House dust mite-driven neutrophilic airway inflammation in mice with TNFAIP3-deficient myeloid cells is IL-17-independent

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Summary
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: Tnfaip3fl/fl ×Lyz2+/-cre (Tnfaip3LySM-KO) mice were crossed to Il17raKO mice, generating Tnfaip3LySMIl17raKO mice and subjected to an HDM-driven airway inflammation model.

Results: Both eosinophilic and neutrophilic inflammation observed in HDM-exposed WT and Tnfaip3LySM-KO mice respectively were unaltered in the absence of IL-17RA. Production of IL-5, IL-13 and IFN-γ by CD4+ T cells was similar between WT, Tnfaip3LySM-KO and Il17raKO mice, whereas mucus-producing cells in Tnfaip3LySMIl17raKO mice were reduced compared to controls. Strikingly, spontaneous accumulation of pulmonary Th1, Th17 and γδ-17 T cells was observed in Tnfaip3LySM-KOIl17raKO 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 Tnfaip3LySM-KOIl17raKO 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 Tnfaip3LySM-KOIl17raKO 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. Granulocytic cells observed in pulmonary inflammation of asthmatic patients can comprise eosinophils, neutrophils or a mixture of both cell types. Eosinophilic inflammation of asthmatic patients can comprise eosinophils, neutrophils and higher morbidity. Neutrophils and Th17 cells are likely contributors to asthma symptoms, because (a) it induces airway remodelling by promoting fibroblast proliferation, (b) reduces apoptosis of smooth muscle cells and (c) increases the expression of mucin genes in airway epithelial cells. Th17 cells primarily produce IL-17, and IL-17 further contributes to asthma symptoms, because (a) it induces airway remodelling as antigen load, expression of costimulatory molecules, and thereby controls NF-κB signalling. TNFAIP3 is also implicated in Th2 signalling to the development of neutrophilic asthma.

KEYWORDS
asthma, IL-17A, neutrophils

MATERIALS AND METHODS

2.1 Mice

Male and female C57BL/6 mice harbouring a conditional Tnfaip3 allele between LoxP-flanked sites were crossed to transgenic mice expressing the Cre recombinase under the LysM promoter, generating Tnfaip3fl/flxLyz2+/cre mice, in which Tnfaip3 will be deleted in cells that express or have expressed LysM. Tnfaip3fl/flxLyz2+/− mice (Tnfaip3LysM−/− KO mice), Tnfaip3fl/flxLyz2+/+xIl17ra−/− mice (Tnfaip3LysMKOIl17ra−/− KO mice) and Tnfaip3fl/flxLyz2+/+xIl17ra−/− 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).

2.2 HDM-induced allergic airway inflammation

During intranasal (i.n.) exposures, mice were anesthetized using isoflurane. On day 0, mice were sensitized with 10^7 HDM cells expressing the Cre recombinase under the LysM promotor,21 generating Tnfaip3fl/flxLyz2+/cre mice, in which Tnfaip3 will be deleted in cells that express or have expressed LysM. Tnfaip3fl/flxLyz2+/− littermates (wild-type WT mice) were used as controls. Tnfaip3LysM−/− mice were crossed with conventional Il17ra−/− mice,22 creating Tnfaip3fl/flxLyz2+/+xIl17ra−/− mice (Tnfaip3LysM−/− KO mice) and Tnfaip3fl/flxLyz2+/+xIl17ra−/− 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).

2.3 Cell suspension preparation

Bronchoalveolar lavage was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mmol/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 minutes 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% NH4Cl, 1% KHCO3, and 0.04% NA2EDTA in Milli-Q). MLN and spleen were isolated for flow cytometry, for which they were homogenized through a 100-μm cell strainer.
2.4 Flow cytometry procedures

Flow cytometry surface and intracellular staining procedures have been described previously.23 Monoclonal antibodies used for flow cytometric analyses are listed in Table S1. 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 hours at 37°C. Data were acquired using a LSR II flow cytometer (BD Biosciences) with FACS Diva™ software and analysed with FlowJo version 9 (Tree Star Inc software, Ashland, OR, USA).

