed after completion of the HDM exposure protocol. (**A**) Flow cytometry data is shown of intracellular cytokine expression within Th cells of broncho-alveolar lavage (BAL) of representative HDM exposed mice. (**B**) Quantification of BAL Th cell cytokines IL-13, IL-5, IL-17 and IFNγ as determined by flow cytometry. Results are presented as mean ± SEM of $n = 6$ per group and are representative of two independent experiments. *$P < 0.05$, **$P < 0.01$.

Tnfaip3$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to either HDM-sensitized *Il17ra*^{KO} or *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 3B**).

In conclusion, lack of IL-17RA-signalling did not alter Th2 cytokines in HDM-sensitized mice, which correlated with the previously seen eosinophilic infiltrate. In contrast, IL-17-production and IFNγ-production by Th cells significantly increased in HDM-sensitized mice lacking myeloid TNFAIP3 with absent IL-17RA-signalling.

Myeloid TNFAIP3-deficient mice have IL-17RA-independent increases of neutrophil chemokines upon HDM-sensitization.

IL-17 may contribute to neutrophil chemokine (C-X-C motif) ligand (CXCL)1[31, 32], CXCL2[23, 33], and CXCL12 release[34]. Since neutrophilic inflammation persisted in lungs of HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice, we assessed mRNA expression levels of these chemokines. HDM-sensitized lungs of *Tnfaip3*$^{LysM\text{-}KO}$ mice expressed increased amounts of *Cxcl1, Cxcl2* and *Cxcl12 mRNA* compared to HDM-sensitized WT mice (**Figure 4A**). Surprisingly, *Cxcl1* and *Cxcl12* mRNA expression did not differ between HDM-sensitized lungs of *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice and *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 4A**). In contrast, lung

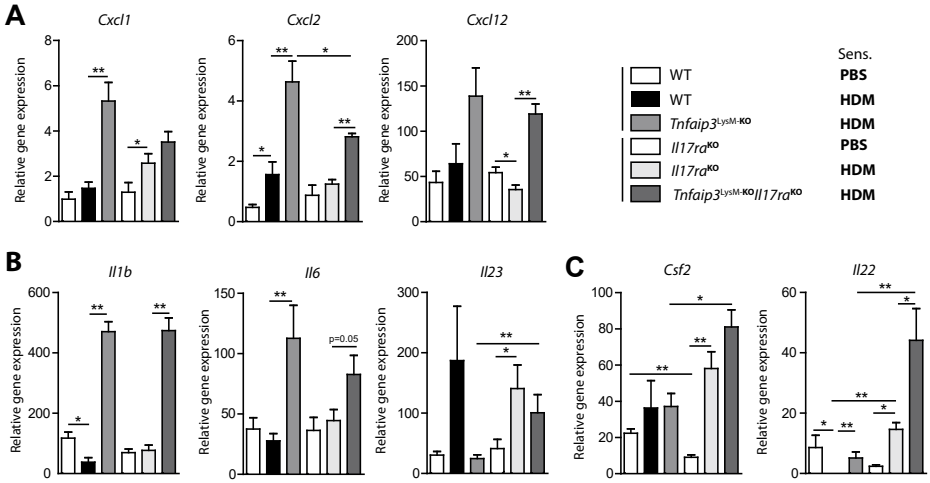

Figure 4: Myeloid TNFAIP3-deficient mice have IL-17RA-independent increases of neutrophil chemokines upon HDM-sensitization.

Total lung homogenates of HDM-challenged *Tnfaip3*^{LysM}*Il17ra* were analysed by RT-PCR. (**A-C)** Quantification of neutrophil chemokines *Cxcl1, Cxcl2, Cxcl12* gene expression (A), pro-inflammatory cytokines *Il1b, Il6* and *Il23* gene expression **(B)** and Th17-associated cytokines *Csf2* and *Il22* gene expression **(C)** in lung homogenates of PBS and HDM challenged *Tnfaip3*^{LysM}*Il17ra* mice. Results are presented as mean ± SEM of $n = 6$ per group. *$P < 0.05$, **$P < 0.01$.