2.5 Lung histology

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

2.6 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, Waltham, MA, USA). 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 Table S2. 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.

2.7 Statistical analysis

All data were 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).

3 RESULTS

3.1 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 Tnfap3Lvsm−/− KO mice17,18 with conventional ll17raKO mice.22 It has been demonstrated that aged Tnfap3Lvsm−/− mice develop arthritis19 and that ll17raKO mice have altered monocyte24 and neutrophil25,26 homeostasis. We therefore first examined whether abrogation of IL-17RA-signalling in Tnfap3Lvsm−/− 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 Tnfap3Lvsm−/− and Tnfap3Lvsm−/−Il17raKO mice showed splenomegaly in comparison with WT and ll17raKO control mice (Figure 1A), whereas total splenic cell counts were only increased in 8- and 18-week-old Tnfap3Lvsm−/−Il17raKO mice (Figure 1B). Monocytes and neutrophils (gated as shown in Figure S1) were significantly increased in 8-week-old Tnfap3Lvsm−/− mice in comparison with WT mice (Figure 1C,D), however only neutrophils were significantly increased in 18-week-old Tnfap3Lvsm−/− mice compared to WT mice (Figure 1D), confirming previous findings.18 Interestingly, both neutrophils and monocytes were significantly increased in 18-week-old Tnfap3Lvsm−/−Il17raKO mice compared to Tnfap3Lvsm−/− mice (Figure 1C,D). Despite elevated monocyte and neutrophil numbers in Tnfap3Lvsm−/−Il17raKO mice, the macroscopic and microscopic arthritis phenotype was similar between Tnfap3Lvsm−/− mice and Tnfap3Lvsm−/−Il17raKO mice (Figure S2).

As IL-17 controls its own expression in CD4+ T cells,25 we assessed conventional TCRγδ T cells and γδ T cells in spleens (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 ll17raKO mice compared to WT mice (Figure 1F). Splenic RORγt+ Th17 cells were elevated in 8-week-old Tnfap3Lvsm−/−Il17raKO mice compared to Tnfap3Lvsm−/− mice, but this was no longer seen in 18-week-old mice (Figure 1G). Only 18-week-old ll17raKO mice and Tnfap3Lvsm−/−Il17raKO mice had increased splenic γδ T cell numbers compared to respective Il17raWT controls (Figure 1H). Splenic CD8+ T cells were reduced in Tnfap3Lvsm−/− KO mice and Tnfap3Lvsm−/−Il17raKO mice compared to respective Tnfap3Lvsm−/− 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.

3.2 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 Tnfap3Lvsm−/−Il17ra mice to an HDM-driven airway inflammation model (Figure 2A). As previously shown,17 HDM-sensitization and challenge induced a predominant eosinophilic inflammation in WT mice compared to PBS-sensitization,
whereas Tnfaip3LysM-KO mice developed a primarily neutrophilic inflammation in the bronchoalveolar lavage (BAL) (Figure 2B). Absence of IL-17RA-signalling did not significantly alter eosinophilic or neutrophilic inflammation in HDM-sensitized Tnfaip3LysM-KO mice compared to HDM-sensitized WT mice (Figure 2B). BAL DCs were increased in both HDM-sensitized WT mice and II17raKO mice compared to their respective PBS-sensitized littermates (Figure 2B). However, in HDM-sensitized Tnfaip3LysM-KO mice, DC numbers were reduced compared to HDM-sensitized WT mice and were increased in HDM-sensitized Tnfaip3LysM-KOII17raKO mice compared to HDM-sensitized Tnfaip3LysM-KO mice (Figure 2B). The absence of IL-17RA did not significantly alter the number of

Figure 1 Loss of IL-17RA-signalling combined with myeloid TNFAIP3 deficiency increases splenic monocytes, neutrophils and γδ T cells with progressing age. Tnfaip3LysMII17ra mice were analysed at 8 and 18 wk 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.
BAL macrophages in comparison with IL-17RA sufficient controls (Figure 2B).