Cxcl2 mRNA expression was partially reduced in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice as compared to HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 4A**). As absence of IL-17RA-signalling only moderately influenced chemokine expression, we evaluated other pro-inflammatory cytokines that can promote their expression, such as IL-1β[31, 35], IL-6[36] and IL-23[37]. HDM-treated *Tnfaip3*$^{LysM\text{-}KO}$ mice demonstrated elevated *Il1b* and *Il6* expression as compared to HDM-treated WT controls (**Figure 4B**). Abrogated IL-17RA-signalling in HDM-exposed *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice resulted in similar *Il1b* and *Il6* cytokine expression as *Tnfaip3*$^{LysM\text{-}KO}$ mice (**Figure 4B**). In contrast, *Il23* expression was markedly increased in HDM-exposed *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to *Tnfaip3*$^{LysM\text{-}KO}$ controls (**Figure 4B**).

Next to IL-17A, Th17 cells can produce other cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF)[38] and IL-22[39, 40], which are known to regulate neutrophil chemokines CXCL1/CXCL2 and directly attract neutrophils respectively. mRNA expression of *Csf2* and *Il22* were augmented in HDM-sensitized *Il17ra*^{KO} mice compared to PBS-sensitized *Il17ra*^{KO} mice (**Figure 4C**). Only *Il22* gene expression was further increased in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice lungs compared to HDM-sensitized *Il17ra*^{KO} mice (**Figure 4C**). Both lung *Csf2* and *Il22* mRNA expression was enhanced in HDM-sensitized *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice compared to HDM-exposed

Tnfaip3$^{LysM\text{-}KO}$ mice (**Figure 4C**), which corresponded to the number of Th17 cells (**Figure 3B**).

In summary, myeloid TNFAIP3-deficient HDM-sensitized mice had elevated lung mRNA expression of the neutrophil chemokines *Cxcl1, Cxcl2* and *Cxcl12,* despite abrogated IL-17RA-signalling. IL-17RA-signalling partially contributes to *Cxcl2* expression in response to HDM-sensitization in myeloid TNFAIP3-deficient mice. Neutrophil chemo-attractants are probably maintained in the absence of IL-17RA-signalling by equal quantities of IL-1β and IL-6, that are most likely derived from activated myeloid cells.

DISCUSSION

IL-17 is implicated in severe and uncontrolled asthma, as patients who suffer from severe asthma display increased levels of IL-17 in lung tissue[41]. Recently we have shown that the presence of intrinsically activated myeloid cells, obtained through TNFAIP3/A20 ablation, induces development of neutrophilic inflammation accompanied by increased Th17 cells in contrast to Th2 cell-driven eosinophilic inflammation induced in control mice[18]. To investigate whether neutrophilic inflammation development as observed in HDM-treated *Tnfaip3*^{LysM} mice is dependent on IL-17-signalling, *Tnfaip3*^{LysM} mice were crossed to IL-17RA-deficient mice.

Surprisingly, absence of IL-17RA-signalling had only limited effects on neutrophilic inflammation, and neutrophil chemo-attractants in our HDM-driven airway inflammation mouse model. Ablation of IL-17RA-signalling increased the number of Th1 and Th17 cells, whereas Th2 cell differentiation and eosinophilic inflammation was not hampered. Strikingly, the presence of mucus-producing cells was severely reduced in mice with deficient IL-17RA-signalling and TNFAIP3-deficient myeloid cells.

The IL-17RA subunit forms a heterodimer with either the IL-17RC or IL-17RB subunit. IL-17RA/C heterodimer is used by IL-17A, IL-17F, and IL-17A/F and the IL-17RA/B heterodimer is activated by IL-17E (also known as IL-25)[20]. Ablation of the IL-17RA subunit will therefore affect the signalling of IL-17A, IL-17F, IL-17A/F, and IL-25. We observed that neutrophilic inflammation and neutrophil chemo-attractants persisted in the absence of IL-17RA-signalling, indicating that neutrophilia can develop without the presence of the described IL-17R family members IL-17A, IL-17F, and IL-25. This is in contrast to other reports that showed dependency of neutrophil influx on IL-17RA-signalling not only in asthma and COPD, but also in pulmonary bacterial and viral infections[9, 11, 23, 42-44]. Neutrophil chemo-attractants CXCL1, CXCL2 and CXCL12 were not altered upon ablation of IL-17RA-signalling indicating that these chemo-attractants can be induced by factors independent of IL-17RA-signalling. Similar quantities of Th17-promoting cytokines IL-1β and IL-6 were found in the lungs of HDM-exposed *Tnfaip3*$^{LysM\text{-}KO}$ and *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO}

mice, whereas IL-23 expression was increased in HDM-exposed *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} as compared to *Tnfaip3*$^{LysM\text{-}KO}$ mice. IL-1β has been shown to induce CXCL1 as efficiently as IL-17 by mouse embryonic fibroblasts[31]. Furthermore, IL-1β-deficient mice have defective neutrophil mobilization upon group B streptococcus infection, most likely caused by strongly reduced CXCL1 and CXCL2 production[35]. Likewise, IL-6 can induce CXCL1 transcription in endothelial cells[36]. This could indicate that pulmonary IL-1β and IL-6 expression in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice can induce CXCL1 expression by lung epithelial cells, independent of IL-17RA-signalling.

Ablation of IL-17RA-signalling alone only slightly increases the presence of IL-17-expressing T cells, however combined with *Tnfaip3*-deficient myeloid cells, pulmonary Th17 cells were massively enhanced in allergen-exposed *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. Increased pulmonary IL-23-expression, high levels of IL-1β and IL-6, and defective negative feedback normally provided by IL-17 in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice, could be responsible for this massive increase. It is known that IL-23 expression by myeloid cells, such as DCs and macrophages drives clonal expansion of Th17 cells[45], whereas IL-17 acts as a negative feedback to control its own expression[26]. Strikingly, only IL-23, and not IL-1β and IL-6, was specifically increased in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice when compared to *Tnfaip3*$^{LysM\text{-}KO}$ mice, suggesting that IL-17RA-signalling also controls IL-23 production.

We found limited effects of defective IL-17RA-signalling on all features observed in HDM-mediated allergic airway inflammation including Th2 differentiation and eosinophilic inflammation. This implicates that IL-17A, IL-17A/F, IL-17F and IL-25 are dispensable for Th2-mediated eosinophilic inflammation upon HDM treatment. Blockade of IL-17A also did not influence eosinophilic inflammation and Th2 cytokine secretion upon exposure to the HDM Der f allergen[42]. This is in contrast to ovalbumin (OVA)-mediated allergic airway models, where reduced eosinophilic inflammation, Th2 cytokines, and airway hyper responsiveness (AHR) were observed in either IL-17RA-deficient or IL-17-deficient mice[28, 29]. This suggests that the importance of IL-17 depends on the allergen/model used. While IL-17-depletion during HDM challenges has no effect on eosinophilia and Th2 cytokines[42], blockade of IL-17 during challenge in OVA-mediated models promotes Th2-mediated eosinophilic inflammation[28]. Treatment with recombinant IL-17 promotes inflammatory resolution upon OVA-mediated airway inflammation[30], indicating that IL-17 during the resolution phase can be beneficial.