HDM-sensitized WT and Il17raKO mice exhibited enhanced small airway mucus-producing goblet cells and inflammatory cells compared to their PBS-sensitized controls (Figure 2C). HDM-sensitized Tnfaip3LysM-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 Tnfaip3LysM-KOIl17raKO mice compared to HDM-sensitized Il17raKO 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 Tnfaip3LysM-KOIl17raKO mice compared to HDM-sensitized Tnfaip3LysM-KO mice (Figure 2E). HDM-sensitized Il17raKO 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 (Figure S3).

In conclusion, absence of IL-17RA-signalling did not significantly alter eosinophilic or neutrophilic airway inflammation in respectively HDM-treated Il17raKO and Tnfaip3LysM-KOIl17raKO 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 Tnfaip3LysM-KO and WT mice upon HDM sensitization, these populations remarkably increase with loss of IL-17RA-signalling.

3.3 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.27-29 As eosinophilia and neutrophilia were only moderately affected by the loss of IL-17RA in HDM-sensitized Tnfaip3LysM-WT and Tnfaip3LysM-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 Il17raKO and Tnfaip3LysM-KOIl17raKO mice compared to their respective controls with functional IL-17RA-signalling (Figure 3B). HDM-sensitized Tnfaip3LysM-KOIl17raKO mice that had reduced IL-13+ and IL-5+ Th cells compared to HDM-sensitized Il17raKO mice (Figure 3B). As previously shown,17 BAL IL-17+ Th cells increased in HDM-sensitized Tnfaip3LysM-KO mice compared to HDM-sensitized WT controls. Already in PBS-sensitized Il17raKO mice, an increase in BAL IL-17+ Th cells was observed compared to PBS-sensitized WT mice, which was even more enhanced in HDM-sensitized Tnfaip3LysM-KOIl17raKO mice (Figure 3B). BAL IFN-γ-producing Th cells were only increased in HDM-sensitized Tnfaip3LysM-KOIl17raKO mice compared to either HDM-sensitized Il17raKO or Tnfaip3LysM-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 IFN-γ-production by Th cells significantly increased in HDM-sensitized mice lacking myeloid TNFAIP3 with absent IL-17RA-signalling.

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

IL-17 may contribute to neutrophil chemokine (C-X-C motif) ligand (CXCL)1,30,31 CXCL2,32 and CXCL12 release.33 Since neutrophilic inflammation persisted in lungs of HDM-sensitized Tnfaip3LysM-KOIl17raKO mice, we assessed mRNA expression levels of these chemokines. HDM-sensitized lungs of Tnfaip3LysM-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 Tnfaip3LysM-KOIl17raKO mice and Tnfaip3LysM-KO mice (Figure 4A). In contrast, lung Cxcl2 mRNA expression was partially reduced in HDM-sensitized Tnfaip3LysM-KOIl17raKO mice as compared to HDM-sensitized Tnfaip3LysM-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,30,34 IL-635 and IL-23.36 HDM-treated Tnfaip3LysM-KO mice demonstrated elevated Il1b and Il6 expression as compared to HDM-treated WT controls (Figure 4B). Abrogated IL-17RA-signalling in HDM-exposed Tnfaip3LysM-KOIl17raKO mice resulted in similar Il1b and Il6 cytokine expression as Tnfaip3LysM-KO mice (Figure 4B). In contrast, Il23 expression was markedly increased in HDM-exposed Tnfaip3LysM-KOIl17raKO mice compared to Tnfaip3LysM-KO controls (Figure 4B).