Next to airway type-2 inflammation, goblet cell hyperplasia was also almost completely absent in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice. This suggests that the presence of Th2 cytokines in WT mice, or Th17 cytokines in *Tnfaip3*$^{LysM\text{-}KO}$ are essential for goblet cell hyperplasia. Indeed, mucus production by goblet cells is induced by Th2 cytokines IL-4, IL-13[46-49], and Th17 cytokines IL-17A[7] and IL-17F[50]. Furthermore, IL-25 (e.g. IL-17E) is also implicated in goblet cell hyperplasia[51, 52]. The combination of OVA-specific Th2 and Th17 cells was shown to induce more mucus-producing goblet cells than OVA-specific Th2

cells alone[53]. This indicates that both Th2 and Th17 cytokines can induce hyperplasia of mucus-producing cells separately and can even take over each other function, as combined absence of Th2 cytokines and abrogated IL-17RA-signalling in *Tnfaip3*$^{LysM\text{-}KO}$*Il17ra*^{KO} mice completely hampers the induction of goblet cell hyperplasia. Furthermore, mucus production by goblet cells in *Il17ra*^{KO} mice develops independent of IL-25.

In conclusion, our results show that neutrophilic airway inflammation induced by activated TNFAIP3/A20-deficient myeloid cells can develop in the absence of IL-17RA-signalling. Increased pulmonary pro-inflammatory cytokines IL-1β and IL-6 quantities are not influenced by IL-17RA-deficiency in mice with activated myeloid cells after HDM exposure. Both IL-1b and IL-6 can induce the expression of neutrophil chemo-attractants, contributing to neutrophilic airway inflammation independently of IL-17 signalling.

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Conflict of interest

The authors declare no conflict of interest.

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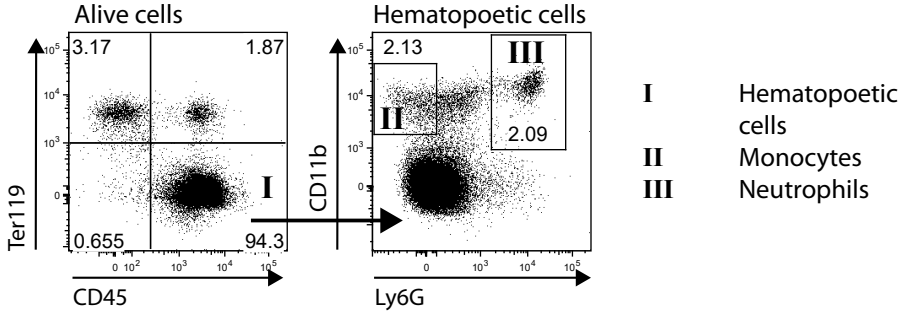
Supplementary Table 1: Antibodies used for flow cytometry.