Next to IL-17A, Th17 cells can produce other cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF)27 and IL-22,38,39 which are known to regulate neutrophil chemokines CXC11/CXCL2 and directly attract neutrophils respectively. mRNA expression of Csf2 and Il22 was augmented in HDM-sensitized Il17raKO mice compared to PBS-sensitized Il17raKO mice (Figure 4C). Only Il22 gene expression was further increased in HDM-sensitized Tnfaip3LysM-KOIl17raKO mice lungs compared to HDM-sensitized Il17raKO mice (Figure 4C). Both lung Csf2 and Il22 mRNA expression were enhanced in HDM-sensitized Tnfaip3LysM-KOIl17raKO mice compared to HDM-exposed Tnfaip3LysM-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-sensitisation. 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.
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. 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. To investigate whether neutrophilic inflammation development as observed in...
We observed that neutrophilic inflammation and neutrophil chemotaxis persisted in the absence of IL-17A, IL-17F, IL-17A/F and IL-25. We observed that neutrophilic inflammation and neutrophil chemotactic activity 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.9,10,22,41-43 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-6 and IL-23 were found in the lungs of HDM-exposed Tnfaip3^LysM-KO and Tnfaip3^LysM-KO/Il17a^KO mice, whereas IL-23 expression was increased in HDM-exposed Tnfaip3^LysM-KO/Il17a^KO as compared to Tnfaip3^LysM-KO mice. IL-1β has been shown to induce CXCL1 as efficiently as IL-17 by mouse embryonic fibroblasts.30 Furthermore, IL-1β-deficient mice have defective neutrophil mobilization upon group B streptococcus infection, most likely caused by strongly reduced CXCL1 and CXCL2 production.34 Likewise, IL-6 can induce CXCL1 transcription in endothelial cells.35 This could indicate that pulmonary IL-1β and IL-6 expression in Tnfaip3^LysM-KO/Il17a^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-KO/Il17a^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-KO/Il17a^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, whereas IL-17 acts as a negative feedback to control its own expression. Strikingly, only IL-23, and not IL-1β and IL-6, was specifically increased in Tnfaip3−/−mice when compared to Tnfaip3+/+ 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. This is in contrast to ovalbumin (OVA)-mediated allergic airway models, where reduced eosinophilic inflammation, Th2 cytokines and airway hyperresponsiveness (AHR) were observed in either IL-17A-deficient or IL-17A-deficient mice.77,78 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, blockade of IL-17 during challenge in OVA-mediated models promotes Th2-mediated eosinophilic inflammation.77 Treatment with recombinant IL-17 promotes inflammatory resolution upon OVA-mediated airway inflammation, 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−/−mice. This suggests that the presence of Th2 cytokines in WT mice, or Th17 cytokines in Tnfaip3−/− mice, is essential for goblet cell hyperplasia. Indeed, mucus production by goblet cells is induced by Th2 cytokines IL-4, IL-13, and Th17 cytokines IL-17A and IL-17F.49 Furthermore, IL-25 (e.g., IL-17E) is also implicated in goblet cell hyperplasia.50,51 The combination of OVA-specific Th2 and Th17 cells was shown to induce more mucus-producing goblet cells than OVA-specific Th2 cells alone.52 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−/−mice completely hampers the induction of goblet cell hyperplasia. Furthermore, mucus production by goblet cells in LysM-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-1β 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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**FIGURE 4** Myeloid TNFAIP3-deficient mouse have IL-17RA-independent increases in neutrophil chemokines upon HDM-sensitization. Total lung homogenates of HDM-challenged Tnfaip3−/− mice 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−/− mice. Results are presented as mean ± SEM of n = 6 per group. *P < 0.05, **P < 0.01
CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
HV, TD, RWH and MK designed the experiments. HV, TD, IB, JvH and FA performed experiments and analysed data. HV, TD, RWH and MK wrote the manuscript. All authors read and approved the final manuscripts.

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**SUPPORTING INFORMATION**

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

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