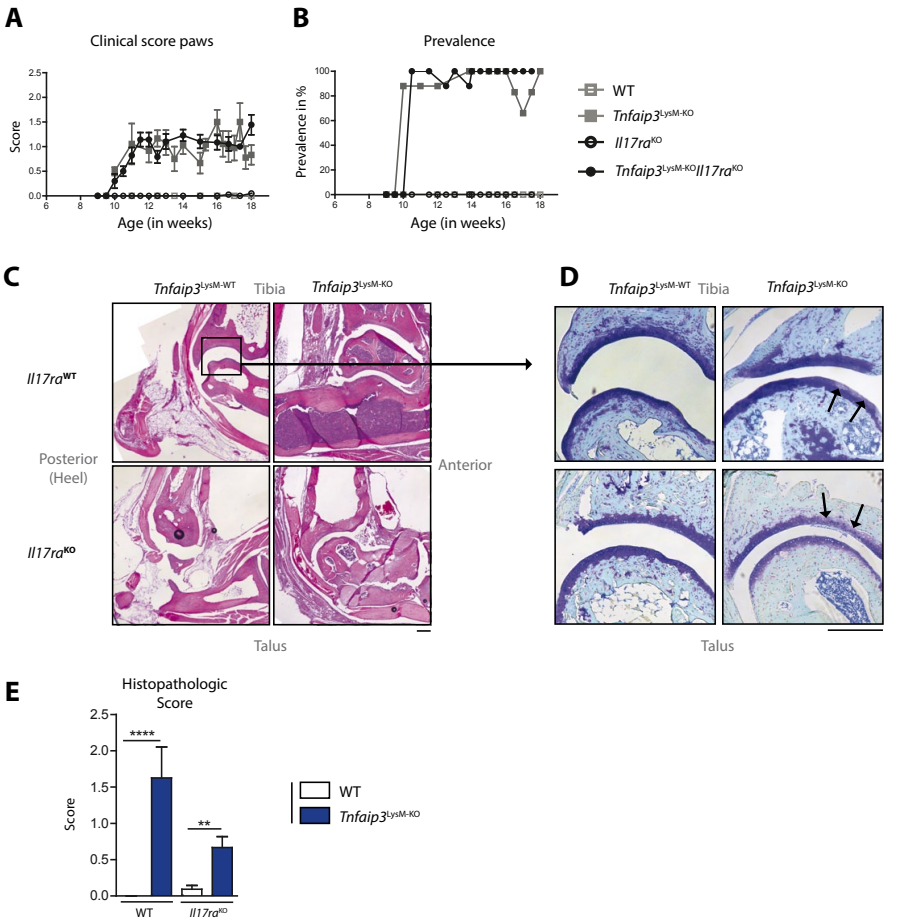
Antibody	Conjugate	Clone	Company
CD3	APC-eF780	17A2	ebioscience
CD4	PE-CF594	RM4-5	BD
CD4	BV711	RM4-5	BD
CD8	PE-Cy7	53-6.7	ebioscience
CD11b	PerCP-cy5.5	M1/70	BD Bioscience
CD11c	PE Texas Red	N418	ebioscience
CD25	Pe-Cy7	PC61.5	ebioscience
CD27	Biotin	LG.7F9	ebioscience
CD44	FITC	IM7	ebioscience
CD44	APC-cy7	IM7	BD
CD45	PE Texas Red	I3/2.3	Abcam
CD64	APC	X54-5/7.1	BD
CD64	PE	X54-5/7.1	BD
CD86	PE Cy7	GL1	BD
CD103	APC	2E7	ebioscience
FcƐRI	Biotin	MAR-1	ebioscience
GR1	Pe-cy7	1A8	BD
IFNγ	eF450	XMG1.2	ebioscience
IL-5	PE	TRFK-5	BD
IL-5	APC	TRFK-5	BD
IL-13	AF647	eBio13A	ebioscience
IL-17A	AF700	TC11-18H10.1	BD
MHC-II	Alexa Fluor 700	M5/114.15.3	ebioscience
RORγt	PE	Q31-378	BD
Streptavidin	PerCP-Cy5.5		BD
Streptavidin	APC-eF 780		ebioscience
Streptavidin	Brilliant Violet 786		BD
TCRγδ	Biotin	UC7-13D5	ebioscience
Ter119	APC	TER-119	ebioscience

Supplementary Table 2: The RT-PCR primers have been listed with the gene name, corresponding encoded protein and forward/reverse sequence.

Gene name	Encoded Protein	Forward sequence	Reverse sequence
Csf2	GM-CSF	AAAGGGACCAAGAGATGTGGC	GTTTGTCTTCCGCTGTCCAAG
Cxcl1	CXCL1	CTGGGATTCACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC
Cxcl12	CXCL12	GTCAGCCTGAGCTACCGATG	TTCTTCAGCCGTGCAACAATC
Cxcl2	CXCL2	CGCTGTCAATGCCTGAAG	GGCGTCACACTCAAGCTCT
Gapdh	GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA
Il1b	IL-1β	AGTTGACGGACCCCAAAAG	TTTGAAGCTGGATGCTCTCAT
Il22	IL-22	GTGACGACCAGAACATCCAG	TCCACTCTCTCCAAGCTTTTTC
Il23	IL-23	CCAGCGGGACATATGAATCT	TGGATACGGGGCACATTATT
Il6	IL-6	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTCATACA
Muc5a	Mucin 5AC	ACCACTTTCTCCTTCTCCACAC	CCCCTGAGGACCCTACACTC

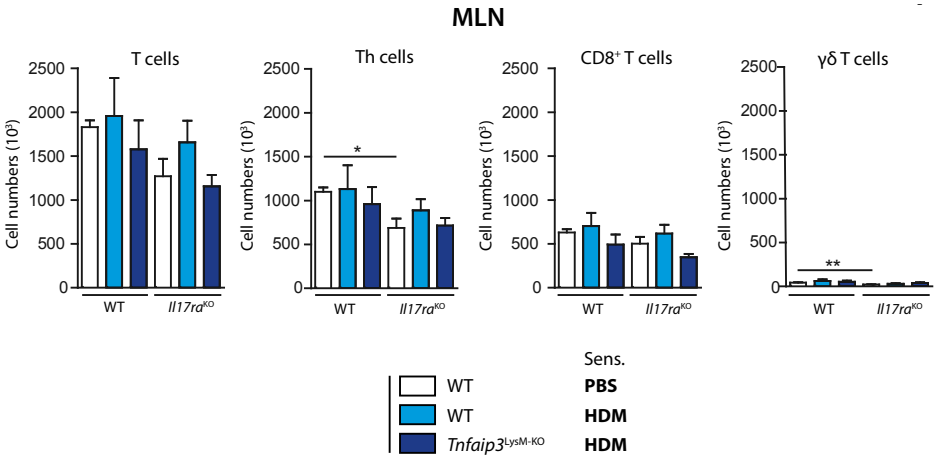


Supplementary Figure 1: Flow cytometric gating strategy of myeloid cells. An example is shown of a spleen derived from a WT mouse.



Supplementary Figure 2: Aged myeloid *Tnfaip3*-deficient mice develop arthritis, regardless of IL-17RA-signalling.

Mice were analysed at 18 weeks of age when arthritis had spontaneously developed. (**A-B**) Paws were scored biweekly for severity (**A**) and the derived prevalence graph (**B**) of spontaneous arthritis is shown. (**C**) Hematoxylin and eosin (H&E) stained paraffin embedded, EDTA decalcified, talo-tibial joints at a magnification of 50x is shown. (**D**) Tolluidine blue stain of the talo-tibial cartilage region is depicted at a magnification of 200x. Arrows represent areas of cartilage loss. Scale bars (200µm) are at the lower right corner of the microscopic images. (**E**) Histopathologic score of talo-tibial joints as assessed by combining H&E and toluidine blue stain scores. Results are presented as mean ± SEM of n = 4-10 per group and are pooled from several experiments. **$P < 0.01$, **$P < 0.001$, ****$P < 0.0001$.



Supplementary Figure 3: HDM exposure does not lead to increased T cells or γδ T cells in the MLN of myeloid *Tnfaip3*-deficient and IL-17RA-signalling deficient mice.
Quantification of total CD3+ T cells, Th cells, CD8+ T cells and γδ T cells in cell suspensions of the mediastinal lymph node (MLN) using flow cytometry. Results are presented as mean ± SEM of $n = 6$ per group. *$P < 0.05$, **$P < 0.01$.

SUPPLEMENTARY MATERIAL METHODS:

Assessment of joint swelling

To assess the degree of joint swelling, we evaluated hind and front paws swelling of mice from 6 weeks of age giving a maximum score of 2 per paw (Corneth et al, Arthritis Rheum, 2014), rising to a theoretical total of 8 per mouse. Mice were removed from the experiment with scores of 6 to alleviate suffering. Disease severity and incidence were monitored.

Histology

Ankle joints were fixed in formalin, decalcified in EDTA and embedded in paraffin. The tissues were stained with Hematoxylin and Eosin (H&E) to visualize the structure of bone. Toluidine blue staining was done to assess the cartilage integrity (Schmitz et al, Osteoarthritis Cartilage, 2010). Each histology slide was arbitrarily scored for tissue inflammation and cartilage integrity by two independent blinded researchers.